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In situ production and functional properties of exopolysaccharides from acetic acid

bacteria in baking applications

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List of Abbreviations

AAB	acetic acid bacteria
ANOVA	analysis of variance
ATP	adenosine triphosphate
BC	before christus
BU	Brabender Units
BW	buckwheat
Ca	calcium
CD	coeliac disease
CFU	colony forming units
Cl	chloride
CO	corn
CoA	coenzyme A
CO_2	carbon dioxide
Cu	copper
Da	Dalton
DATA	diacetyl tartaric acid
DHA	dihydroxyacetone
DLG	Deutsche Landwirtschafts-Gesellschaft
DO	dissolved oxygen concentration
DY	dough yield
e. g.	for example
EPS	exopolysaccharides
etc.	and so on
F.	Fagopyrum
FBC	fat binding capacity
Fe	iron
frc	fructose
Frc equ	fructose equivalents (entity for determination of EPS from sourdoughs)
FTF	fructosyltransferase
g	g-force
G.	*Gluconobacter*
g	gram
GDH	glucose dehydrogenase
GF	gluten-free
GI	glycemic index
glc	glucose
GTF	glycosyltransferase

h	hour
HCl	hydrochloric acid
HDL	high density lipoproteins
HePS	heteropolysaccharides
HoPS	homopolysaccharides
HPLC	high-performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
IEL	intraepithelial lymphocyte counts
J	joule
k	kilo (10^3)
K.	*Kozakia*
K	potassium
L.	*Lactobacillus*
l	liter
LAB	lactic acid bacteria
M	Mega (10^6), molar
m	milli (10^{-3}), meter
Mg	magnesium
MI I	golden millet
MI II	wild browntop millet
min	minute
Mn	manganese
MUFA	monounsaturated fatty acids
n	nano (10^{-9})
N.	*Neoasaia*
na	not applicable
Na	sodium
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
nd	not defined
OA	oat
OD_{590}	optical density at 590 nm
P	phosphorus
RDA	recommended daily amount
RI	rice
rpm	roots per minute
RY	rye
S.	*Saccharomyces*
Se	selenium
SP	spelt
SSL	sodium stearoyl-2-lactylate
St	standard
sucr	sucrose
T	Temperature
TMW	Institute of Technical Microbiology,

	Technische Universität München, Germany
Trp	tryptophan
TTA	total titrable acid
TTB	total titrable base
USA	United States of America
WHC	water holding capacity
WKP	water kefir powder
WT	wheat
w/v	mass/volume
w/w	mass/mass
WW	whole wheat
Zn	zinc
μ	micro (10^{-6})

1 Theoretical Background and Objective of Work

Baking is one of the most common food heating methods and is used to present cereals in an attractive, palatable and digestible form. The resulting products are mainly bread, rolls, cakes, biscuits and other baked goods, whereas bread serves as one of the most popular staple foods and is consumed in many countries all over the world.

1.1 Bread

Bread is baked from a dough basically consisting of flour, water, leavening agents and salt and serves as an important supplier of nutrients: It contains high-molecular carbohydrates in the form of starch, as well as proteins, dietary fibre, vitamins, minerals, trace elements and little fat ([Becker, 1995]).

Due to the presence of gluten proteins which form a macromolecular network during dough mixing, wheat flour usually is the main ingredient in dough and bread formulations. The gluten proteins confer properties of elasticity and extendability to the dough which are essential for a high bread quality ([Anjum et al., 2007]). Other common flours used in bread making are rye and spelt. In case of gluten sensitivity, mixtures of buckwheat, rice, corn or millet and several additives can be employed instead, but still with quality losses. As leavening agents, yeasts and/or sourdough are traditionally used, however they can be supplemented with or replaced by chemical raising agents ([Holmes and Hoseney, 1987]).

In Germany, more than 300 different bread recipes and about $1,200$ small baked products exist. Although the consumption of bread has reduced by about 50% during the last 100 years, the daily ration per person currently lies at about $220\,\mathrm{g}$, which totals $80\,\mathrm{kg}$ per year ([Seibel, 1991], [Becker, 1995]). Therefore in Germany the bread consumption is among the highest within the European countries.

1.1.1 Historical overview of bread making

There are different opinions about the history of bread. One says that the first bread was baked around $10,000$ years before Christ (BC) and the art of bread making was made popular throughout the world by the Egyptians ([Mondal and Datta, 2008]). What is certain is that bread baking as well as sourdough fermentations have been discovered by chance and that bread has been playing a major role in human nutrition since ancient times.

Although breadmaking is such an old traditional craft, over the past 150 years the small artisan bakeries formerly present in every village were displaced by the bakery industry ([Decock and Cappelle, 2005]). Whereas in the 19th century, wheat bread baking included slow mixing and long proving times, resulting in a fully developed structure and aroma, the industrial bakeries aimed to save time and process costs. This resulted in a loss of flavours, along with an extensive use of additives like enzymes, emulsifiers, hydrocolloids, acidifiers or leavening agents ([Haros et al., 2002], [Kohajdová et al., 2009]). However, in recent years, there has been an increased interest in tastier and more authentic, artisan-style breads among a large part of consumers ([Decock and Cappelle, 2005]).

1.1.2 Bread ingredients: cereal grains and flours

Biologically, a cereal grain kernel contains three main parts: the endosperm, the bran and the germ. The endosperm consists of the starchy endosperm (70 - 80 % of the kernel) and the aleurone layer, which is rich in protein and also contains fat, enzymes, vitamins and phytate ([Belitz et al., 2009]). Phytate has been shown to reduce the availability of several minerals ([Davies and Nightingale, 1975]). For millers, the aleurone layer counts as part of the bran, which is rich in proteins, dietary fiber, vitamins, minerals and essential fatty acids ([Bartnik and Jakubcyk, 1989]). The cereal germ contains lipids (especially unsaturated fatty acids), tocopherol (wheat and corn), B-vitamins, proteins, minerals and enzymes. In wholemeal flours, the principal anatomical components of the cereal grain are present in the same proportions as in the intact kernel. During milling of refined flours, the bran and germ are separated from the starch endosperm and the aleurone layer is removed. Depending on the extraction rate, which corresponds to the ash content, the concentrations of essential nutrients, e.g. minerals and proteins, decrease ([Anglani, 1998]). This results in a substantial loss of B-vitamins, minerals and micronutrients ([Pedersen and Eggum, 1983], [Belitz et al., 2009]). In breadmaking, ash influences the buffering capacity of sourdough and therefore leads to a higher content of total titrable acid (TTA)([Decock and Cappelle, 2005]). Due to its buffering properties and high nutrient contents, ash is important for bacterial growth during sourdough fermentation. In the following subsections, some selected cereals and pseudocereals are further descibed. Wheat, spelt and rye, which are discussed at first, are cereals and therefore contain gluten. Subsequently described are oat, rice, corn, buckwheat and the millets, which belong to pseudocereals and are therefore gluten-free.

Wheat

Wheat (*Triticum aestivum* ssp. *vulgare*) is the largest cereal grain crop in the world and is mainly grown in China, India, North and South America, Russia, Europe, the Mediterranean countries and Australia ([Belitz et al., 2009]). The wheat crop is adaptable to a range of environmental conditions, whereby it repays heavy nitrogenous manuring ([Kent and Evers, 1984]). Cultivation of wheat has two different seasons: Winter wheat germinates in autumn and is harvested in spring, while spring wheat is sown in spring and harvested before the first frosts. Wheat can be generally classified depend-

ing on the milling behaviour into hard or soft and depending on the protein content into strong or weak. Due to its pleasant flavour, long shelf-life and gluten-forming proteins, wheat is the most important cereal in bread making ([Goesaert et al., 2005], [Chavan and Chavan, 2011]). Furthermore, it is consumed as bulgur, pasta and flat breads. For breads with a large loaf volume, good crumb texture and good keeping properties, strong wheat flours with a high protein content are preferred ([Madden, 2001]).

After harvest, the wheat kernels are separated from the husk by threshing and, in contrast to rice, processed to various extents before consumption. From the wheat germ, a highly regarded oil is made, which is rich in unsaturated fatty acids and vitamin E ([Bourne, 1989]). The endosperm is then milled into differently refined flours.

During dough making, the prolamine and glutelin of the wheat endosperm constitute the gluten complex, which mainly provides dough structure. The carbohydrates are primarily water-insoluble pentosans, which bind and retain high amounts of water. Furthermore, the lipids and certain gluten proteins form a lipoprotein complex and gliadins and glutenins aggregate due to the formation of noncovalent bonds like hydrogen bonds, ionic bonds and hydrophobic bonds ([Chavan and Chavan, 2011]). These effects are responsible for the unique cohesive and viscoelastic flow properties of wheat dough, which enable gas retention on expansion and determine the baking ability ([Gan et al., 1995], [Belitz et al., 2009], [Roccia et al., 2009], [Chavan and Chavan, 2011]).

In the wheat endosperm, the lysine content and concentration of sulfur containing amino acids is low ([Pedersen and Eggum, 1983], [Roccia et al., 2009]). Protein and tryptophan levels are similar to quinoa and spelt, but higher than in rice, corn, oat, rye and millet flour ([Comai et al., 2007]). Available carbohydrates present in wheat flour are maltose, followed by sucrose, glucose and fructose. Furthermore, some trisaccharides like maltotriose and raffinose can be found ([Chavan and Chavan, 2011]). It has been shown that wholemeal winter wheat flour contains fewer lipids than wholemeal spelt flour, whereas free lipids account for most of this difference ([Ruibal-Mendieta et al., 2002]). In addition to this, the proportion of monounsaturated fatty acids (MUFA) in spelt flour is twice as high as in wheat flour ([Marques et al., 2007]). In a study carried out by Skrabanja et al. ([Skrabanja et al., 2001]), white wheat bread and flour contained fewer proteins compared to white and wholemeal spelt flour. This was verified by Pruska-Kedzior et al. ([Pruska-Kedzior et al., 2008]), who showed that common wheat flours have a lower protein content than spelt flour. In Table 1.1, the chemical composition of wheat, whole wheat and whole spelt flours is compared in detail.

Spelt

Spelt (*Triticum aestivum* ssp. *spelta*) is an old European breadmaking cereal and a close relative of wheat ([Bonafaccia et al., 2000]). From the 5th century until the beginning of the 20th century, it was the predominant bread cereal ([Schober et al., 2006]). Spelt tolerates poorly drained and low-fertility soils ([Ranhotra et al., 1995]) and is therefore suited for organic farming ([Skrabanja et al., 2001]). However, due to a 98.5 % identity of α-gliadin between spelt and wheat, it is toxic to coeliacs ([Kasarda and D'Ovidio, 1999]). As it has lower yields and is harvested as a hulled grain which must be expensively

Table 1.1 – Dry matter, lipids and ash [%], fatty acid contents [% (w/w) of total lipids], α-tocopherol and mineral composition [mg/100 g] of sieved wheat, whole wheat and whole spelt flours. Averages of nine dehulled spelt compared to five soft winter wheat varieties shown. nd: not defined; na: not applicable. Adapted from Ruibal-Mendieta et al. ([Ruibal-Mendieta et al., 2005])

	sieved wheat	whole wheat	whole spelt
dry matter	84.71 ± 0.37	88.08 ± 0.42	88.57 ± 0.31
α-tocopherol	nd	3.24 ± 0.66	2.88 ± 0.37
lipids	1.39 ± 0.12	2.48 ± 0.16	2.92 ± 0.17
ash	0.42 ± 0.04	1.49 ± 0.07	1.83 ± 0.11
palmitic	19.42 ± 0.03	18.95 ± 0.05	16.44 ± 0.02
oleic	9.35 ± 0.03	10.48 ± 0.04	15.75 ± 0.08
linoleic [% w/w of lipids]	66.19 ± 0.07	63.31 ± 0.09	61.64 ± 0.09
α-linolenic	3.60 ± 0.00	5.24 ± 0.01	3.77 ± 0.01
Cu	na	na	na
Fe	0.63 ± 0.11	2.18 ± 0.27	3.11 ± 0.42
Mn	0.49 ± 0.04	2.75 ± 0.22	2.84 ± 0.17
Zn	0.61 ± 0.27	1.92 ± 0.25	3.05 ± 0.25
Na	8.80 ± 2.47	9.19 ± 2.96	8.48 ± 1.97
K	109 ± 10	373 ± 14	372 ± 27
Ca	18.99 ± 1.13	32.01 ± 2.86	28.36 ± 5.17
Mg	18.00 ± 3.45	96.31 ± 4.57	127.41 ± 5.20
P	72.90 ± 27.9	210.50 ± 62.1	292.30 ± 24.5

dehulled before milling ([Ruibal-Mendieta et al., 2002], [Ruibal-Mendieta et al., 2005]), spelt has widely been displaced by modern wheat in the past ([Kling, 1993]). Recently, it has regained importance in sourdough bread baking, especially for craft bakers and speciality breads ([Madden, 2001], [Schober et al., 2002], [Abdel-Aal, 2003]). This can be explained by its several advantages over wheat ([Ruibal-Mendieta et al., 2005]), which are described in Section 1.1.2 and in the following: During a study with different spelt and wheat cultivars, it has been shown that spelt has a higher protein content and higher soluble dietary fibre than wheat ([Bonafaccia et al., 2000]). In the same study, spelt flour had a much higher water absorption capacity than wheat flour. Breads produced from whole spelt flour showed a higher protein content compared to bread made from common wheat flour ([Bonafaccia et al., 2000]). The gluten properties and protein size classes of spelt flour differ depending on cultivar, location and environmental conditions, which is similar to wheat gluten ([Schober et al., 2006]). The protein content and the ratio of proteic tryptophan (Trp), an essential amino acid, is similar to wheat, whereas the free Trp is more than twice as high as in wheat ([Comai et al., 2007]). In a study assessing the glycemic index (GI) of white spelt bread, no differences could be found compared to white wheat bread ([Marques et al., 2007]). Zörb et al. ([Zörb et al., 2007]) identified sucrose, 1-kestose, raffinose, maltose, kestotetraose, fructose, glucose, xylose and stachyose in spelt and spelt wholemeal flour, whereas the first mentioned four sugars were three- to two-fold higher in the wholemeal. The total free sugars in wholemeal spelt flour amounted 17.1 mg/g dry weight and were therefore higher than in wholemeal wheat flour (12.5 mg/g dry weight), whereas neither xylose nor stachyose were detected in wheat.

In Table 1.1, a comparison of dry matter, tocopherol content, lipid and ash composition of wheat, whole wheat and whole spelt flour analysed by Ruibal-Mendieta et al. ([Ruibal-Mendieta et al., 2005]) is shown.

Rye

Rye (*Secale cereale*) nowadays is the second-most important bread grain after wheat. It was first domesticated in Germany from about the fourth century BC and was then brought to southern Europe ([Kent and Evers, 1984]). The rye plant grows well in temperate climate and has a high tolerance for pests, diseases and light acid soils ([Belitz et al., 2009]). In contrast to wheat, rye has reduced requirements of fertilizer and pesticides and can therefore be grown in organic farming ([Hansen et al., 2004]). In addition to its use as a bread cereal, a small amount of rye is used for malting, brewing and distilling ([Kent and Evers, 1984]).

The structure of a rye grain resembles that of wheat, but the average size is slightly smaller and the shape is longer ([Kent and Evers, 1984]). Similar to wheat, rye kernels are separated from the husks by threshing. Its main chemical constituents are starch, dietary fiber (mainly comprising arabinoxylans and pentosans), proteins and minerals ([Bourne, 1989], [Hansen et al., 2004]). Addtionally, a whole range of bioactive compounds like cinnamic acids, alkylresorcinols, lignans, sterols and vitamins are present in the grain ([Andersson et al., 2009]).

Rye flour with 87 % dry matter contains 72.8 % starch, 6.59 % proteins, 1.01 % lipids and 0.87 % ash, related to dry matter basis ([Verwimp et al., 2004]). Compared to wheat flour, it has lower protein and starch contents, but contains more ash, lipids, arabinose, xylose, dietary fibre and lysine ([Seibel, 1975],[Verwimp et al., 2004]), as well as more free sugars, especially sucrose (0.7 g/100 g). In addition to that, traces of glucose, fructose, raffinose and stachyose have been determined ([Henry and Saini, 1989]).

In rye dough, solubles like arabinoxylans, pentosans, dextrins, sugars and soluble proteins are responsible for water absorption ([Bourne, 1989]). Due to the lack of gluten formation, they are highly important for the dough structure and for baking quality, as they increase dough viscosity and enhance bread volume ([Drews and Seibel, 1976], [Delcour et al., 1991], [Vinkx and Delcour, 1996]). Water absorption of arabinoxylans is improved by acidification during sourdough fermentation, which also causes inactivation of starch degrading α-amylases ([Hansen et al., 2004]). Furthermore, fermentation of rye sourdough leads to the degradation of the arabinoxylans into small, more soluble chains ([Escrivá and Martinez-Anaya, 2000]).

Oat

There are two species of oats: *Avena (A.) sativa* and *A. byzantina.* In Europe, types of *A. sativa* predominate. Oat cultivation dates back to about 100 BC ([Hammes et al., 2005]). The oat crop is more successful than wheat in wet climates, although it does not stand cold very well ([Kent and Evers, 1984]). As the adherent husk of the oat grain is inedible by humans, it is removed in a special shelling process. In contrast to wheat, the bran of the kernel (groat) is relatively thin and has only minor effects on the milled product. Therefore oatmeal is manufactured as whole groat meal ([Kent and Evers, 1984]).

Oat prolamins, which represent the aqueous ethanol soluble proteins, are termed avenins. Avenins do not form a gluten network when mixed with water and are not toxic to patients with celiac disease. Nevertheless, oats are not uniformly recommended for a gluten-free diet, as most commercially available products are contaminated with gluten-containing grains ([Ciclitira and Moodie, 2003], [Green and Jabri, 2003], [Green and Cellier, 2007]). Furthermore, some celiac disease patients have avenin-reactive mucosal T-cells and are thus sensitive towards oats ([Arentz-Hansen et al., 2004]).

Oat starch is a compound starch and contains granules of up to 10 µm. The nutritive value of oat flour has been investigated by Hager et al. ([Hager et al., 2012]) and is summarized in Table 1.2. In another study, free sugar contents of oats have been analyzed. The results showed that the average contents of free sugars of the varieties Dal, Froker and Cayuse amounts 1.3 % and is composed of sucrose (0.49 %), raffinose (0.21 %), maltose (0.02 %), stachyose (0.08 %), fructose (0.03 %) and glucose (0.06 %)([MacArthur and D'Appolonia, 1979]).

In oats, β-glucans predominate the composition of hemicelluloses in the endosperm ([Belitz et al., 2009]). Positive effects of β-glucans on blood cholesterol levels ([Katina, 2005]) as well as a prebiotic function have been reported ([Angelov et al., 2006]). Furthermore, oats have been identified as suitable substrates supporting high cell viability during cold

storage for different probiotic strains ([Må rtensson et al., 2002]). During fermentation of oat concentrates with different lactic acid bacteria, contents and maximum viscosities of the soluble fibre concentrates decreased ([Lambo et al., 2005]), whereas other studies have not found a change in β-glucan content during fermentation ([Angelov et al., 2006]).

Although oat flour is not typically used for bread making due to the lack of gluten structure, it has been successfully included into a wheat bread structure ([Polaki et al., 2010]).

Rice

The history of rice (*Oryza sativa*) cultivation started about $5,000$ years ago. At first, it was cultivated in tropical Southeast Asia and was then brought to Central and South America ([Zhou et al., 2002]). Now it is the staple food of many different countries like China, India ([Belitz et al., 2009]), Asia ([Bourne, 1989], [Zhang et al., 1997]) and Brasil ([Heinemann et al., 2005]). In 2006, about 634 million tons of rice have been produced all over the world. China and India produced 184.07 and 136.51 million tons, respectively ([Belitz et al., 2009]). Rice can be grown either on dry land or under water, while the common practice of flooding the fields serves as a means of irrigation and controlling weeds ([Kent and Evers, 1984]).

Rice grains are slightly smaller than wheat; the proportion of husk averages about 20% ([Kent and Evers, 1984]). During milling, the hull is removed from the paddy rice and brown rice is obtained. Then, the brown rice is polished to remove the bran coats, the silvery cuticle, the germ and the aleurone layer. Through a final rubbing-off step, white rice is obtained ([Zhou et al., 2002]). As white rice is low in vitamin content and minerals, about 25% of the worlds harvest is subjected to a parboiling process, during which minerals and vitamins diffuse to the inner endosperm ([Belitz et al., 2009]). In a study performed by Heinemann et al. ([Heinemann et al., 2005]), parboiled milled rice had 18% more ash and higher contents of potassium and phosphorus, but lower contents of manganese, calcium and zinc when compared to milled rice.

The chemical composition of rice grains varies widely and depends on environment, soil and variety ([Zhou et al., 2002]). In general, brown rice is regarded as having the lowest protein content and also low fibre and lipid contents as compared to other common grains ([Zhou et al., 2002]). However, the yield of utilizable protein and digestible energy in rice are the highest among common cereals ([Juliano, 1985], [Zhou et al., 2002]). Rice has a relatively high content of essential amino acids and a rather low content of glutamic acid and other non-essential amino acids ([Bourne, 1989]). In non-parboiled milled rice, the elements calcium, phosphorus, iron, magnesium, potassium, zinc (Zn), copper (Cu), manganese (Mn) and selenium (Se) were found, whereas the Se, Zn, Mn and Cu levels of a raw $50\,g$ portion would contribute to about 25%, 13%, 12% and 11% of recommended daily amount (RDA)([Heinemann et al., 2005]).

Due to its low contents of sodium, protein, fat and fibre, the absence of gluten, the high content of easily digested carbohydrate and its hypoallergenic properties, rice is favoured as an alternative to wheat for persons suffering from inflamed kidneys, hypertension and coeliac disease ([Kent and Evers, 1984], [Ju et al., 2001], [Gujral and Rosell, 2004]).

Table 1.2 – Starch, protein, dietary fibre, phytate, fat and ash content [% (w/w)], fatty acid profile [% (w/w) of total lipids], polyphenols [mg/100g] and mineral composition [mg/kg] of gluten free and wheat flours. All values are based on fresh weight of samples. Adapted from Hager et al. ([Hager et al., 2012])

	Wheat	Wholewheat	Rice	Oat	Buckwheat	Corn
Total starch	68.01±2.34	57.24±0.26	77.52±0.42	69.38±1.66	61.35±2.15	71.52±0.42
Protein	11.54±1.07	9.89±0.17	7.33±0.03	6.91±0.08	12.19±0.38	5.50±0.19
Total dietary fibre	3.44±0.01	11.42±1.27	0.43±0.15	4.05±0.40	2.18±0.11	2.62±0.45
Phytate	0.16±0.03	0.77±0.01	0.21±0.01	0.27±0.01	0.64±0.06	0.09±0.03
Polyphenols	13.04±0.23	82.20±0.42	14.16±2.45	22.16±0.16	465.47±22.41	97.85±0.64
Fat	1.81±0.05	3.63±0.104	0.90±0.06	6.74±0.80	4.21±0.74	2.48±0.46
Myristic 14:0	1.48±0.014	0.10±0.000	0.44±0.002	0.24±0.001	0.11±0.000	0
Palmitic 16:0	19.74±0.076	16.97±0.011	22.43±0.014	20.62±0.001	15.78±0.03	12.62±0.01
Stearic 18:0	10.41±0.094	0.75±0.000	2.45±0.012	1.71±0.007	2.08±0.001	2.07±0.00
Oleic 18:1, 9c	31.14±0.006	12.73±0.007	40.01±0.019	41.85±0.004	36.53±0.012	26.08±0.01
Linoleic 18:2 9, 12	23.74±0.034	60.79±0.020	29.38±0.003	26.56±0.011	33.01±0.010	54.73±0.01
α-Linolenic 18:3 9, 12, 15	1.74±0.004	5.04±0.002	1.91±0.009	0.71±0.014	3.78±0.005	2.08±0.00
Eicosenoic 20:1 11	1.61±0.016	0.72±0.001	0.53±0.007	1.06±0.001	3.27±0.007	0.26±0.00
Saturated fatty acids	38.94±0.038	18.97±0.004	26.35±0.035	23.42±0.001	21.43±0.021	15.21±0.00
Unsaturated fatty acids	60.06±0.044	80.72±0.001	73.25±0.038	71.78±0.005	60.06±0.044	84.29±0.00
Ash	0.92±0.02	1.32±0.01	0.51±0.01	0.82±0.01	1.65±0.01	0.37±0.03
Ca	1797.7±10.5	307.7±4.5	50.7±0.5	224.3±2.1	148.2±1.7	33.2±1.1
Mg	244.0±1.0	782.7±2.5	338.0±4.0	392.7±4.5	1736.0±13.0	315.7±1.5
Na	38.1±3.7	19.9±0.2	15.1±0.3	16.7±3.5	10.8±2.6	4.8±0.3
K	1520.3±8.5	3997.7±29.5	973.7±4.5	1743.7±16.5	4022.7±24.5	1487.0±7.0
Fe	13.4±0.1	26.9±0.2	6.0±0.3	16.4±0.1	28.5±0.0	9.1±0.1
Cu	1.51±0.0	4.0±0.0	2.2±0.0	2.7±0.1	5.1±0.0	0.9±0.0
Mn	8.25±0.1	23.4±0.1	7.3±0.02	27.7±0.4	11.8±0.0	1.5±0.0
Zn	7.59±0.00	17.5±0.4	17.8±0.2	11.3±0.1	18.8±0.0	6.6±0.1
Cl	825.6±42.6	998.0±23.7	350.6±31.5	670.0±48.5	144.0±31.0	484.2±23.1
P	908.7±3.5	2040.7±3.5	954.7±16.5	1476±25	2787.0±14.0	813.7±8.5

Corn

In the beginning, corn or maize (*Zea mays*) was cultivated in tropical Southeast Asia and Central and South America about $5,000$ years ago ([Belitz et al., 2009]). In Mayan and Aztec culture, it was an essential item and played a great role not only in nutrition, but also in religious beliefs and festivities ([FAO, 1992]). Nowadays, corn is the third-most important cereal grain in the world ([FAO, 1992]) and serves as a staple food for large groups of people in Latin America, Asia and Africa ([Bourne, 1989]). Furthermore, it is used for animal feeding and for the manufacture of starch, syrup, sugar, beer, industrial spirit, paper and whiskey ([Kent and Evers, 1984], [de Vasconcelos et al., 2013]). The green plant is turned into silage and used in the dairy and beef industries. In recent years, corn has gained high importance for bioethanol production, e. g. in the USA, where approximately $95\,\%$ of total bioethanol is produced from corn starch ([Taylor et al., 2006]).

The corn plant is a cross pollinating species. The grains develop in the ears, or cobs. In each ear, 12 to 16 rows with about 300 to $1,000$ kernels grow. The grains are much larger than those of other cereals ([Kent and Evers, 1984]). For traditional processing, the germ of the corn grains is removed and the endosperm is either ground to grist for corn porridge (*polenta*) or to flour for flat cakes (*tortillas*)([Belitz et al., 2009]).

The nutritive value of corn is lower than that of wheat (Table 1.2). Corn lacks nicotinic acid and has a relatively low protein content. The protein is deficient in lysine, tryptophan and isoleucine, but high in leucine, which is implicated with isoleucine deficiency ([Patterson et al., 1980], [Kent and Evers, 1984]). The corn kernel mainly consists of starch, whose composition is genetically controlled and depends on the variety. In common corn, the starch contains amylose and amylopectin, whereas waxy maize starch consists of $100\,\%$ amylopectin ([FAO, 1992]). Resistant starch present in high amylose corn starch has been shown to prevent colon cancer, type II diabetes, obesity and cardiovascular diseases ([Englyst and Cummings, 1985], [Robertson et al., 2005], [Behall et al., 2006], [Dronamraju et al., 2009]). Besides starch, other simple sugars like glucose, sucrose and fructose are present in amounts between 1 and $3\,\%$. Whereas the maize germ is relatively rich in minerals, the endosperm contains less than $1\,\%$ ash. From the germ, a highly regarded oil can be made, which is rich in oleic and linoleic acids ([FAO, 1992]).

Buckwheat

Buckwheat (*Fagopyrum esculentum, Fagopyrum tataricum*) is also a member of the group of pseudo-cereals. The buckwheat plant is more bushlike, unpretentious to living environments and suitable for ecological growing ([Krkošková and Mrázová, 2005]), because it absorbs less water and lower amounts of nutrients than other cereals ([Li and Zhang, 2001]). It is mainly cultivated in Russia, China, USA, Canada, France, Germany, Italy and Poland ([Bonafaccia et al., 2003]). The seeds are triangular with a black hull covering the light green to white kernel. The hull is of lower density than water and can be easily removed. By isoelectric precipitation and chemical methods, the slightly bitter taste of *F. tataricum* seeds can be lowered ([Li and Zhang, 2001]).

Buckwheat seeds have a lower protein content than oat flour, but this is still higher than in rice, wheat, millet and corn ([Krkošková and Mrázová, 2005]). Its proteins are gluten-free ([Christa and Soral-Śmietana, 2003]) and have a high biological value, although they are not very well digested ([Ikeda and Kishida, 1993]). As the lysine/arginine and methionine/glycine ratios in buckwheat protein are low, it is supposed to have a cholesterol-lowering effect ([Carroll and Kurowska, 1995]). However, the amino acid composition in different parts of the seed differs and the distribution of proteins is not very uniform ([Li and Zhang, 2001]). Buckwheat contains minerals such as potassium, magnesium, phosphorus, iron, calcium, copper, zinc, selenium, barium, boron, iodine, platinum and cobalt, especially in the outer layers of the seed and hulls ([Li and Zhang, 2001], [Bonafaccia et al., 2003]). Buckwheat flour is also rich in vitamins, especially of the B-group ([Fabjan et al., 2003]). The composition of a buckwheat flour analysed by Hager et al. ([Hager et al., 2012]) is shown in Table 1.2. The flavonoids, flavones, phytosterols, fagopyrins and thiamin-binding proteins found in buckwheat are known or supposed to have anti-allergic, antiviral, anticancer, antioxidant and cholesterol-lowering effects, reduce high blood pressure and play a protective role against coronary heart disease and arteriosclerosis ([Hertog et al., 1995], [Chao et al., 2001], [Li and Zhang, 2001], [Christa and Soral-Śmietana, 2003], [Fabjan et al., 2003], [Kandaswami et al., 2005]). Besides this, a prebiotic potential of a buckwheat diet in rats has been shown ([Prestamo et al., 2003]).

The millets

The term millets is applied to various small-seeded grasses belonging to the subfamilies *Eragrostoideae* and *Panicoideae*. The most important species include pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine corocana*), proso millet (*Panicum miliaceum*) and foxtail millet (*Setaria italica*), whereas Golden German is a variety of foxtail millet ([May et al., 2007]). Browntop millet (*Urochloa ramosa*, also known as *Brachiaria ramosa* or *Panicum ramosum*) belongs to the minor millets and is sown for feeding game birds in the southeastern United States ([Anderson and Martin, 1949], [Cooper, 2007], [Amadou et al., 2013]). Together with sorghum, millets are the most drought-tolerant cereal grain crops ([Subramanian et al., 1986]) and were first cultivated in subtropical and tropical regions of Asia and Africa at around 4,000 BC ([Hammes et al., 2005]). Today, about 95 % of the millet cultivation area is located in these countries ([Léder, 2004]). Grains can be used in porridges, steam-cooked products, boiled rice-like products, beverages, snacks and breads ([Léder, 2004]). The oil which is obtained from the seeds is used for skin and hair care products ([Henning, 2005]).

Millet grains provide protein, fatty acids, minerals, vitamins, dietary fibre and substantial levels of phenolic compounds ([Taylor et al., 2006], [Amadou et al., 2013]). Their fiber content is lower than that of wheat and rye ([Belitz et al., 2009]), whereas in general, the composition and nutritional value of millets is similar to rice ([Léder, 2004]). Millet protein contains high levels of essential amino acids, especially the sulphur containing amino acids methionine and cysteine ([Amadou et al., 2013]). In the grains, also anti-nutrient components like polyphenols, tannin, phytic acid, phytate, goitrogens and

oxalic acid are present. Phytic acid is believed to lower the blood cholesterol level and phytate is associated with reduced cancer risk ([Amadou et al., 2013]). Furthermore, millet containing foods are potentially prebiotic ([Amadou et al., 2013]).

The chemical composition of a yellow foxtail millet flour with 89.78 % dry matter contained 73 % total starch, 11.41 % protein, 1.92 % dietary fibre, 0.68 % ash and 2.9 % lipids ([Mohamed et al., 2009]). The main sugars in a 70 % alcohol-soluble extract of foxtail millet are sucrose, glucose, maltose, fructose and raffinose ([Wankhede et al., 1979]). They amounted 33.4, 13, 9, 8.6 and 8.6 % of total carbohydrate composition of the extract, respectively. Furthermore, small amounts of maltotriose, xylose, higher oligosaccharides and other unidentified sugars were found. Although the protein content of foxtail millet flour is comparable with that of wheat and corn, the methionine fraction amounts 0.8 - 3.0 g/100 g protein and threonine levels range from 3.3 - 4.0 g/100 g protein ([Kent and Evers, 1984], [Mohamed et al., 2009]). A protein concentrate of Korean foxtail millet and proso millet has been shown to elevate plasma adiponectin and HDL cholesterol and to cause major decreases in insulin levels relative to a casein diet in type 2 diabetic mice ([Choi et al., 2005], [Park et al., 2008]). For different millets including foxtail millet, free radical quenching potential has been reported ([Kamara et al., 2012]).

1.1.3 Structure formation during the bread making procedure

During bread production, different physicochemical changes occur to turn the ingredients from their liquid (water) and solid (e. g., flour) state into a bread loaf with crumb and crust. For different types of flours, different types of handling during the single processing steps are necessary. At this, wheat has unique properties among the cereal flours and will solely form a viscoelastic dough with gas-holding properties and a desired structure when mixed with water ([Autio and Laurikainen, 1997], [Bindrich et al., 2008]). If not stated differently, the structure formation during the bread making procedure is described for wheat breads in the following.

Dough mixing and kneading

Mixing and kneading of the dough are the first two steps in every baking process. They are performed either by hand or by using a kneading machine. During mixing, the ingredients are blended and homogenized, whereas kneading relates to the development of the dough structure through the input of mechanical work. The gluten proteins swell and include the starch kernels, which are embedded and surrounded by water ([Autio and Laurikainen, 1997], [Freund and Hermann, 2003]). Due to their large molecule size and low charge density, gluten proteins interact by both hydrogen and hydrophobic bonds and build a three-dimensional network via disulphide bridges and noncovalent bonds, such as hydrogen bonds, ionic bonds and hydrophobic bonds ([Hoseney and Rogers, 1990a], [Bindrich et al., 2008], [Chavan and Chavan, 2011]). This network is a decisive prerequisite for the gas holding capacity and elastic and processing properties of the dough. When kneading is completed, the dough has a viscoelastic structure and incorporates small air bubbles

([Autio and Laurikainen, 1997]). The included air is necessary for oxidational processes and yeast growth, and for forming of gas nuclei for the carbon dioxide that is generated during fermentation ([Hoseney and Rogers, 1990a],[Cauvain and Young, 1998], [Dobraszczyk and Morgenstern, 2003]).

Dough properties are influenced by various factors, among them are the choice and quality of ingredients or the process parameters ([Autio and Laurikainen, 1997]). For example, a certain ratio between big and small starch grains is advantageous for optimum baking properties ([Ternes, 2011]). If sourdough is added to wheat doughs, other rheological changes result and doughs become softer, less elastic and easily extendable. The increased softness is a possible consequence of a higher repulsion among gluten proteins at low pH values ([Angioloni et al., 2005]). Concerning the process parameters influencing dough properties, there is an optimum energy input via mixing speed and energy for bread volume (Figure 1.1): On the one hand, the energy input must be higher than a certain value for a proper development of the gluten network. On the other hand, it should not exceed a critical level above which gluten structure is damaged, doughs become more humid, start sticking to the wall of the bowl and gas holding capacity is lowered ([Autio and Laurikainen, 1997]).

Figure 1.1 – Relation between energy input during kneading and loaf volume of baked wheat bread. x-axis: kneading energy (kJ/kg). After Frazier et al. ([Frazier et al., 1979])

The positive effect of wheat gluten on dough structure has also been shown for pound cakes, where it increased viscosity and cake volume ([Wilderjans et al., 2008]).

For the preparation of rye doughs, sourdough usage is required to inhibit the amylases and therefore to avoid an early liquefaction of the dough ([Bindrich et al., 2008]). Furthermore, the formation of a gluten network is hindered by pentosans ([Belitz et al., 2009]). During kneading, rye dough ingredients are mainly mixed and pentosans need time to swell, which leads to smaller kneading intensities but longer kneading times ([Bindrich et al., 2008]). Resulting doughs are sticky and plastic.

Dividing, moulding and sheeting of dough

By dividing and moulding, the gas cell structure of dough is modified, because small cells burst and coalesce into larger ones ([Anon., 1995]). The sheeting process has a great impact on final dough quality by organizing the protein network in the dough ([Feillet et al., 1977]). Further, air bubble size can be reduced through repeated sheeting. For wheat doughs, this results in a decreased extendability and resistance ([Stenvert et al., 1980]). This plays a role in the production of multi-layered pastries,

for example, which require stronger doughs.

For rye dough, dividing, moulding and sheeting can be performed within less processing steps due to its sticky and plastic properties ([Bindrich et al., 2008]).

Dough proving

During dough proofing, yeasts ferment the sugars set free by flour originating amylases, and ethanol and carbon dioxide are produced. The emerging gas acts as leavening agent, while it is trapped by the gluten, and a desired liquid-gas-solid structure is developed ([Gan et al., 1990], [Bindrich et al., 2008]). At the end of proving, the extendable gluten network surrounds numerous gas bubbles with a diameter between $10 - 100\,\mu m$ ([van Vliet, 1999]) and the dough has expanded up to the $4 - 5$ fold of the original volume ([Giannou et al., 2003], [Chavan and Jana, 2008], [Belitz et al., 2009]). As a greater pressure is needed to expand small gas cells than larger ones, the initial size of gas cells and the pressure within the cells mainly determine whether they expand or not. Therefore proofing provides a link between the bubble structure created in the mixer and the final baked loaf structure ([Clarke et al., 2002]), whereby the time required for a proper dough leavening varies and depends on the flour variety, the ingredients, the yeast concentration and oven temperature.

Baking of bread

The third major unique property of wheat flour doughs is their ability to set in the oven during baking ([Hoseney and Rogers, 1990b]). Depending on the temperature, major structural changes occur during bread baking. Between $20 - 40\,°C$, yeasts propagate, fermentation processes take place and carbon dioxide and ethanol are produced. The initial expansion of bread volume during heating is driven by the carbon dioxide stored in a dissolved state in the dough membranes. The high viscosity retains expanding air nuclei and ensures a uniform disperson of the ingredients ([Wilderjans et al., 2008]). Ethanol and water vaporize from the outer bread layers and further increase the bread volume ([de Vries et al., 1989], [Fan et al., 1999]). At a certain time point, the starch-gluten matrix fails to enclose the gas cells completely, leaving areas between adjacent cells that contain only a liquid film with surface active materials at the gas/liquid interface ([Gan et al., 1990]). Rupture of this film finally leads to an open sponge structure as found in ready baked bread ([Gan et al., 1995]). The temperature range over which volume expansion ends and bread pores become connected lies between $53 - 72\,°C$, relating to the onset of starch gelatinization, which differs depending on the type of amylose and therefore on the flour variety ([He and Hoseney, 1991]). Wheat, rye and barley starch are likewise in terms of gelatinization ([Ternes, 2011]). At this temperature, the swollen starch takes up the $20 - 40$ fold volume of water and dough viscosity is strongly increased. After a part of the amylose has left the starch kernel and is dissolved in water, the kernel breaks ([Belitz et al., 2009]). Starch crystals melt and form a polymer network. This coincides with the death of yeasts (at about $60\,°C$) and the denaturation of enzymes and gluten proteins, leading to a water migration from gluten proteins to

starch ([Mondal and Datta, 2008], [Belitz et al., 2009]).

A further temperature rise above 110 °C in the oven enables Maillard reactions, which describe non-enzymatic browning reactions between amino acids and reducing sugars ([Kent and Evers, 1984], [Mondal and Datta, 2008]). Furthermore, dextrins, as well as caramelisation and roasting products are formed in the outer layer. These reactions are essential for the colour and aroma of the bread crust ([Rehman et al., 2006]).
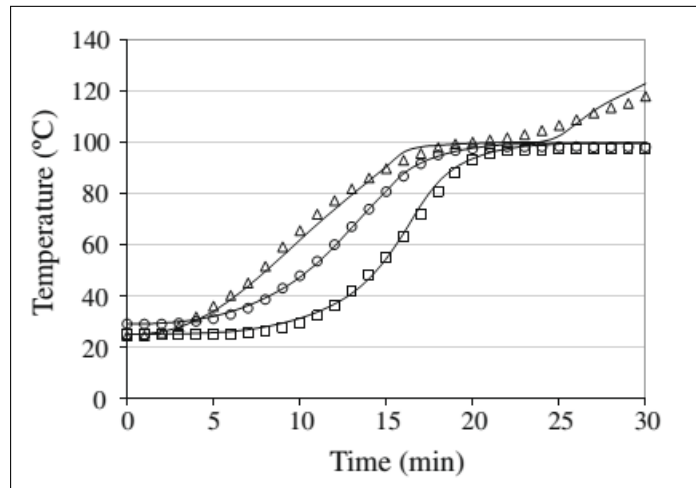
Figure 1.2 – Temperature profiles of the core (□), intermediate (◯) and surface (△) region of bread during baking at 220 °C under natural convection. lines: simulated; symbols: experimental (after Purlis and Salvadori ([Purlis and Salvadori, 2010])).

[Purlis and Salvadori, 2010] show simulated and experimental temperature profiles of the core, intermediate and surface region of bread baked for 30 min at 220 °C under natural convection (Figure 1.2). Due to a slow heat transfer within the dough from the outer layers to the inner zone, the temperature remains at a lower level in the middle of the loaf. Furthermore, expanded carbon dioxide and water vapor act as insulating agent preventing high temperatures of bread crumb ([Mondal and Datta, 2008]). The temperature gradient in the outer layer of the loaf reaches from over 200 to 120 °C. In the middle, the temperature remains at around 100 °C, which is why water evaporates mainly in the outer layer of the loaf. Due to diffusion processes, water content can even be higher in the fresh baked bread crumb than in the dough ([Belitz et al., 2009]).

1.1.4 Coeliac disease causing a challenge for bread making

Coeliac disease (CD) is an immune-mediated enteropathy caused by a permanent intolerance of the small bowel mucosa for dietary gluten in genetically susceptible individuals ([Trier, 1991]). It affects about 1 % of the population ([Green and Jabri, 2003], [West et al., 2003], [Catassi and Fasano, 2008]) and is associated with gastrointestinal symptoms like villous atrophy (Figure 1.3), hypertrophy of crypts ([Collin, 2002]) and

malabsorption of nutrients ([Green and Cellier, 2007]), e. g. iron deficiency. Symptoms range from typical intestinal features (chronic diarrhea, weight loss, etc.) to atypical extraintestinal features like anemia, osteoporosis and neurological disturbances ([Catassi and Fasano, 2008]). Occasionally, silent forms are also discovered by serological screening ([Catassi and Fasano, 2008]). The risk of atypical features and misdiagnosis can be long-term complications like infertility, lymphoma and carcinoma of the small bowel ([Brousse and Meijer, 2005], [Catassi and Fasano, 2008]).

(a) Villous atrophy at diagnosis   **(b)** Recovery of villi after gluten-free diet
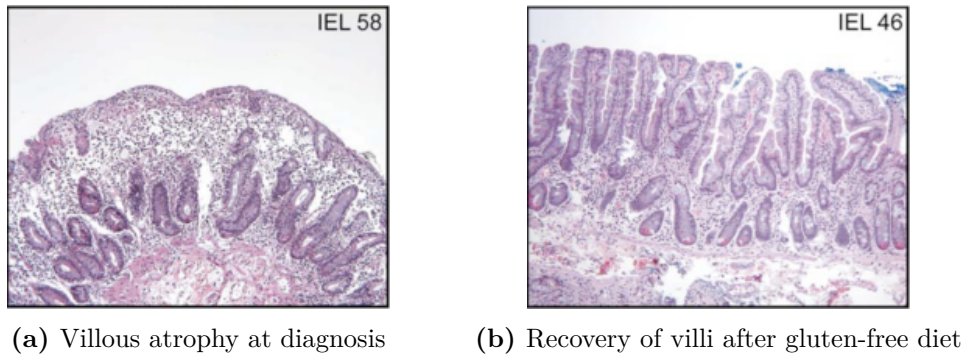
Figure 1.3 – Histology of intestinal mucosa of a patient suffering from coeliac disease. IEL: intraepithelial lymphocyte counts (after Arentz-Hansen et al. ([Arentz-Hansen et al., 2004])).

CD is induced by wheat, rye, spelt and barley proteins, whereas some patients are also sensitive towards oats ([Mäki et al., 2003], [Moroni et al., 2009]). Until today, the only effective treatment is a strict gluten free diet. However, gluten free cereals lack gluten proteins in even the smallest quantities ([Cauvain and Young, 1998]). This leads to a major technological challenge in bread baking, because the viscoelastic properties of gluten are necessary for elasticity, water and gas holding capacity of the dough and thus for bread structure (see Section 1.1.3). Gluten-free bread is usually made from starch, protein-based ingredients, hydrocolloids and a gluten-free base flour ([McCarthy et al., 2005]). Until today, a poor sensory and textural quality, low gas holding ability and a high staling rate of these breads are a challenge for food scientists ([Galle et al., 2012]).

1.1.5 The role of sodium chloride in bread making and for human health

Sodium chloride, which is commonly known as salt, is a useful food ingredient. In bakery products, it fulfills the following broad functions: flavor enhancement, control of yeast fermentation and therefore decrease in gas production, and improvement of dough handling properties ([Vetter, 1981], [Brady, 2002], [Hutton, 2002]). Secondary functions include to assist preservation and to reduce spoilage ([Brady, 2002]). A high sodium chloride content toughens the gluten proteins and improves the tolerance of dough towards mixing, which leads to a more stable and stiff dough ([Galal et al., 1978],

[DeSimone et al., 2013]). Moreover, salt addition slows heat-induced reactions such as starch gelatinisation and protein coagulation ([Angioloni and Dalla Rosa, 2005]).

However, there is clear evidence that current salt intake levels largely explain the high prevalence of hypertension due to sodium content ([Karppanen and Mervaala, 2006], [Lozada et al., 2007], [DeSimone et al., 2013], [Mohan and Prabhakaran, 2013]). It has been shown that a one-third decrease in the average salt intake is accompanied by a clear fall in the population average of blood pressure, and a 75 % to 80 % decrease in both stroke and coronary heart disease mortality ([Karppanen and Mervaala, 2006]). Therefore, a reduction of dietary sodium is widely recommended ([Gibson et al., 2000]). As 75 % of salt intake comes from processed foods and meals away from home, salt content in ready made food products should be significantly lowered ([Loria et al., 2001], [Kenney, 2006],[DeSimone et al., 2013], [Mohan and Prabhakaran, 2013]). At this, cereal products, and in particular bread as a commonly eaten food, are a major source of dietary sodium ([DeSimone et al., 2013],[Hager, 2013]).

Studies have shown that from a technological point of view, a 50 % or 75 % sodium reduction would be feasible without significant effects on rheological properties or bread-making performance. Takano and Kondou ([Takano and Kondou, 2002]) have shown that in white bread, even a complete replacement of sodium chloride by sodium gluconate or potassium gluconate did not cause a difference in loaf volume, shelf live or overall desirability. At this, an improvement of the taste quality in low-salt breads counts as the critical factor ([Lynch et al., 2009]). One approach to meet the sensorial challenge of reducing sodium is to employ salt replacers. These are ingredients who do not contain sodium but taste salty, e. g. potassium chloride or ammonium chloride ([Busch et al., 2013]). Another approach is the usage of salt boosters or salt enhancers, which do not taste salty themselves, but make the salt receptor more sensitive ([Busch et al., 2013]). For example, ingredients that increase umami notes are used as sodium reduction tools, because the flavour profile is rebalanced and therefore saltiness perception may be increased ([Busch et al., 2013]). In another study it was demonstrated, that several savory compounds of different chemical classes and with different sensory impact are able to provide a significant increase of perceived saltiness through multisensory interactions ([Batenburg and Velden, 2011]).

On the other hand, sodium reduction in bread can also be balanced by employing designed product structures attempting to optimize the delivery of salt to the taste buds ([Stieger, 2011], [Busch et al., 2013], [Konitzer et al., 2013]).

1.1.6 Quality requirements for modern bread and bread staling

Bread quality has different aspects for producers and consumers. For producers, good dough handling and baking properties and an efficient production process are paramount. These parameters are greatly influenced by the processing conditions, e. g. mixing, moulding, sheeting, fermentation and baking, and also by the quality and proportion of ingredients and additives ([Autio and Laurikainen, 1997]). For customers, the quality of the final product mainly influences their buying decision. Although the perception of bread quality is very personal and strongly depends on the type of bread, many

people would agree that bread should generally be tasty and not spoiled. The taste of bread can be improved by using sourdough, which has been fermented by a complex microflora of yeasts and lactic acid bacteria, and therefore confers specific flavour compounds. It has also been shown that some lactic acid bacteria produce antifungal compounds retarding bread spoilage ([Lavermicocca et al., 2003], [Gerez et al., 2009]). Furthermore, mould spoilage is influenced by the quality of raw materials. However, the most common requirement for bread is that it should retain all of the attributes which it had when it left the oven – it should remain fresh ([Bowles, 1996]). During bread staling, which relates to a loss of product freshness, the crust adsorbs moisture and looses its crispiness and glossyness. Aroma compounds evaporate or are entrapped, predominantly by amylose helices occuring in the crumb ([Belitz et al., 2009]). Furthermore, the crumb structure changes and becomes firm, brittle and dry. Although this phenomenon has been intensively studied for decades, a full scientific and technological understanding of bread staling has not been reached yet ([Chinachoti, 2003]). It is known that it is mainly caused by two separate subprocesses: the moisture transfer from crumb to crust and the starch retrogradation ([Pateras, 1998]). During the retrogradation of gelatinized starch, it is gradually transitioned from the amorphous state to a more ordered structure ([Courtin and Delcour, 2002], [BeMiller and Huber, 2008]). The solubility decreases and the structure becomes rigid and shrunken ([Katina, 2005]). These processes begin as soon as the baking process is finished and the product starts to cool ([BeMiller and Huber, 2008]), whereas amylose retrogradation is mainly completed when the product has cooled down to room temperature and stabilizes the crumb ([Goesaert et al., 2005], [Belitz et al., 2009]). The process is depicted in Figure 1.4. Retrogradation of amylopectin is believed to involve primarily association of its outer branches, leading to a double helix structure, and to require a much longer time ([BeMiller and Huber, 2008], [Goesaert et al., 2005], [Pateras, 1998]). This is the process during which the crumb looses its elasticity and becomes stale ([Belitz et al., 2009]).

The staling rate depends on several variables, such as the molecular ratio of amylose to amylopectin and structures of these molecules, product formulation, baking process, storage conditions and the level and type of other ingredients, like water, solutes (salts and sugar), flour hydrocolloids (pentosans), proteins and lipid materials ([Pateras, 1998], [Courtin and Delcour, 2002], [BeMiller and Huber, 2008], [Belitz et al., 2009]).

For example, starch retrogradation is accellerated at $4\,^\circ\mathrm{C}$ and retarded at $15\,^\circ\mathrm{C}$, whereas on the other hand, too high temperatures promote the growth of moulds ([Decock and Cappelle, 2005]).

A sufficient water availability is important to mobilize long polymer chain segments, as it acts as plasticizer ([Pateras, 1998]).

Bacterial α-amylase cleaves branched oligosaccharides from amylopectin and hinders the formation of crystalline structures ([Belitz et al., 2009]). Also the introduction of additional branch points into the amylose and amylopectin molecules by branching enzymes can reduce starch retrogradation. Furthermore, there exist hemicellulases, pentosanases and xylanases with an anti-staling activity, although their mechanism of action is not completely clear ([Haros et al., 2002]). As the optimum activity for many of these en-
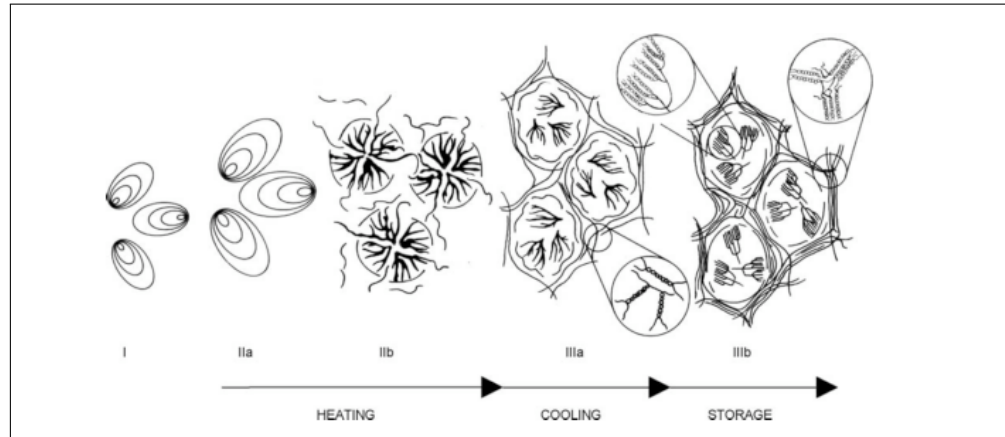
Figure 1.4 – Schematic representation of changes in a starch-water mixture. (I) Native starch granules; (II) gelatinisation during heating: (a) swelling, (b) amylose leaching and partial granule disruption, resulting in the formation of a starch paste; (III) retrogradation: (a) gelation/amylose retrogradation and formation of an amylose network during cooling, (b) amylopectin retrogradation and formation of ordered or crystalline amylopectin molecules during storage (from [Goesaert et al., 2005]).

zymes is below 55 °C, they can easily be used in breadmaking, where usually a proving stage at room temperature is applied ([Roller and Dea, 1992]).

Emulsifiers like distilled, saturated monoglycerides, sodium stearoyl-2-lactylate (SSL) and diacetyl tartaric acid esters of monoglycerides (DATA esters) form insoluble inclusion complexes with amylose or interact with amylopectin and/or protein and therefore decrease the staling rate ([Goesaert et al., 2005], [Pateras, 1998]).

Besides, sugar addition to starch-based baked goods has been shown to reduce staling, probably by locally increasing the glass transition temperature and thereby suppressing the recrystallization of polymer molecules ([I'Anson et al., 1990], [Levine and Slade, 1990]).

On the other hand, a higher salt level can maintain bread freshness for a longer time by lowering the water activity and therefore increasing the energy necessary for chemical and physical reactions ([Angioloni and Dalla Rosa, 2005]).

Furthermore, addition of hydrocolloids, such as carboxymethylcellulose, hydroxypropylmethylcellulose, sodium alginate, xanthan, κ-carrageenan and exopolysaccharides, e. g. fructans from acetic acid bacteria, have been identified to delay staling ([Kohajdová et al., 2009], [Jakob et al., 2012b]). Although the mechanism of action of different hydrocolloids may vary due to their different origin and chemical structure, they mainly act through their high water retention capacity ([Guarda et al., 2004]). As exopolysaccharides (EPS) are also produced by some sourdough lactic acid bacteria, sourdough addition can be a helpful measure for a longer shelf-life of bread and a substitute for additives like xanthan and guar gum ([Tieking et al., 2003]). Additionally, EPS produced in situ have been identified as more effective than externally added EPS ([Arendt et al., 2007]).

1.2 Bread dough: sourdough technique

Sourdough has been used to leaven and acidify wheat and rye breads since ancient times. Its usage has beneficial effects not only on the flavour, texture and shelf life, but also on the nutritional properties of bread and other baked goods ([Katina et al., 2005], [Gänzle et al., 2007], [Guerzoni et al., 2011]). By sourdough application, the whole grain or fractions of cereal grain can be modified and the bran fraction, which is very rich in dietary fibre, minerals, vitamins and many phytochemicals can be explored for bread baking ([Katina et al., 2005], [Chavan and Chavan, 2011]). This way, levels of various bioactive compounds and mineral bioavailability are increased and the glycemic index of baked products is decreased. The mouthfeel and palatability of wholemeal bread, other cereals like rye and oat and even gluten-free products are improved ([Katina et al., 2005]), whereas antinutritional compounds like phytate and also allergens are decreased ([Guerzoni et al., 2011]). Furthermore, the level of preservatives can often be reduced by applying sourdough technology, because many sourdough bacteria have been shown to produce antibacterial and antimold compounds ([Chavan and Chavan, 2011]).

Different types of sourdough are distinguished dependent on their way of spontaneous or intended development, the fermentation parameters and their usage. Still, the underlying microbial activity and its associated dough-ripening processes are the same.

1.2.1 Different types of sourdough

Sourdough can either develop spontaneously by leaving a dough made of flour and water for a certain period, or by inoculating it with starter preparations, whose use ensures a more constant product quality. After fermenting the predough, in which the most competitive organisms (lactic acid bacteria and yeasts) have become dominant, it can be added to a final bread dough and/or be used as an inoculum for a new fermentation. The traditional process of inoculating a new dough with a readily fermented product is called backslopping. These produced doughs are termed Type I sourdoughs ([Böcker et al., 1995]). After a few propagation steps of a new fermented dough, a characteristic microflora develops. Specified ripening conditions, e. g. temperature, play a great role in maintaining the desired microflora over the different refreshments ([Spicher and Stephan, 1999]). In bakery practice, Type I sourdoughs can be sustained like this over many years or even decades ([Spicher and Schröder, 1978], [Ehrmann et al., 2011]). During the industrialization of baking procedure, two other types of sourdough have developed: Type II sourdoughs relate to an industrial type of sourdough, where adapted strains are used to start a fermentation. Type III sourdoughs can be dried, e. g. by spray or drum drying, and are therefore preferred by industrial bakeries, because variations in end products are eliminated ([Böcker et al., 1995]).

1.2.2 Microbial composition of sourdough

Besides the inoculum, the microbial composition of sourdough is determined by endogenous and exogenous factors. Endogenous factors include the type and concentration of carbohydrates, nitrogen sources, lipids, free fatty acids, the availability of minerals and enzyme activities ([Hammes et al., 2005]). Exogenous factors are mainly process parameters like dough yield and water activity, oxygen availability, fermentation time and number of propagation steps ([Hammes et al., 2005]). During a certain time of fermentation, the sourdough microflora develops, which mainly consists of lactic acid bacteria (LAB) and yeasts, their ratio being 100:1 ([Ottogalli et al., 1996]). In flour, an aerobic mesophilic bacterial count between 10^5 and 10^7 CFU/g is present ([Brandt and Gänzle, 2006]), whereas in mature sourdoughs, LAB reach up to $3 \cdot 10^9$ CFU/g dough and yeasts between 10^6 and $5 \cdot 10^7$ CFU/g dough ([Hammes et al., 2005]). Heterofermentative LAB of the genus *Lactobacillus* (*L.*), which are able to produce lactic acid, acetic acid, ethanol and carbon dioxide dominate compared to homofermentative LAB, whose main fermentation product is lactic acid ([De Vuyst et al., 2002], [Corsetti et al., 2003]). Less frequently found are *Leuconostoc*, *Weissella* and *Pediococcus* species. From wheat and rye sourdough, the strains most often isolated are *L. sanfranciscensis, L. plantarum, L. pontis, L. acidophilus, L. alimentarius, L. fermentum, L. curvatus, L. panis* and *L. brevis* ([De Vuyst and Vancanneyt, 2007]). Among the yeasts, the genera *Saccharomyces* and *Candida* are most frequently isolated ([De Vuyst and Neysens, 2005]).

1.2.3 Functions of sourdough

Despite the many positive functions of sourdough usage on bread products, not all details about its mechanism of action have been fully understood yet ([Arendt et al., 2007]). This relates to the microbial ecology and physiology of sourdough ([Gobbetti, 1998]), as well as to its influence on the structure of dough and bread. The main processes involved in sourdough fermentation are acid formation, proteolysis and the degradation of carbon compounds ([Martinez-Anaya, 1996]), leading to beneficial effects on aroma, taste, texture (volume, evenness and colour of bread crumb, character of crust, grain of bread), shelf life and nutritional properties of baked goods ([Gänzle et al., 2007], [Plessas et al., 2007],[Rehman et al., 2007]). During sourdough fermentation, the arabinoxylans from wheat (and rye), which have a higher water solubility than oat β-glucans ([Belitz et al., 2009]), are degraded into small, more soluble chains ([Escrivá and Martinez-Anaya, 2000]).

Acid production

Based on enzyme activity and bacterial metabolism, organic acids are produced during sourdough fermentation. These lower the pH value of typical wheat sourdoughs to around $3.6 - 3.8$ ([Brummer and Lorenz, 1991]), whereas $600 - 800 \, \text{mg}/100 \, \text{g}$ sourdough of lactic acid and $80 - 160 \, \text{mg}/100 \, \text{g}$ sourdough of acetic acid are usually detectable ([Barber et al., 1992], [Hansen and Hansen, 1994]). By acidification

below pH 4.0, the gluten network in the dough is altered, because a sizable positive net charge occurs. Gluten swells and mild hydrolysis of starch takes place ([Zeleny, 1947], [Axford et al., 1979], [Barber et al., 1992]). In wheat dough, water uptake is increased by adding organic acids in the absence of salt ([Tanaka et al., 1967], [Maher Galal et al., 1978]). The increased electrostatic repulsion promotes the solubilisation of proteins and therefore prevents the formation of new bonds ([Clarke et al., 2004]). The reduction of disulfide bonds potentially enables greater access by proteolytic enzymes and therefore allows more efficient proteolysis ([Thiele et al., 2002]), whereby the increased protease and amylase activity and lead to a reduction in staling ([Arendt et al., 2007]). A higher acetate concentration is advantageous for the aroma profile and the microbial stability of sourdough breads due to its antifungal properties ([Schieberle, 1996], [Kaditzky and Vogel, 2008]). In rye sourdough, acidification is necessary to promote the solubilisation of pentosans and therefore enhance water binding in the dough. Besides this, the very high alpha-amylase activity is inhibited ([De Vuyst and Neysens, 2005]).

Increased acidity has further been determined as an effective means to limit the germination of *Bacillus* spores, which lead to rope spoilage. In this respect, the most effective acids are propionic acid and acetic acid ([Rosenquist and Hansen, 1998]).

As another result of acidification, the glycemic index (GI) of sourdough enriched products is lowered, which is nutritionally interesting as a low GI is associated with decreased risk of diabetes and cardiovascular diseases ([Katina et al., 2005], [De Angelis et al., 2009]).

Acidification is mainly regulated by the amount of fermentable carbohydrates ([Katina, 2005]), whereas white flours contain only $1.55 - 1.84\%$ of free sugars. However, during mixing, the endogenous amylase activity leads to a rapid increase of initial maltose levels ([Martinez-Anaya, 2003]). The amylase activity of wheat flour depends on the extraction rate and flour quality. The bran fraction has the highest activity. For this reason, wholemeal flour, which has a higher bran content, shows higher enzyme activities. In general, a higher water content, the usage of wholemeal flour and a higher temperature improve the acidification of sourdoughs ([Brummer and Lorenz, 1991]).

Another way to enhance the production of acetic acid, and therefore acidification, is to increase oxygen availability. This is because it stimulates heterofermetative lactobacilli and shifts the metabolic pathway from ethanol to an acetate route ([Martinez-Anaya, 2003]).

However it should also be considered that high acid concentrations can cause off-flavor and compromise crumb structure ([Kaditzky and Vogel, 2008]).

Proteolysis

The degradation of various cereal proteins to peptides and amino acids in the sourdough system is mainly caused by flour enzymes, followed by microbial enzymes of flour and sourdough bacteria ([Thiele et al., 2002]). Proteolysis has contraproductive effects: On the one hand, precursors for enhanced aroma formation are provided, but on the other hand, structure formation is impaired.

Prieto et al. ([Prieto et al., 1990]) have shown that the content of total free amino acids increases by 64 % during 15 min of mixing an unfermented wheat dough. As cereal proteinases, mainly aspartic proteinases and carboxypeptidases from wheat and rye flours, have been shown to be more active at low pH values (pH 4−6), their activity is increased during sourdough fermentation ([Wu and Hoseney, 1989], [Thiele et al., 2002], [Chavan and Chavan, 2011]). In this relation, LAB contribute to proteolysis by creating the optimal pH. Furthermore, they add their own enzymes in a strain-specific manner ([Spicher and Nierle, 1988]): Homo- and heterofermentative LAB show qualitative and quantitative differences in sulphur-containing, cyclic and hydroxy amino acids.

The proteolytic degradation observed during sourdough fermentation includes cereal prolamins and the gluten macropolymer, which is solubilised and depolymerised ([Thiele et al., 2004]). Therefore the rheological properties of doughs and the texture of final breads are changed. A decreased resistance to extension, a large reduction of elasticity and dough firmness, an increase in extendability and degree of softening has been observed for fermented doughs ([Di Cagno et al., 2002], [Clarke et al., 2004]). Further, the dough viscosity is decreased ([Kawamura and Yonezawa, 1982]) and weak and sticky doughs can be a result ([Katina, 2005]). As the gluten proteins have a great impact on gas holding capacity of the dough, the impact of sourdough addition on gas formation must also be considered ([Arendt et al., 2007]). However, despite the gluten depolymerization, sourdough enriched breads lead to larger loaf volumes compared to straight dough process ([Corsetti et al., 1998]). The proteolysis of gluten subunits has further been proposed to prevent staling ([Corsetti et al., 1998]).

Besides, a chance of gluten detoxification and the reduction of certain allergen compounds by proteolysis due to selected LAB exists for future foods ([De Angelis et al., 2006], [Arendt et al., 2007],[Guerzoni et al., 2011]).

Another positive effect of proteolysis is that the increased amino acid content provides precursors for the formation of aroma volatiles by the Maillard reaction during baking. Further, the amino acids resulting from proteolytic degradation can be used as flavour precursor compounds by microorganisms ([Gobbetti et al., 1995], [Thiele et al., 2002]). This might be the main reason why sourdough usage increases the amount of volatile compounds in bread and leads to a broader range of aroma molecules compared to the reference produced in a direct process ([Decock and Cappelle, 2005]). However, yeasts present during the dough fermentation can reduce the free amino acid concentration through consumption ([Thiele et al., 2002]). Therefore an accumulation of amino acids must exceed the demand of microbes to show positive effects on bread taste.

Using wholemeal flour has a positive influence on amino acid liberation, probably due to a higher activity of cereal aspartylic proteinase and other cereal proteases in the outer layers of the cereal kernel ([Loponen et al., 2004]). Furthermore, the fermentation conditions, e. g. dough yield and temperature, influence the proteolytic process ([Spicher and Nierle, 1988]).

Leavening

The leavening effects of sourdough are attributed to the degradation of sugars present in the flour. High glucose concentrations lead to increased leavening effects by supporting the gas production, but depress maltose consumption ([Korakli, 2002]). Yeast cell concentration and the type of yeast mainly determine gas production rates ([Rehman et al., 2006]). The introduction of *Saccharomyces cerevisiae* as a superior leavening agent instead of sourdough or brewing yeast was one of the major factors enabling industrial wheat bread production in the 19th century ([Katina, 2005], [Rehman et al., 2006]).

However, yeast leavening and carbon dioxide production are also influenced by LAB. For example, in rye sourdough, a major contribution to leavening can be made by LAB alone ([Nout and Creemers-Molenaar, 1987], [Gänzle et al., 1997]), which is why Type I sourdoughs do not necessarily require the addition of baker's yeast as leavening agent ([Chavan and Chavan, 2011]). Another study showed that the associative growth of *Saccharomyces* (*S.*) *cerevisiae* and *L. sanfranciscensis* is accelerated threefold until a comparable carbon dioxide production by the yeasts is reached ([Gobbetti, 1998]). It was also observed that associative growth of *L. plantarum* and *S. cerevisiae* does not only increase the carbon dioxide production, but also improves the gas retention capacity of the dough. This is attributed to the lactic acid produced by such facultatively heterofermentative LAB. On the other hand, an increased acetic acid production which can be promoted by the addition of fructose (6 g/kg) slightly affects carbon dioxide production and increases the gas production rate ([Gobbetti, 1998]). A higher acidity of wheat sourdough breads compared to wheat yeast breads has been identified as the reason for a higher gas cell-total area and a better developed gluten network effectively retaining the carbon dioxide ([De Angelis et al., 2009]).

Exopolysaccharide formation

Among sourdough LAB, the production of exopolysaccharides (EPS) from sucrose is widespread ([Tieking et al., 2003]). Although these sugar polynoms are sometimes associated with spoilage of foods, e.g. tube closures in the sugar industry, slimes in spoiled soft drinks or the formation of biofilms, they also exhibit interesting features for the food industry ([Korakli and Vogel, 2006]). In bread, they are known to enhance rheological properties of dough and texture, nutritional value, shelf life and machinability of wheat, rye and also gluten-free bread ([Tieking et al., 2005], [Galle et al., 2010], [Rühmkorf et al., 2012]). These positive effects may be caused by the hydrocolloid character of EPS, which causes changes in water absorption ([Chavan and Chavan, 2011], [Jakob et al., 2013]). Therefore they may replace plant and microbial hydrocolloids, like guar and xanthan, currently used to increase water binding capacity of baked goods. The higher water content improves starch gelatinization and retards staling ([Katina, 2005]). As EPS are not digested by pancreatic enzymes, they are available for metabolism by intestinal microorganisms ([Tieking et al., 2003], [Arendt et al., 2007], [Waldherr et al., 2008], [Chavan and Chavan, 2011]). Korakli et al. ([Korakli, 2002])

showed that up to 66 % of levan from *L. sanfranciscensis* is retained during sour-dough fermentation and can be determined in the final bread. The levan produced by *L. sanfranciscensis* was digested by bifidobacteria and therefore had a prebiotic effect ([Korakli et al., 2002]). An enrichment of *Bifidobacterium spp.* when fed with levan-type EPS from *L. sanfranciscensis* has been confirmed ([Bello et al., 2001]).

There exist two different classes of exopolysaccharides: Heteropolysaccharides (HePS) have two to eight (ir)regularly repeating units composed of glucose, fructose, galactose and rhamnose ([De Vuyst and Degeest, 1999]) and are synthesized from intracellular sugar nucleotide precursors. They are formed in small amounts of up to 2 g/l. Homopolysaccharides (HoPS) consist of only one repeating unit and are synthesized extracellularly ([Tieking et al., 2003]). They are produced from sucrose and can be found in large amounts of up to 40 g/l ([Korakli and Vogel, 2006]). HoPS can be of the fructan- or glucan-type, containing either D-fructose or D-glucose. During their formation, the osidic bond of sucrose is cleaved either by a fructosyl- or glycosyltransferase and the energy set free enables the efficient transfer of the sugar residue via a covalent glycosyl-enzyme intermediate ([Monsan et al., 2001], [Korakli and Vogel, 2006]). The key reactions involved in these metabolic pathways are shown in Figure 1.5.
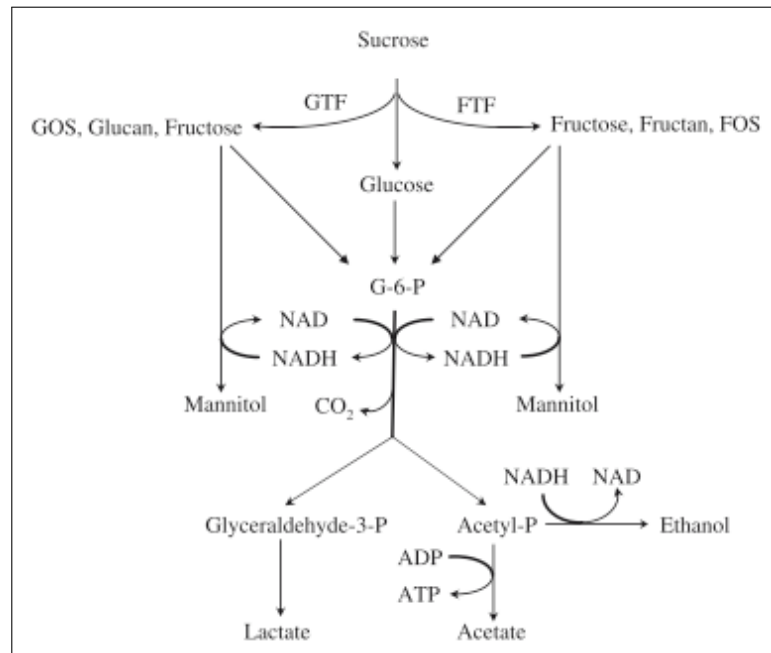
Figure 1.5 – Sucrose metabolism via FTF or GTF in heterofermentative LAB. If the liberated monosugar is used as an electron acceptor for NAD regeneration, mannitol is formed and an additional ATP is generated via the acetate kinase reaction (from Korakli and Vogel ([Korakli and Vogel, 2006])).

Examples for glucans are dextran, mutan and alternan. Dextran contains α-1,6 linked glycosyl-units. Fructans are distinguished into levan and inulin which contain β-2,6 and

β-2,1 osidic bonds, respectively ([Monsan et al., 2001]).

Most bacteria produce either HoPS or HePS. Only few, such as *Serratia marcescens*, *Aeromonas salominicida* and *Kozakia baliensis*, have been reported to produce two or more different EPS ([Kwon et al., 1994], [Moonmangmee et al., 2008]).

The functions for the microorganisms ascribed to EPS are mainly of a protective nature, as they can serve as defence against desiccation and predation by protozoans or affect the diffusion properties to provide inaccessibility to antibiotics ([Kumar et al., 2007]). EPS also play a role in the formation of cell aggregates, initiation of flocculation and biofilm formation. As most microorganisms are not able to degrade the EPS they produce, these polysaccharides are probably not formed as a nutrient reserve ([Cerning, 1990]). On the other hand, EPS formation causes a decrease in sucrose concentration, which could have osmotic and energetic advantages for the producing bacteria ([Korakli and Vogel, 2006]).

Although the application of EPS produced by LAB is very common in the dairy industry, the research on EPS in sourdoughs and their impact on bread quality is very limited ([Arendt et al., 2007], [Ketabi et al., 2008]). However, about 20 % of over one hundred *Lactobacillus* strains screened by Tieking et al. ([Tieking et al., 2003]) were found to produce EPS from sucrose. A typical sourdough consists of two to five strains, which is why it seems likely that EPS are produced during any given sourdough fermentation and therefore contribute to the positive effects of sourdough.

1.3 Acetic acid bacteria with potential for sourdough applications

The first systematic attempts to study acetic acid bacteria (AAB) were carried out in 1868 by Louis Pasteur, who recognized that specific microorganisms were necessary to produce vinegar. He named these bacteria, which he associated with the conversion of alcohol to acetic acid, *Mycoderma aceti* ([Pasteur, 1864]). About 30 years later, in 1898, the first species belonging to the acetic acid bacteria has been characterized with *Acetobacter aceti* ([Beijerinck, 1898]).

Acetic acid bacteria are gram-negative, obligate aerobic and ellipsoidal to rod-shaped bacteria ([Cleenwerck and De Vos, 2008]). Their common feature is the incomplete oxidation of a wide range of alcohols and sugars via alternative pathways to organic acids, aldehydes and ketones, which are excreted near-quantitatively into the surrounding media ([Deppenmeier et al., 2002]). This makes them a source of valuable compounds like vitamin C, acetic acid, cellulose, gluconic acid, L-ribose and dihydroxyacetone (DHA), which are increasingly important in dermatology, medicine and cosmetics ([Kim et al., 1996], [Stasiak and Blazejak, 2009]). Some strains are also known to fix nitrogen ([Pedraza, 2008]) and/or to produce exopolysaccharides ([Velázquez-Hernández et al., 2011], [Jakob et al., 2012a], [Jakob et al., 2012b], [Jakob et al., 2013]). Although acetic acid bacteria, namely *Gluconobacter*, were heavily investigated for their capability of incomplete oxidation, only little is known about their EPS metabolism.

Acetic acid bacteria are widespread in nature ([Cleenwerck and De Vos, 2008]) and can be isolated from vinegar ([De Vero and Giudici, 2008]), sugar, flowers, fruits ([Kersters and Vancanneyt, 2005]), coffee plants ([Jimenez-Salgado et al., 1997]), Kombucha, mealy bugs from sugar cane ([Franke et al., 2000]), ragi, palm brown sugar ([Lisdiyanti et al., 2003]), coconut toddy ([Kadere et al., 2008]), pollen ([Jojima et al., 2004]) and honey bees ([Lambert et al., 1981]). Until today, the taxonomy of AAB has not been fully established due to limited knowledge of the AAB phylogenesis and difficulties in isolating and preserving these bacterial strains ([De Vero and Giudici, 2008]). At the moment, the family *Acetobacteraceae* includes 10 genera ([Pedraza, 2008]), among which *Gluconobacter (G.)*, *Neoasaia (N.)* and *Kozakia (K.)* are the most recognized ones ([Stasiak and Blazejak, 2009]).

1.3.1 The genus *Gluconobacter*

Members of the genus *Gluconobacter* are distinguished from *Acetobacter* and most *Gluconacetobacter* through their inability to oxidize acetate and lactate to carbon dioxide ([De Ley and Swings, 1984]). Furthermore, *Gluconobacter* use ubiquinone-10 instead of ubiquinone-9 as electron carrier in their cytoplasmic membranes ([Yamada et al., 1997]). If motile, they exhibit polar flagella ([Asai, 1968]). The species of the genus *Gluconobacter* were phylogenetically divided into two groups. The first group corresponds to the *G. oxydans* group and includes *G. oxydans* and *G. albidus* and the second group corresponds to the *G. cerinus* group, relating to *G. cerinus*, *G. frateurii* and *G. thailandicus* ([Yamada and Yukphan, 2008]).

Gluconobacter can generally be maintained on minimal media, which contain sugar or polyols and yeast extract, ammonium ions or casitone as nitrogen source, or in highly concentrated sugar solutions. However, the organisms require pantothenic acid, para-aminobenzoic acid and nicotinic acid ([De Ley and Swings, 1984]). Maximum biomass is produced with D-mannitol and D-sorbitol. Good growth was also achieved on glycerol and D-fructose and with D-glucose under pH control ([De Ley and Swings, 1984]), whereas even in a complete medium, *Gluconobacter* are incapable of rapid growth ([Macauley et al., 2001]). They are highly sensitive towards dissolved oxygen concentration (DO), which is why a high aeration rate stimulates growth and metabolic activities ([Macauley et al., 2001]). However, above 30 % saturation, acid production is not affected by DO ([Buse et al., 1992]).

Gluconobacter prefer temperatures between $25 - 30\,°C$ and pH values between $5.5 - 6$, although some species are able to grow below pH 3.7 ([Gupta et al., 2001], [Macauley et al., 2001], [Deppenmeier et al., 2002]). For *G. oxydans* 621H, for example, good growth rates at pH 2.5 have been described in complex medium ([Olijve, 1978]). In contrast, *Gluconobacter* strains isolated from coconut toddy were able to grow at 15, 25 and 30 °C and at pH 7.0 and 4.5 ([Kadere et al., 2008]). No growth was observed at 45 °C and at the pH values 2.5, 8.0 and 8.5. For pH 3.0 and 40 °C, growth was strain-dependent ([Kadere et al., 2008]).

Gluconobacter are not pathogenic towards man or animals ([Gupta et al., 2001]). Although they are applied in a wide range of biotechnological applications, many of

their metabolic pathways are yet not fully understood. It is known that the oxidation of various sugars and polyols takes place via alternative pathways, mostly catalyzed by membrane-bound dehydrogenases which are connected to the respiratory chain. As the reactive centers of these enzymes are oriented towards the periplasmic space, transport of substrates and products across the cell membrane is not necessary ([Deppenmeier and Ehrenreich, 2009]). This enables rapid accumulation of products in the surrounding medium ([Deppenmeier et al., 2002]). One of the pathways used relates to the direct oxidation of sugars, steroids and aliphatic and cyclic alcohols, resulting in almost quantitative yields of oxidation product. The second pathway includes initial oxidation of polyols and reduction of ketogluconates and other ketosugars by cytosolic NAD(P)-dependent oxidoreductases ([Klasen et al., 1995]). This is followed by phosphorylation to glucose-6-phosphate by intracellular dehydrogenases and oxidation via the pentose phosphate pathway or the Entner-Doudoroff-pathway ([De Ley and Swings, 1984], [Prust, 2004]). The enzymes of the pentose phosphate pathway have their optimum at pH 7.0 and are inactivated at pH 4.5 ([Ohrem and Voss, 1995]). Therefore, for the production of biotechnological compounds a lower pH is advantageous, as almost all CO_2 produced is set free via the pentose phosphate pathway, although nutrient requirements increase at low pH values ([Olijve and Kok, 1979a]). This is not applicable to other enzymes, such as the membrane bound dehydrogenase enzymes, which have different operational optimums ([Yamada et al., 1979]). Prust ([Prust, 2004]) has shown that *Gluconobacter* lack phosphofructokinase, which is the key enzyme of the glycolysis, as well as succinyl-CoA synthetase and succinic dehydrogenase, both are necessary to perform the citric acid cycle. As the succinic dehydrogenase belongs to complex II of the respiratory chain, the remaining enzymes can only be used to produce intermediates for anabolism, e. g. amino acids. Therefore, *Gluconobacter* possess no essential amino acids ([Prust, 2004]).

As an important example for reactions catalyzed by specific dehydrogenases, the production of gluconate and ketogluconates from glucose should be mentioned. The oxidation of glucose to gluconate is driven by glucose dehydrogenase (GDH) bound in the membrane. The GDH and 2-ketogluconate dehydrogenase can then further oxidize gluconate to 2-ketogluconate, 2,5-diketogluconate and 5-ketogluconate ([Prust, 2004], [Klasen et al., 1995], [Deppenmeier and Ehrenreich, 2009]). For *G. oxidans* it was shown that the oxidation of gluconate takes place only under controlled pH at pH 5.5 and when glucose has almost been exhausted ([Weenk et al., 1984]). Gluconate and 2-ketogluconate can also be taken up into the cytoplasma, where the latter component is reduced to gluconate. After phosphorylation, the resulting 6-phosphogluconate is further metabolized.

Some *Gluconobacter* species are able to produce levans from sucrose, which are promising functional compounds for food applications ([Jakob et al., 2012a]). It was shown that depending on the molecular structure, isolated levans from *Gluconobacter* lead to increased volume and a clear softening effect of wheat breads, as well as retarded staling within one week storage ([Jakob et al., 2012b], [Jakob et al., 2013]). In Figure 1.6, the proposed structure of levans isolated from *G. frateurii*, *G. cerinus*, *N. chiangmaiensis* and *K. baliensis* is shown.
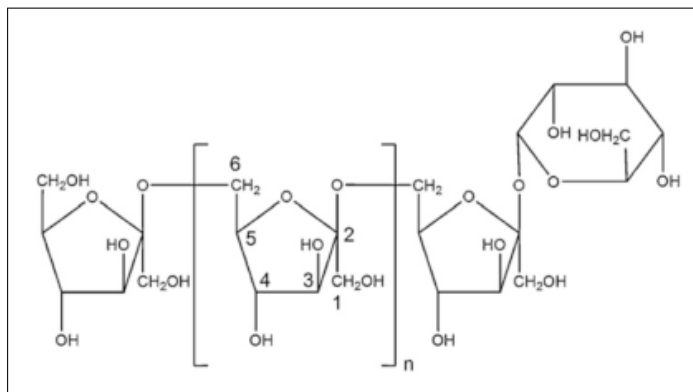
Figure 1.6 – Molecular structure of levans isolated from *G. frateurii*, *G. cerinus*, *N. chiangmaiensis* and *K. baliensis* as proposed by Jakob et al. ([Jakob et al., 2013])

1.3.2 The genus *Neoasaia*

The genus *Neoasaia (N.)* is one of the six monotypic genera of the family *Acetobacteraceae*, in which only one species has been described so far ([Yamada and Yukphan, 2008]). The first isolate *N. chiangmaiensis*, which was found on a flower of red ginger in Thailand by Yukphan et al. ([Yukphan et al., 2005]), was proposed as the type strain of this genus.

Members of the genus *Neoasaia* are characterized as follows: Cells are non-motile and form pink, shiny and smooth colonies. They are able to produce acetic acid from ethanol, but like *Gluconobacter*, they cannot further oxidize it to carbon dioxide and water ([Cleenwerck and De Vos, 2008]). Yukphan et al. ([Yukphan et al., 2005]) found no oxidation of acetate and lactate, but growth on glutamate agar, mannitol agar, 30 % glucose (w/v) and 0.35 % acetic acid. *Neoasaia* is neither able to grow in the presence of 1.0 % potassium nitrate (w/v), nor Methanol and was not found to produce a water-soluble brown pigment, which distinguishes it from *Swaminathania* and *Gluconacetobacter*. The production of dihydroxyaceton from glycerol is weak. On D-mannitol, ammoniac nitrogen is weakly assimilated, but not on D-glucose and ethanol. Acid is produced from D-xylose, *meso*-erythritol, glycerol, melibiose, sucrose, raffinose and ethanol. For D-arabinose, L-rhamnose, D-mannitol and dulcitol, acid production was weak and for D-fructose and D-sorbitol, it was delayed ([Yukphan et al., 2005]).

In opposite to Yukphan et al. ([Yukphan et al., 2005]), *N. chiangmaiensis* was identified as a producer of exopolysaccharides when grown in sucrose-enriched medium by Jakob et al. ([Jakob et al., 2013]): They identified a levan (Figure 1.6) with two different molecular weight fractions, one ranging from $100-575$ MDa (about 20 % of molecules) and one in the range of $22-98$ MDa (about 75 % of molecules). The isolated levan lead to increased volume and a clear softening effect on fresh wheat breads and was able to retard bread staling during one week of bread storage ([Jakob et al., 2012b]).

1.3.3 The genus *Kozakia*

The genus *Kozakia (K.)* is another member of the six monotypic genera belonging to the family *Acetobacteraceae* ([Yamada and Yukphan, 2008]), as no additional strains have been found until today. It has first been described by Lisdiyanti et al. ([Lisdiyanti et al., 2002]), who isolated four unknown acetic acid bacterial strains from palm brown sugar and ragi collected in Bali and Yogyakarta, Indonesia. Since then, more isolates have been found only on sapodilla fruits collected in Thailand by Kommanee et al. ([Kommanee et al., 2009]). As type strain, *K. baliensis* DSM 14400 was proposed ([Lisdiyanti et al., 2002]).

Lisdiyanti et al. ([Lisdiyanti et al., 2002]) described the genus *Kozakia* as follows: Cells are strictly aerobic, catalase-positve, oxidase-negative, gram-negative and non-pigmented. They are non-motile and rod-shaped, whereas the size of a single cell lays between $0.6 - 0.8$ by $2.0 - 3.0\,\mu$m. *K. baliensis* does not produce water-soluble brown pigments from D-glucose or on calcium carbonate containing agar slants and is able to grow at pH 3.0 and 30 °C. The strain does not produce cellulose, gelatinase, hydrogen sulphide, indole or ammonia from L-arginine and does not reduce nitrate ([Lisdiyanti et al., 2002]). From ethanol, acetic acid is produced and in opposite to *Gluconobacter*, a weak activity for the oxidation of acetate and lactate to carbon dioxide and water was discovered ([Cleenwerck and De Vos, 2008]). On mannitol medium with vitamins, but not on ethanol, ammonium sulfate is assimilated, whereas some isolates also assimilate it on glucose medium with vitamins. *Kozakia* cells can grow in the presence of 0.35 % acetic acid, relating to pH 3.5. On mannitol agar, growth occured, whereas on glutamate agar or on 30 % D-glucose, no growth was observed. *K. baliensis* produces dihydroxyacetone from glycerol. Furthermore, D-gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate, but not 2,5-diketo-D-gluconate are set free from D-glucose. The strain is able to produce acid from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, melibiose, raffinose, *meso*-erythritol, glycerol and ethanol, whereas no acid production was discovered for L-rhamnose, D-fructose, L-sorbose, lactose, D-mannitol, D-sorbitol or dulcitol as carbon source. From D-arabinose and sucrose, the acid production depended on the strain. Methanol is not used. From sucrose and D-fructose, a large amount of levan-like mucous substance is excreted into the surrounding medium ([Yamada and Yukphan, 2008], [Kommanee et al., 2009]).

Jakob et al. ([Jakob et al., 2013]) identified the levan from *K. baliensis*, whose proposed structure is shown in Figure 1.6. Having a very high molecular weight of 2466 MDa, it is the largest fructan polymer reported so far. The isolated, lyophilised levan lead to increased volume and a clear softening effect on fresh wheat breads and was able to delay bread staling during one week storage ([Jakob et al., 2012b]).

Moonmangmee et al. ([Moonmangmee et al., 2008]) found that *K. baliensis* also produces 3.72 g/l of a heteropolysaccharide when grown in a medium containing 1 % glycerol as carbon source. It consists of glucose and galactose and has an apparent molecular mass of higher than 700 kDa.

1.4 Water kefir: a source for acetic acid bacteria and novel hydrocolloids

The term water kefir relates to a home-made beverage prepared by fermenting a sucrose solution with dried figs and lemon pieces upon addition of kefir grains. After one or two days of fermentation at room temperature, a cloudy, carbonated and straw coloured drink has developed, which is acidic, low in sugar and slightly alcoholic.

The water kefir grains host a variety of different bacteria and yeasts embedded in an exopolysaccharide-matrix forming transparent, mucilaginous, jelly crystals (microstructure of grains shown in Figure 1.7). The most abundant bacteriae belong to *Lactobacillus*, *Leuconostoc* and *Acetobacter* ([Gulitz et al., 2011]). Furthermore, yeasts of the species *Saccharomyces*, *Lanchancea*, *Hanseniaospora* and *Zygotorulaspora* have been identified ([Gulitz et al., 2011]). These microbiota makes water kefir a valuable source of bacterial strains with interesting physiological properties, e. g. EPS-production or 4-keto-D-arabonate formation ([Adachi et al., 2010], [Jakob et al., 2012a], [Jakob et al., 2013]).

The polysaccharide matrix of water kefir grains is described to contain dextran, an α-1,6 linked glucose polymer ([Pidoux, 1989], [Galli et al., 1995]). During kefir production, grains assault as waste as the consortium grows.
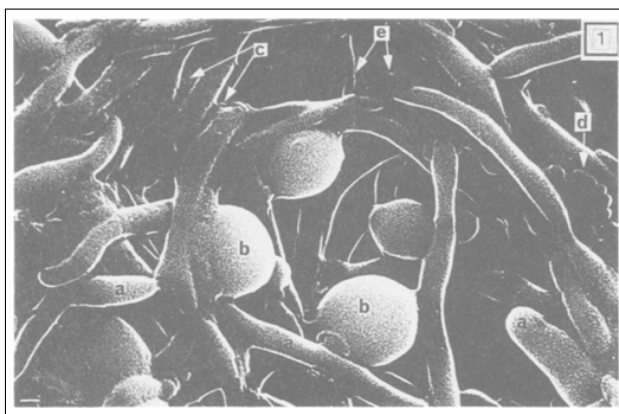
Figure 1.7 – Scanning electron microscopy of the outer part of a sugary kefir grain. a) network of pseudomycelia; b) blastospores; c) adhesive substance sticking together the filaments; d) cocci; e) bacilli (after [Pidoux, 1989]).

Water kefir needs to be distinguished from milk kefir which is a fermented milk product and originates in the Caucasus Mountains in Central Asia ([Yilmaz et al., 2002], [Plessas et al., 2007]). Several attempts for the use of milk kefir grains as a sourdough starter have been made ([Plessas et al., 2005], [Plessas et al., 2007], [Plessas et al., 2011]) and good rising ability, good overall quality and increased shelf-life have been noticed, whereas a higher concentration of volatile aroma compounds could not be verified in all studies. Water kefir grains have recently been used as a biodegradable coating for the storage of blackberries ([Oliveira et al., 2013]). Another study has investigated flavour

modification by fermenting tomato with watery kefir ([Wignyanto and Vida, 2007]). However, until now no studies have been carried out on the hydrocolloid function of processed water kefir grains or on their use as a baking agent.

1.5 Objective of work

The food and biotechnological industry is constantly searching for novel hydrocolloids with special structures and for promising microbial strains for biotechnological applications ([Roller and Dea, 1992]). Furthermore, a more sustainable waste treatment and a more efficient use of natural resources is the aim of many studies. In the production of baked goods, the focus is on the improvement of shelf-life, volume, texture and taste of both gluten-containing and gluten-free breads, for which sourdough usage has been identified as a prerequisite for high quality bread ([Katina, 2005], [Arendt et al., 2007], [De Angelis et al., 2009], [Guerzoni et al., 2011]). Recently, Jakob et al. ([Jakob et al., 2012b]) have shown that several *Gluconobacter* strains isolated from water kefir produce high amounts of fructans, which cause an increased volume, clear softening effect and retarded staling of fresh wheat breads during one week storage. However, the isolation of exopolysaccharides is expensive and energy- and time-consuming ([Kumar et al., 2007]) and purified fructans are not yet approved as food additives.

Therefore the objective of this thesis was to explore EPS-forming acetic acid bacteria and their EPS for food applications. By implementing *in situ* production of EPS with suitable acetic acid bacteria on cereal substrates, a new generation of gluten-containing and gluten-free sourdoughs, which contain effectual EPS levels, should be developed. Furthermore, fermentations in laboratory media with different sugar compositions should be carried out to explore possibilities for other industrial applications and to get a deeper insight into bacterial metabolism and EPS-formation. During this, one main aim of this thesis was to fix optimum fermentation parameters for EPS production in laboratory media, as well as in various gluten-containing and gluten-free sourdoughs. Furthermore, the potential of water kefir granules, that mainly consist of bacterial exopolysaccharides and assault as waste during water kefir production, should be explored as a novel hydrocolloid in bread making.

2 Materials and Methods

2.1 Selected acetic acid bacterial strains

Based on a pre-screening of different AAB strains ([Gulitz et al., 2011], [Jakob et al., 2012b]), six exopolysaccharide-producing strains of the genera *Gluconobacter* (*G.*), *Neoasaia* (*N.*) and *Kozakia* (*K.*) were chosen for the experiments (Table 2.1).

Table 2.1 – Bacterial strains selected for fermentations in nutrient laboratory media.

Bacterial strain	TMW number	Other collection	Origin
G. oxidans	2.339		Water kefir
G. frateurii	2.767		Water kefir
G. cerinus	2.878	DSM 9533T	Cherries
N. chiangmaiensis	2.1086	NBRC 101099 (type strain)	Flower of red ginger
K. baliensis	2.1087	DSM 14400 (type strain)	Palm brown sugar
G. albidus	2.1191		Water kefir

Strains were streaked on No. 5 agar plates (recipe shown in section 2.3) from glycerol stock stored at $-80\,°C$. No. 5 agar was produced based on the recipe shown in Section 2.4 with $15\,g/l$ agar (Merck). After 2 - 3 days of aerobic cultivation at $30\,°C$, plates were stored in a cooling room at $4\,°C$ until further use.

2.2 Preculturing of strains for fermentations

For experiments in liquid laboratory media, preculturing was always performed in liquid No. 5 medium (Section 2.3) under aerobic conditions ($180\,rpm$) at $30\,°C$. For dough fermentations and fermentations with different carbon sources, unchanged No. 5 medium was only used to grow the *Gluconobacter* species, whereas for *Neoasaia*, mannitol was replaced by xylose (Fluka) and for *Kozakia* by glucose (GERBU Biotech) to reduce lump formation. Samples of these two genera were further exposed to ultra sound treatment ($1 \cdot 10$ cycles at $75\,\%$ intensity in $30\,s$, Bandelin electronic) before measuring the optical density of precultures at 590 nm (OD_{590}) using a Novaspec II (Pharmacia Biotech). If OD_{590} was higher than 0.3, samples were dissolved with peptone water before measuring. The OD_{590} was needed to assess cell count in order to calculate the inoculation volume for main cultures based on the following equation:

$$\text{Inoculation volume} \, [\text{ml}] = \frac{\text{Target OD}_{590} \cdot \text{Volume of main culture} \, [\text{ml}]}{\text{OD}_{590} \, \text{of preculture}} \qquad (2.1)$$

Before main cultures were inoculated, cells of all strains were washed with peptone water made from $8.5 \, \text{g/l}$ sodium chloride (VWR Prolabo) and $1 \, \text{g/l}$ peptone from casein (Merck). They were resuspended in peptone water for fermentations in lab media and in tap water for sourdough fermentations.

2.3 Robustness of EPS formation in different laboratory media

In the set of experiments, an extended screening of the chosen strains was performed in different laboratory media to gain a further insight into their individual levels of EPS formation. The seven nutrient media chosen for the experiments were prepared based on the following recipes:

1. Mannitol medium (GM medium)([Buchert and Viikari, 1988]): mannitol ($25 \, \text{g/l}$), yeast extract ($5 \, \text{g/l}$)(both from Carl Roth), peptone from casein ($3 \, \text{g/l}$),

2. GfK medium (after Bauer et al. ([Bauer et al., 2005]), slightly modified): glucose ($50 \, \text{g/l}$), yeast extract ($5 \, \text{g/l}$), KH_2PO_4 ($1 \, \text{g/l}$, Sigma-Aldrich), K_2HPO_4 ($2 \, \text{g/l}$, GERBU Biotech), $(NH_4)_2SO_4$ ($5 \, \text{g/l}$, Merck), $MgSO_4 \cdot 7\,H_2O$ ($0.5 \, \text{g/l}$, Merck),

3. Gluconate medium (SoG medium)([Adachi et al., 1979]): mannitol ($10 \, \text{g/l}$): sodium gluconate ($20 \, \text{g/l}$) (Merck), yeast extract ($3 \, \text{g/l}$), peptone from casein ($2 \, \text{g/l}$), glycerol ($3 \, \text{g/l}$, GERBU Biotech),

4. No. 5 medium (after Shinjoh and Hoshino ([Shinjoh and Hoshino, 1995]) with slight modifications): mannitol ($10 \, \text{g/l}$), yeast extract ($15 \, \text{g/l}$), $MgSO_4 \cdot 7\,H_2O$ ($2.5 \, \text{g/l}$), glycerol ($0.5 \, \text{g/l}$),

5. Homohiochii medium (Spicher medium)([Kitahara et al., 1957]): peptone from casein ($10 \, \text{g/l}$), meat extract ($2 \, \text{g/l}$, Merck), yeast extract ($7 \, \text{g/l}$), sodium gluconate ($2 \, \text{g/l}$), $C_2H_3NaO_2 \cdot 3\,H_2O$ ($5 \, \text{g/l}$, Merck), $C_6H_{14}N_2O_7$ ($5 \, \text{g/l}$, Roth), KH_2PO_4 ($2.5 \, \text{g/l}$), $MgSO_4 \cdot 7\,H_2O$ ($0.2 \, \text{g/l}$), $MnSO_4 \cdot H_2O$ ($0.1 \, \text{g/l}$, Merck) $FeSO_4 \cdot 7\,H_2O$ ($0.05 \, \text{g/l}$, Merck) $C_3H_7NO_2S \cdot HCl$ ($0.5 \, \text{g/l}$, Merck) Tween 80 ($1 \, \text{g/l}$, GERBU Biotech) glucose ($7 \, \text{g/l}$), fructose ($7 \, \text{g/l}$, GERBU Biotech), maltose ($7 \, \text{g/l}$, Merck), if necessary for solid medium: agar ($15 \, \text{g/l}$),

6. mMRS-medium (modified after De Man et al. ([De Man et al., 1960])): peptone from casein ($10 \, \text{g/l}$), meat extract ($5 \, \text{g/l}$), yeast extract ($5 \, \text{g/l}$), K_2HPO_4 ($2.6 \, \text{g/l}$), KH_2PO_4 ($4 \, \text{g/l}$), NH_4Cl ($3 \, \text{g/l}$, Roth), $C_3H_7NO_2S \cdot HCl$ ($0.5 \, \text{g/l}$), Tween 80 ($0.5 \, \text{g/l}$), 1 ml vitamin-mix ($MgSO_4 \cdot 7\,H_2O$ ($10 \, \text{g/l}$), $MnSO_4 \cdot H_2O$ ($1.8 \, \text{g/l}$), thiamine ($0.01 \, \text{g/l}$, Sigma), folic acid ($0.01 \, \text{g/l}$, Sigma), pyridoxine hydrochloride ($0.01 \, \text{g/l}$, Fluka), D-pantothenic acid $\cdot$ ½ Ca ($0.01 \, \text{g/l}$, Sigma), cobalamin ($0.01 \, \text{g/l}$, Sigma-Aldrich), glucose ($5 \, \text{g/l}$), fructose ($5 \, \text{g/l}$, GERBU Biotech), maltose ($10 \, \text{g/l}$, Merck),

7. GM medium with vitamins: base recipe of GM medium enriched with 1 ml/l of vitamin mix from MRS medium.

To all media in this experiment, 100 g/l sucrose was added. All ingredients except the carbon sources were suspended in distilled water and pH = 6 was adjusted using a glass electrode (InLab 412, pH 0 0-14, Mettler-Toledo, Germany), before autoclaving at 121 °C for 20 min. In Spicher medium, pH was adjusted to 5.4 following the instructions of the recipe. Carbon sources were solved in distilled water, autoclaved separately and mixed with the other ingredients after cooling down to avoid Maillard reactions.

Fermentations took place in 2 l glass flasks filled with 190 ml liquid medium. Flasks were inoculated with 10^8 CFU/ml and cultivated aerobically (180 rpm) at 30 °C. Sampling took place after 0, 6, 24, 30, 48, 54 and 72 h, relating to t_0, t_6, t_{24}, t_{30}, t_{48}, t_{54} and t_{72}.

Sample material was stored at -20 °C. It was defrosted and centrifuged (11 800 g, 4 °C, 15 min) to remove bacterial cells before EPS analysis, which was performed as described in Section 2.6.1. Strains which showed high EPS production in the different media were chosen for further experiments.

2.4 Fermentations in liquid nutrient media with different carbon sources in addition to sucrose

With four bacterial strains which were identified as strong EPS-producers in the previous experiments, fermentations in liquid nutrient media were performed in order to estimate the influence of additional carbon sources on EPS formation, growth and bacterial metabolism.

2.4.1 Influence of different carbon sources on growth

In these experiments, the influence of different carbon sources on growth, EPS- and other metabolites formation should be examined for *G. frateurii*, *G. albidus*, *N. chiangmaiensis* and *K. baliensis*.

No. 5 medium (Section 2.3) was chosen as base recipe and 80 g/l sucrose was added to allow EPS synthesis. As additional carbon source, various sugars were added. The following labelling is used in the following:

- A: no additional carbon source

- B: mannitol (10 g/l)

- C: glucose (10 g/l)

- D: fructose (10 g/l)

- E: maltose (10 g/l)

- F: sodium gluconate (10 g/l)

To gain insight into the influence of different sugar compositions on growth in the log-phase, fermentations were performed in 500 ml glass flasks filled with 60 ml liquid medium (A-F). Based on Equation 2.1, cultures were inoculated with the volume of preculture necessary to obtain $OD_{590} = 0.05$. Flasks were incubated aerobically (180 rpm, 30 °C). Every hour for a period of 12 h, OD_{590} was measured in order to estimate growth. Before measuring OD_{590} of *N. chiangmaiensis* and *K. baliensis*, cell aggregates of samples were destroyed with ultrasonic treatment (Section 2.2).

2.4.2 Influence of different carbon sources on bacterial metabolism

In order to analyse bacterial metabolism, cultures were inoculated in duplicates with $1.5 \cdot 10^7$ CFU/ml and incubated aerobically, following the description in Section 2.3. Sampling took place immediately after inoculation and after 6, 24, 30, 48 and 72 h (relating to t_0, t_6, t_{24}, t_{30}, t_{48} and t_{72}) by taking 7 ml of main cultures.

To assess bacterial growth during 72 h of fermentation, serial dilutions were produced in peptone water (recipe in Section 2.2). Duplicates of 50 µl from the 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated on No. 5 agar plates using a spiral plater (Eddy Jet). Samples taken after 0 and 6 h were diluted 10^{-2}, 10^{-3} and 10^{-4}-fold before plating, due to a lower cell count. Plates were inoculated aerobically at 30 °C for 2 - 3 days and colonies were counted manually. If given cell counts amount $< 2 \cdot 10^5$ CFU/ml, this means that no colonies have been counted on plates with the lowest dilution (10^{-4}), as one CFU per 50 µl of a 10^{-4} dilution would mean $2 \cdot 10^5$ CFU/ml sample. Therefore smaller cell counts than these could not be detected by this method.

Remaining material was stored at -20 °C. It was defrosted and centrifuged (11 800 *g*, 4 °C, 15 min) to remove bacterial cells before further use.

EPS were isolated in duplicate according to the method described in Section 2.6.1. Isolated EPS were quantified by weighing on a precision scale and expressed as mean values and standard deviations.

The methods for the quantification of sugars and acetic acid are given in Section 2.6.2 and 2.6.3.

PH values of liquid media were measured with a glass electrode (InLab 412, pH 0–14, Mettler-Toledo, Germany) at all sampling times. Data of pH measurements were subjected to an analysis of variance (ANOVA) investigating the linear regression by Dr. Hannes Petermeier (Lehrstuhl für Mathematische Modellierung biologischer Systeme-Fachgebiet Biostatistik, Technische Universität München, Liesel-Beckmann-Straße 2, 85350 Freising, Germany) with the following linear model:

$$
\begin{aligned}
\text{pH} = \beta_0 &+ \beta_1 \cdot Gf + \beta_2 \cdot Kb + \beta_3 \cdot Nc + \beta_4 \cdot B + \beta_5 \cdot C + \beta_6 \cdot D \\
&+ \beta_7 \cdot E + \beta_8 \cdot F + \beta_9 \cdot t_6 + \beta_{10} \cdot t_{24} + \beta_{11} \cdot t_{30} + \beta_{12} \cdot t_{48} + \beta_{13} \cdot t_{72}
\end{aligned}
\tag{2.2}
$$

where β_0 summarizes the strain *G. albidus* in medium A at time t_0. The variables of interest were strain, medium and time. Therefore *Gf*, *Kb* and *Nc* relate to strains, *B*, *C*, *D*, *E* and *F* relate to liquid media and t_6, t_{24}, t_{30}, t_{48} and t_{72} relate to sampling times. β_1 to β_{13} are the coefficients of the [0,1] coded dummy variables for strain, medium

and time as stated above. They can be switched from 0 to 1 to investigate the relation between β_0 and the respective superposition of the strain-media-time-combination effect. By using this approach of statistical analysis, differences between the parameters could be pointed out to support the discussion of metabolic interdependencies.

2.5 Sourdough fermentations

Sourdough fermentations were carried out following two different experimental setups. In a first experiment, cultivation conditions under which AAB strains were able to grow and become dominant were assessed by testing different flours and oxygen conditions partly with mannitol addition. In the subsequent second experiment, the flours and fermentation conditions found suitable for AAB cultivation, i. e. growth after 24 h and less than 10 % contaminants, were used. In that second experiment, doughs were enriched with sucrose to determine EPS production.

2.5.1 Selected flours

The flours selected for dough fermentations are shown in Table 2.2. WT, WW, SP and RY flour contain gluten, whereas in MI I, MI II, CO, RI and BW flour, no gluten is present. OA takes a special position, because allthough many celiac disease patients can eat OA flour, some patients show oat intolerance ([Arentz-Hansen et al., 2004]). Nutrient contents of the used RI, MI II and BW flour are shown in Table 2.3. MI II flour was further specified by the supplier as containing 9.6 % protein, 58 % carbohydrates (including 0.7 % sugars), 17.7 % dietary fibre and 3.5 % lipids, which were composed of 2.9 % unsaturated fatty acids and 0.5 % saturated fatty acids.

2.5.2 Total titrable acid and total titrable base of doughs

To analyse the buffer capacity flours, total titrable acid (TTA) and total titrable base (TTB) were analyzed. The TTA and TTB were determined using a glass electrode (InLab 412, pH 0–14, Mettler-Toledo, Germany). To determine TTA, the consumption of 0.1 M NaOH in ml per 25 g sample (5 g flour suspended in 20 g distilled water) up to a final pH value of 8.5 ± 0.1 was measured. The TTB was determined accordingly as the consumption of 0.1 M HCl in ml per 25 g sample up to a final pH value of 4.5 ± 0.1. Dough samples were analyzed once to give only a rough estimation.

2.5.3 Sourdough fermentations under different conditions

Aerobic doughs were produced by mixing 5 g flour, 19 ml tap water, 1 ml suspended bacteria (cultivation described in Section 2.2) and optional 0.5 g mannitol in 250 ml glass flasks in order to obtain a dough yield of 500 (Equation 2.4) and 10^8 CFU/ml dough. To produce anaerobic doughs, 50 ml test tubes were used instead of glass flasks. A summary of flours and strains fermented with and without maltose under different

Table 2.2 – Selected gluten-containing (+) and gluten-free (-) flours used for sourdough fermentations with acetic acid bacteria and their suppliers.

Abbreviation	Flour Variety & Type	Gluten +/-	Supplier
WT	Wheat type 550	+	Rosenmehl
WW	Organic whole wheat	+	Tagwerk Freising
SP	Organic whole spelt	+	Eikernmühle GmbH
RY	Rye type 1150	+	Rosenmehl
OA	Whole oat	+/-	Fortin Mühlenwerke GmbH & Co. KG
MI I	Golden millet	-	Ziegler & Co. GmbH
MI II	Wild browntop millet	-	Neuform
CO	Corn	-	Baktat
RI	Rice	-	Ziegler & Co. GmbH
BW	Buckwheat	-	Schälmühle

Table 2.3 – Nutrient content (mg/kg flour) of rice, wild browntop and buckwheat flour used in this study. Data were obtained from an external analysis according to DIN EN ISO 11885 E22 (rice, buckwheat) or from the supplier's specifications (wild browntop).

	Rice	Wild browntop millet	Buckwheat
Al	1.0		32.0
Fe	8.9	0.3	66.3
Mn	22.9	13.5	19.4
Na	11.0	69.0	6.1
Zn	14.4	0.2	27.3
Ca	125.0		450.0
Cu	1.9		6.6
K	2646.0		4602.0
Mg	1.1		1931.0
P		<0.001	
Vit B_6		0.024	

oxygen conditions is given in Table 2.4.

$$\text{Dough yield} = \frac{(\text{flour [g] + water [g]})}{\text{flour [g]}} \cdot 100 \tag{2.3}$$

Aerobic doughs were cultivated at $30\,°C$ under shaking conditions ($180\,$rpm). Anaerobic doughs were mixed for $20\,$s and cultivated without shaking. Sampling was performed after $1\,$h (t_1), $24\,$h (t_{24}) and $48\,$h (t_{48}) of fermentation.

Cell count was determined immediately by plating diluted samples on Homohiochii agar (recipe shown in Section 2.4) according to the method described in Section 2.4.2. Acetic acid bacteria could be easily distinguished from contaminants by their pink colony morphology occuring after a few days storage at room temperature.

Remaining material was kept at $-20\,°C$. Before further analysis, dough extracts were produced by diluting defrosted samples with twice the volume of distilled water, followed by centrifuging at $11\,800\,g$ and $4\,°C$ for $30\,$min to remove bacterial cells and solids.

For doughs where AAB had become dominant, quantification of EPS (as negative control), sugars and organic acids was performed as described in Sections 2.6.1, 2.6.2, and 2.6.3. Further, the pH value of dough extracts was measured. All experiments of this subsection were performed in duplicate.

2.5.4 Fermentations with sucrose addition

All fermentations that contained sucrose were performed under aerobic conditions without mannitol addition. To enable EPS production, 1, 1.5 or $2\,$g sucrose was added to the doughs, relating to 50, 75 and $100\,$g/l dough. An overview of the fermentations with sucrose is given in Table 2.5. Sampling and sample analysis were carried out identically to the fermentations without sucrose addition as described in Section 2.5.1.

2.5.5 Backslopping experiment

To find out whether AAB can remain dominant in sourdoughs during several refreshments, backslopping experiments were carried out with *G. frateurii* and *K. baliensis*.

Aerobic sourdoughs were produced by mixing $2\,$g spelt flour, $0.4\,$g sucrose, $7\,$ml tap water and $1\,$ml bacterial suspension (about $8 \cdot 10^8\,$CFU/ml suspended in tap water) in $100\,$ml glass flasks to obtain a dough yield of 500 and a sucrose content of $50\,$g/l dough. Doughs were incubated at $30\,°C$ under shaking conditions ($180\,$rpm). After $24\,$h, $1\,$ml dough was used to inoculate a new sourdough, whereas this procedure was repeated eight times.

In the beginning of the first fermentation and before each refreshment, $1\,$ml dough was used to determine growth of AAB and contaminants on Homohiochii agar (Section 2.4) according to the method described in section 2.4.2.

2 Materials and Methods

Table 2.4 – Summary of fermentations without sucrose under aerobic (O_2 = y) and anaerobic (O_2 = n) conditions with (y) and without (n) mannitol addition (25 g/l dough). *G. f.*: *G. frateurii*; *G. a.*: *G. albidus*; *N. c.*: *N. chiangmaiensis*; *K. b.*: *K. baliensis*; (+): fermentation was realized; (-): fermentation was not realized.

Strain	O_2	Mannitol	WT	WW	SP	RY	OA	MI I	CO	RI	BW
G. f.	y	y	+	-	+	+	+	+	-	-	-
	y	n	+	-	+	+	+	+	+	+	+
	n	y	+	-	+	+	+	+	-	-	-
	n	n	+	-	+	+	+	+	+	+	+
G. a.	y	y	+	-	+	+	+	+	-	-	-
	y	n	+	-	+	+	+	+	+	+	+
	n	y	+	-	+	+	+	+	-	-	-
	n	n	+	-	+	+	+	+	+	+	+
N. c.	y	y	-	-	-	-	-	-	-	-	-
	y	n	+	+	+	+	+	+	-	-	-
	n	y	-	-	-	-	-	-	-	-	-
	n	n	+	+	+	+	+	+	-	-	-
K. b.	y	y	-	-	-	-	-	-	-	-	-
	y	n	+	+	+	+	+	+	-	-	-
	n	y	-	-	-	-	-	-	-	-	-
	n	n	+	+	+	+	+	+	-	-	-

Table 2.5 – Overview of fermentations with sucrose addition (50, 75 and 100 g/l dough) under aerobic conditions without mannitol. (+): fermentation was realized; (-): fermentation was not realized. Fermentations were done in triplicates except MI I (duplicate) and MI II (single).

Bacterial strain	WT	WW	SP	RY	OA	MI I	MI II	CO	RI
G. frateurii	+	-	+	+	-	+	+	+	+
G. albidus	+	-	+	+	-	+	+	+	+
N. chiangmaiensis	+	+	+	+	+	+	+	-	-
K. baliensis	+	+	+	+	-	+	+	-	-

2.6 Sample preparation for metabolite analysis

Metabolite analyses of experiments in lab media and dough fermentations were carried out identically. From fermentations in lab media, the supernatants obtained after defrosting and centrifuging were used for the analyses (see Section 2.4), whereas from dough fermentations, the readily prepared dough extracts obtained from Section 2.5.1 were taken.

2.6.1 Analysis of EPS

EPS were precipitated by adding two volumes of chilled ethanol ($4\,°C$) to the sample (Figure 2.1). After one night storage at $4\,°C$, samples were centrifuged ($11\,800\,g$ and $4\,°C$, $15\,min$) and ethanol was removed. Precipitated EPS were left for another night at $30\,°C$ under shaking conditions ($180\,rpm$) to evaporate residual ethanol. Then, EPS were dissolved in distilled water, dialyzed against deionized water for $48\,h$ (molecular weight cut-off $12\text{-}14\,kDa$) and lyophilized.

As isolated EPS from experiments in lab media were pure microbial fructans, they were then weighed with a precision scale and expressed as g EPS/ml medium.

Figure 2.1 – Precipitated EPS.

Fructans as part of isolated polysaccharides from sourdoughs were hydrolyzed to fructose monomers by $0.2\,\%$ $HClO_4$ ($70\,\%$ w/v, Merck) and incubation at $100\,°C$ for $1\,h$. Samples were then centrifuged and filtered through $2\,µm$ membrane filters (Phenomenex, Germany). Quantification was carried out by high performance liquid chromatography (HPLC) using a sulfonated styrene-divinylbenzene Rezex ROA column (Phenomenex, Germany) with a flow rate of 0.6 ml/min and an injection volume of $10\,µl$. Mobile phase was $0.005\,M$ H_2SO_4 and temperature was set to $85\,°C$. An external fructose standard was used for calibration and the column was coupled to a refractive index (RI) detector (Gynkotek, Germany). The amount of fructan in sucrose-enriched sourdough was considered as total microbial and flour fructan and expressed as fructose equivalents. In pretrials, a relation of $1\,g$ fructose equivalent (Frc equ) relating to $1\,g$ fructan was determined.

Dry matter of stored EPS

The dry matter of isolated EPS which were stored for several months sealed in plastic containers at room temperature was determined by drying a defined amount of EPS in predried and preweighed plastic tubes at $105\,°C$ for at least $16\,h$. After cooling down to room temperature in a desiccator, tubes were weighed on a fine scale and the dry matter was calculated based on the weight difference before and after drying.

Recovery rate of EPS from sourdoughs

The recovery rate of EPS from doughs was investigated by producing doughs with solutions of EPS from different strains containing 50 mM Frc equ/L. Dough made from wheat, rye, browntop millet, spelt, rice and corn flour were tested for EPS from *G. frateurii*, *G. albidus*, *K. baliensis* and *N. chiangmaiensis*. Oat flour dough was tested only for EPS produced by *N. chiangmaiensis*. Dough extracts were produced according to Section 2.5.3 and EPS were precipitated and purified according to the method explained in Section 2.6.1. Then, EPS were quantified via HPLC and yields were compared to original amounts. Recovery rate was expressed as the relation between recovered EPS and expected EPS in per cent. Two to five replicates of each flour and each strain were produced.

Influence of ethanol ratio on EPS recovery rate from sourdoughs

The influence of excess ethanol during precipitation on EPS recovery was determined by testing different ratios of dough extract to ethanol (1+2; 1+2.5; 1+3; 1+3.5; 1+4) for the precipitation of EPS of different strains in wheat dough. For all flour-EPS-combinations, between 2-4 replicates were obtained.

2.6.2 Quantification of sugars

To perform sugar analysis, $500\,µl$ sample was incubated overnight at $4\,°C$ with $250\,µl$ $ZnSO_4$ ($10\,\%$ w/v) and $250\,µl$ of $0.5\,M$ NaOH. After centrifugation, samples were filtered and quantified using a Rezex RPM column (Phenomenex, Germany) coupled to a RI detector (Gynkotek, Germany). Flow rate was $0.6\,ml/min$, mobile phase was deionized water at $85\,°C$ and the injection volume amounted $20\,µl$. Calibration was performed using external standards.

2.6.3 Determination of organic acids and ethanol

For analysis of acetic acid, lactic acid and ethanol, samples were treated with $5\,\%$ $HClO_4$ ($70\,\%$ w/v) overnight at $4\,°C$, centrifuged and filtered. Metabolites were quantified by HPLC applying a Rezex ROA column with the settings from Section 2.6.1. External standards were used for calibration.

D-Gluconic acid was analysed enzymatically (Roche), using $0.5\,M$ Tris buffer at $pH = 8$ instead of distilled water. An external standard was used for calibration. Analyses were performed in duplicates and mean values were calculated.

2.6.4 Statistical data analysis

Statistical data analysis of dough fermentations was performed by Dr. Hannes Petermeier (Lehrstuhl für Mathematische Modellierung biologischer Systeme - Fachgebiet Biostatistik, Technische Universität München, Liesel-Beckmann-Straße 2, 85350 Freising, Germany). As, apart the flour varieties, all fermentation circumstances were kept as constant as possible, the remaining explanatory variables are fermentation time (t and `time` respectively) and the initial sucrosedosage (s and `sucrosedosage` respectively). To study the influence of both variables, the following model was applied throughout

$$v = \beta_0 + \beta_1 t + \beta_2 s + \beta_3 t^2 + \beta_4 s^2 + \beta_5 s \cdot t \tag{2.4}$$

where v stands for the variable of interest or their logarithm. Variables of interest are `eps`, `gluconic acid`, `acetic acid`, `pH` and the remaining `sucrose` in the dough. The parameters were estimated by means of standard linear models as implemented in the `lm` function of the statistic package R (www.r-project.org, [R Development Core Team, 2013]). Either the variable or its logarithm was used to study the dependency. Residuals were analysed by the quantile-quantile-plot for normal distribution to assess the model's vailidity, under consideration of the response-quantile-plot of the residuals for normal distribution along with the symmetry of the residuals. The models were simplified by skipping all parameters resulting in a p-value above 0.1. Resulting models were checked, whether the prediction results in negative concentrations; if so, models were again evaluated to avoid this. By using this approach of statistical analysis, the unavoidable differences in the fermentations could be pointed out to support the discussion of metabolic interdependencies.

2.7 Water kefir cultivation and preparation of water kefir powder

Water kefir was cultivated as described by Gulitz et al. ([Gulitz et al., 2011]). In a 2 l plastic beaker, 80 g kefir grains were filled. Sucrose (100 g/l), tap water (1 l), two dry figs (Seeberger, non sulfurated and no preservatives) and a slice of organic lemon were added. After three days of fermentation at 21 °C, the supernatant was discarded. Water kefir grains were strained, washed with tap water and strained again. As grains had propagated, 80 g were used for the next cultivation cycle. The residual grains (Figure 2.2) were kept for further experiments.

For the preparation of water kefir powder (WKP), excess grains were autoclaved at 121 °C for 20 min. Then, grains were lyophilized (Labconco, $T = -81$ °C, vacuum set point $= 0.002$ mbar) and milled with a cone mill (DLFU, Buehler Universal) to a particle size below 0.025 mm before further use.

Figure 2.2 – Water kefir grains.

Figure 2.3 – Dry WKP.

2.8 Functional properties of water kefir powder

All further experiments have been performed with the WKP obtained from section 2.7, which was a cream-coloured to white, fluid powder (Figure 2.3). During an internal tasting, it was described as aromatic, slightly sour and fermented. As the WKP was not hygroscopic, it was stored in a plastic tin at room temperature.

The experiments with WKP were generally carried out in triplicates and thus the results were expressed as average and standard deviation of the three experiments.

2.8.1 Determination of dry matter

To determine the dry matter of WKP, three weighing dishes were filled with 15 g sea sand and a glass rod and dried overnight at 103 ± 2 °C. After drying, dishes were cooled down in a desiccator for 1 h and empty weight was measured with a precision scale (SI-234, Denver Instrument)(α_1). Then, 1.000 ± 1 g WKP (α_2) was added to the sea sand and mixed, before dishes were put back into the drying oven. After 24 h, dishes were cooled down in the desiccator for another hour, before dry weight (α_3) was measured. To ensure complete water removal, drying was repeated until a constant weight was reached. The dry matter of WKP was then calculated using the following equation:

$$\text{Dry matter}\,(\%) = \frac{(\alpha_3 - \alpha_1)}{(\alpha_2 - \alpha_1)} \cdot 100 \tag{2.5}$$

2.8.2 Solubility in different solvents

The solubility of WKP was studied in organic and inorganic solvents. As anorganic solvents, distilled water, 0.1 M NaOH and 0.1 M HCl were chosen. As organic solvents, pure ethanol and hexane were used.

In 50 ml test tubes (Sarstedt), 1.000 ± 1 g WKP (α) was filled and mixed with 10 ml solvent for 5 min. Then, tubes were centrifuged at $10\,000$ *g* and 21 °C for 15 min.

To determine the solubility of WKP in organic solvents, the supernatants were poured into pre-dried and pre-weighed glass flasks (α_1). Solvents were vaporized in a rotary evaporator at 70 °C, 120 - 150 rpm and 200 - 500 mbar. Residuals of organic solvents

were evaporated overnight under a fume hood. Finally, samples were lyophilized and weighed.

Residuals of anorganic solvents were transferred into pre-dried and pre-weighed 50 ml test tubes (α_1). To determine the amount of dissolved WKP, samples were also lyophilized (same settings as above) and then weighed (α_2).

As during the drying process all solvent has been removed, the solubility of WKP could be calculated from the difference between empty weight and weight after lyophilization:

$$\text{Solubility}\,(\%) = \frac{(\alpha_2 - \alpha_1)}{(\alpha)} \cdot 100 \tag{2.6}$$

2.8.3 pH in aqueous solutions

The pH-value of WKP in aqueous solutions was determined by suspending 1 and 2 % WKG (w/v) in distilled water. The change of pH was measured with a pH meter (761 calimatic, Knick GmbH, Berlin, Germany) under permanent stirring.

2.8.4 Microscopic observation of dry and wet water kefir powder

Dry WKP and WKP soaked in distilled water for 10 min were transferred to object slides and observed under a microscope (Axiostar plus, Zeiss). Photographs were taken at a 40 x enlargement.

2.8.5 Water holding capacity

Water holding capacity (WHC) was determined for room temperature and 75 °C. It is defined as the capability of a material to retain its own or added water against external forces like decantation, pressure or centrifugation ([Hamm, 1961], [Bouton et al., 1972]).

A mass of 1.000 $\pm$ 1 g WKP was transferred into pre-dried and tared (α) 50 ml test tubes (α_1). A volume of 10 ml distilled water was added and mixed for 30 s to bring all of the sample into suspension. During the next 35 min, one set of samples was left at room temperature and the other one at 75 °C in a water bath (Julabo, MD). Upon centrifugation at 10 000 g and 21 °C for 15 min using a Sigma 6-16K centrifuge, supernatants were carefully removed. Tubes were placed downwards at the angle of $15 - 20$ °C for 30 min and then weighed (α_2). The water binding capacity was calculated as the ratio between additional water and sample:

$$\text{WHC}\,(\text{w/w}) = \frac{(\alpha_2 - \alpha_1)}{(\alpha_1 - \alpha)} \tag{2.7}$$

For a comparison of the results with other hydrocolloids, xanthan (Roth), wheat starch (Mondamin) and hydroxypropylmethylcellulose (HPMC)(Fluka Analytical) were equally analyzed. At this, only 0.500 $\pm$ 1 g xanthan was used due to its extreme swelling capacity.

2.8.6 Freeze-thaw stability of suspended water kefir powder

The freeze-thaw stability was analyzed for different WKP suspensions. About 25 ml of 6, 8, 10 and 12 % WKP solutions (w/v) were transferred into pre-weighed 50 ml test tubes. The full weight was measured and samples were soaked at room temperature for 30 min, before they were frozen at $-20\,°C$. After 24 hours, samples were defrosted at room temperature for 2 h and centrifuged at 10 000 g and 21 °C for 15 min. Supernatants were removed and sample weight was compared to the weight taken before freezing.

2.8.7 Fat binding capacity

The fat binding capacity of WKP was determined using the method described by Schwenke ([Schwenke et al., 1981]). As a reference, soy lecithin (Caelo) and soy protein isolate (NUTRITION Rx) were treated the same way. A mass of 1.000 ± 1 g WKP was transferred into pre-dried and tared (α) 50 ml test tubes (α_1). A volume of 5 ml sunflower oil was added and mixed for 1 min, left still at room temperature for 5 min and mixed again. After another 5 min rest at room temperature, samples were centrifuged at 10 000 g and 21 °C for 15 min. Directly after centrifugation, supernatants were carefully removed using a pipette and samples were weighed (α_2). The fat binding capacity was calculated on the basis of Equation 2.7 as the ratio between retained fat and sample.

2.8.8 Emulsifying activity

The emulsifying activity of WKP was determined in triplicates after the method described by Behnke ([Behnke, 1985]) with slight modifications. Xanthan, soy lecithin and soy protein isolate were used as reference materials. A mass of 2.500 ± 1 g sample was weighed into a glass beaker. Then, 50 ml distilled water were added and mixed until the sample was suspended completely. Due to the high swelling of xanthan, only 0.250 ± 1 g was used. After adding 50 ml sunflower oil (gut und günstig), the suspension was homogenized using an Ultraturrax (ART Moderne Labortechnik, MICCRA D-8) at maximum speed for 1 min. Then, 10 ml suspension was transferred into a graduated 50 ml test tube and centrifuged at 10 000 g and 21 °C for 15 min. The emulsifying activity is shown as the relation between the volume of the emulsion layer to overall volume, which were read from the graduated test tube. Furthermore, emulsions were visually evaluated.

2.8.9 Foaming properties

The foaming properties of WKP were determined in triplicate after Gassmann et al. ([Gassmann et al., 1987]) with slight modifications as described in detail below. As reference materials, soy lecithin and soy protein were used. After suspending 5 g sample in 100 ml distilled water, 60 ml of the suspension were transferred into a graduated beaker and whipped using an Ultraturrax at maximum speed for 1 min. The volume of the foam layer was related to overall volume read from the scale on the beaker.

2.8.10 Gel building activity

Measurements of gelling properties were performed as follows: In a beaker, 1.000 ± 1 g sample was suspended in 50 ml distilled water and left to soak for 25 min at room temperature. Then, samples were brought to a boil under permanent stirring followed by cooling on ice within 30 min. After 1 h rest, gel building activity was assessed. During a second heating process, thermoreversibility of gels was determined. The reference materials chosen for this experiment were agar (Merck) and gelatine (Fluka).

2.9 Wheat breads with water kefir powder

2.9.1 Bread recipes and water dosage

The standard bread recipe for this study included 100 g white wheat flour (Rosenmehl), 50 g fresh baker's yeast (Deutsche Hefewerke Nürnberg), 15 g salt (Ja) and 600 g tap water (20 °C). For bread recipes with WKP, 1, 2 and 5 % of flour (10, 20 and 50 g/kg flour) were replaced by the same mass of WKP. As WKP has a higher WHC than wheat flour, the amount of water in WKP-enriched breads was increased to reach 813 Brabender Units (BU), relating to the same dough strength as in the original recipe with 600 g tap water (Table 2.6).

Table 2.6 – Bread recipes and water dosage for baking experiments.

WKG [g]	flour [g]	water [g]	Brabender Units [BU]
0	1000	600	813
10	990	625	813
20	980	657	813
50	950	730	813

2.9.2 Baking of breads

Bread samples were produced using the straight dough procedure. At the beginning of each day of the baking experiments, freshly opened yeast was used. If WKP was added, it was soaked in the pre-weighed water for 15 min prior to dough preparation. Doughs were made by kneading all ingredients with a Stephan UM12 kneader (Stephan food Service Equipment GmbH) at level 1 for 20 s. During a short break, dough residues were scratched down from the lid, mixer edges and the bowl, before kneading was continued for another 45 s.

The dough was cut to eight 205 g pieces, moulded to loaves and placed in small loaf pans which had been greased (Boeson-Trennwax flüssig-aktiv) before. After proofing

at 30 °C and 80 % humidity for 30 min (Wachtel, PICCOLO I-1), breads were baked at 230 °C for 25 min, with steaming during the first 5 s.

Baked loaves were cooled down at room temperature for 2 – 3 h before further analyses.

2.9.3 Bread evaluation

All obtained breads were weighed and their maximum height was measured manually once per bread.

For the determination of bread volume, two breads per batch were dipped into liquid paraffin, left to solidify and dipped into a water-filled beaker which was placed on a scale. As a result, the bread volume could be read on the scale (Archimedean principle).

The texture profile analysis of bread crumb and crust was performed in a TA.XT plus (Stable Micro Systems, Surrey, UK)(TPA). The six remaining breads per batch were cut into 15 mm thick slices (Graef).

For the analysis of crumb texture, six cylinders per bread with a diameter of 15 mm were cut out. Three cylinders were stored in airtight glass jars to be analysed after 48 h and the others were analysed immediately. For this purpose, a 15 mm diameter plunger at a crosshead speed of 1 mm/s compressed the cylinder two times to a depth of 7 mm (Figure 2.4). The height of the first compression curve measured the resistance to the plunger and represented the crumb firmness.

To analyse crust crispness, six 5 cm long and 5 mm thick stripes were cut off the bread slices of each of the six breads per group. Three stripes were measured instantly and the others were stored in airtight glass jars for 48 h before measuring. With a tensile test, a force profile of bread stripes clamped into the TPA was recorded based on the distance covered by the probe in a certain time. The experiment was interpreted by comparing the maximum power before rupture of the crust stripe (Figure 2.5).

Data were subjected to a bilateral homoscedastic t test ($p < 0.05$) to indicate significant differences.
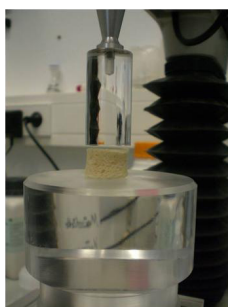
Figure 2.4 – Measuring crumb firmness.
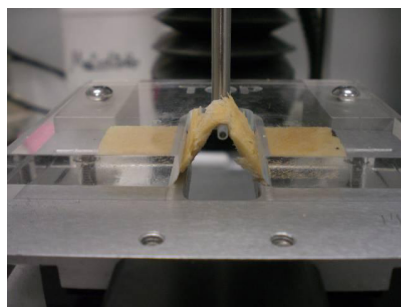
Figure 2.5 – Measuring crust hardness.

2.9.4 Sensory evaluation

Fresh loaves and 48 h old loaves of bread were tasted internally by untrained judges consisting of staff and students from the Institute of Technical Microbiology, Technische Universität München, Germany. Tastings of fresh breads were targeted on the potential of WKP as a saltiness enhancer, because it possesses a savoury aroma. Tastings of old breads focused on the hydrocolloid character of WKP, and thus its effects on bread staling. Therefore, three different bread recipes were prepared and breads were tasted fresh and after 48 h of storage (Table 2.7). Batch D was the original recipe, batch E the original recipe with 30 % sodium reduction and batch F was the 30 % sodium-reduced bread with 2 % WKP-addition.

Breads were baked as described above, cut into 15 mm thick slices and served in a randomized order at room temperature. For fresh breads, smell (aromatic, sour), taste (salty, sweet, sour, aromatic, bland) and popularity were assessed by using a questionnaire, which was a modified version of the DLG-scheme for bread (shown in appendix). For 48 h old breads, musty smell was questioned additionally and sweet taste was left out as no differences in sweetness had been perceived before. The 12 – 15 panellists answered the questions by rating the features of bread sensory quality on a scale from 1 – 6, where 1 stood for weak and 6 for strong.

Table 2.7 – Bread recipes for sensory evaluation.

Sample name	WKG [g]	Flour [g]	Water [g]	Salt [g]	Brabender Units [BU]
D	0	1000	600	15	813
E	0	1000	600	10	813
F	20	980	657	10	813

3 Results

3.1 Robustness of EPS formation in different laboratory media

The six acetic acid bacterial strains chosen in the beginning of this work were at first cultivated in different sucrose-enriched laboratory media in order to test their robustness of EPS production towards different nutrient conditions. This was done to exclude strains requiring very specific media conditions for high EPS formation from further experiments. Different laboratory media were chosen: two nutrient-rich lactobacilli-media (Homohiochii and mMRS medium), a few typical *Gluconobacter* media, among which a medium with and without additional vitamin mix, and a medium with glucose as additional carbon source. This way, two of the six strain were identified as low or very demanding EPS producers and therefore rejected.

For *G. frateurii*, EPS production was highest in SoG medium, where 15.95 ± 0.64 g/l have been reached after 24 h, and 19.05 ± 0.49 g/l after 48 h (Figure 3.1a). The second highest EPS-amounts were found in Spicher medium with a first maximum after 48 h (13.10 ± 0.14 g/l) and a second one after 72 h (13.45 ± 0.78 g/l). In GM, No5 and GM+Vit medium, EPS maxima were reached after 24 to 30 h, but they were not as high as in SoG and Spicher and lay between 12.5 ± 3.25 g/l (GM) and 10.25 ± 0.07 g/l (GM+Vit). In mMRS medium, the EPS maximum was found after 48 h, being at about the same level as in No5 and GM medium. Not less than 0.7 g/l EPS was detected during the whole fermentation with GfK medium. After reaching the peak level, EPS were generally degraded. During the fermentation in Spicher medium, EPS amount was still slightly increasing during the last day of fermentation. In all other media except mMRS and SoG, where EPS contents lay between 8.8 and 12.7 g/l, only 0 to 5 g/l EPS was left after 72 h.

EPS content of *G. cerinus* and *G. oxidans* never exceeded 5 g/l (Figures 3.1b and 3.1c). Therefore both strains were excluded from further experiments.

The highest EPS content of *G. albidus* has been recovered from Spicher medium after 48 h (Figure 3.1d). It lay at 16.25 ± 0.07 g/l and was followed by SoG medium, where 13.45 ± 0.49 g/l were reached at the same time. In GM+Vit, GM, No. 5 and mMRS medium, EPS maxima were reached after 30, 48, 48 and 72 h, respectively, and amounted between 6.38 ± 1.06 (GM+Vit) and 8.5 ± 0.14 g/l (GM). In GfK medium, EPS contents was around 1 g/l during the whole fermentation. EPS concentration decreased strongly after it had remained at maximum level for 18 h in GM+Vit medium. In Spicher, SoG, GM and No. 5 medium, however, EPS degradation was only weak. In mMRS medium, the fermentation was finished when the maximum EPS level had been reached.

In fermentations with *N. chiangmaiensis* EPS concentration remained at about the same level during the first 6 h (Figure 3.1e). In GM+Vit, SoG and GM medium, it
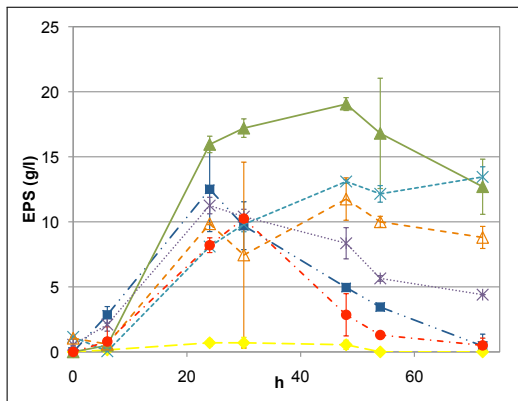
(a) *G. frateurii*
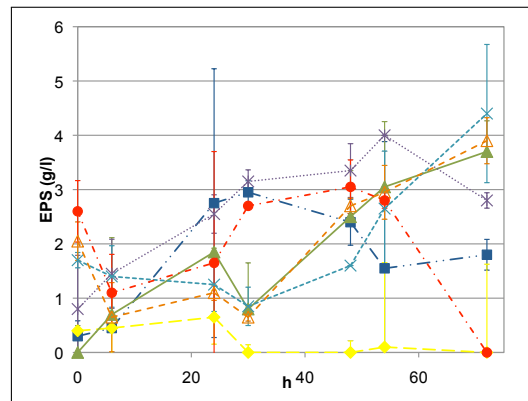
(b) *G. cerinus*
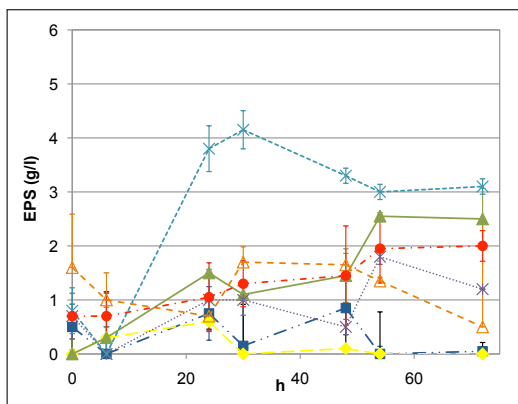
(c) *G. oxidans*
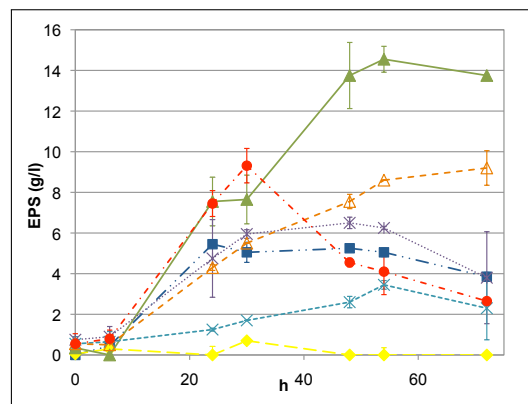
(d) *G. albidus*
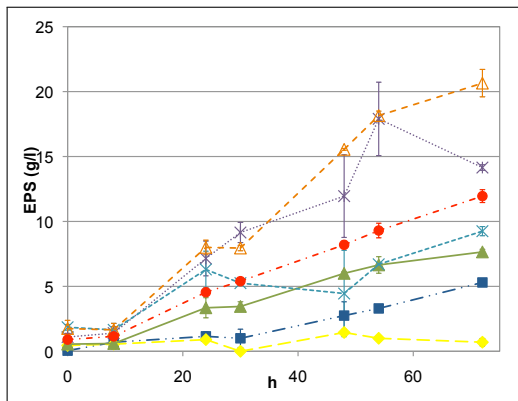
(e) *N. chiangmaiensis*
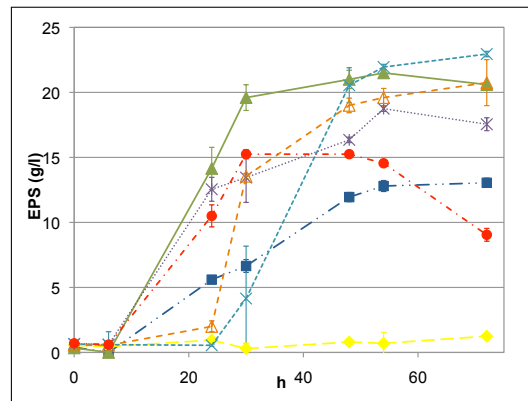
(f) *K. baliensis*

Figure 3.1 – EPS formation of selected acetic acid bacterial strains in different lab media
■: GM; ✳: No5; ◆: GfK; ✕: Spicher; △: mMRS; ▲: SoG; ●: GM+Vit.

then increased almost linearly until 72 h, where it reached 11.95 ± 0.49, 7.65 ± 0.21 and 5.3 ± 0.28 g/l, respectively. The maximum EPS yield was found in mMRS medium after 72 h. It lay at 20.65 ± 1.06 g/l and was followed by 17.9 ± 2.83 g/l in No5 medium after 54 h. In No5 medium, EPS were partly degraded after the maximum, reaching 14.15 ± 0.21 g/l at 72 h. In the other media, no EPS degradation could be recognized, as the maximum values were detected at the end of fermentation. Spicher and GfK medium took a special role. In GfK medium, the EPS maximum was reached after 48 h and amounted 1.45 ± 0.07 g/l. In Spicher medium, a first EPS maximum at 6.3 ± 1.41 g/l was reached after 24 h, followed by a slight drop and another increase up to 9.25 ± 0.35 g/l at 72 h.

Referring to the EPS contents of *K. baliensis*, more than 15 g/l in five out of the seven media tested were formed by this strain (Figure 3.1f): After a start phase of about 6 h, a strong EPS production started in SoG, No. 5 and GM+Vit medium. In these media, 19.8 ± 0.42, 17.3 ± 0.71 and 16.45 ± 0.49 g/l EPS were present after 48 h, respectively. In Spicher and mMRS medium, EPS production started after a longer lag phase of 24 h (mMRS) and 48 h (Spicher). Then, EPS contents increased up to 16.85 ± 0.07 g/l in mMRS and 15.55 ± 0.21 g/l in Spicher medium. In GM medium, EPS production started after 6 h, but was rather slow and lead to a relatively low final EPS content of 14.1 ± 0.99 g/l until t_{72}. In GfK medium, EPS contents never exceeded 0.85 ± 0.99 g/l. After reaching the maximum, EPS were partly degraded in GM+Vit medium. In No5 and SoG medium, a slight degradation of EPS could be observed, too, whereas it remained at maximum level in mMRS. In GM, Spicher and mMRS medium, EPS levels were still increasing when the fermentation was finished.

3.2 Macroscopic properties of EPS formed by acetic acid bacteria in liquid laboratory media

In order to reveal differences between the EPS of different acetic acid bacterial strains, EPS were produced employing different strains under the same conditions and assessed macroscopically. This way, a few obvious differences in colour and texture were discovered. In Figure 3.2, EPS harvested from NaG-medium with 100 g/l sucrose can be seen. The fructan from *G. oxidans* was red-brown and therefore the darkest, followed by *G. cerinus* and *N. chiangmaiensis*. EPS produced by *G. frateurii* and *G. albidus* (picture not shown) were cream-coloured to white, whereas *K. baliensis* produced a shining white EPS in NaG-medium. Concerning texture, isolated fructans from *G. cerinus* and *G. oxidans* were fine and flexible, whereas fructan from *K. baliensis* was slightly brittle, reminding of styrofoam, although it was less firm than this.

3.3 EPS production in media with varying carbon sources

In these fermentations, the influence of additional carbon sources apart from sucrose on EPS formation should be tested, as was suggested by the results from section 2.3. For the experiments, the strains *G. frateurii*, *G. albidus*, *K. baliensis* and *N. chiangmaiensis*

Figure 3.2 – Isolated EPS from different strains. From left to right: *G. cerinus*, *G. oxidans*, *G. frateurii*, *N. chiangmaiensis*, *K. baliensis*. Starter culture was grown overnight in GM-medium and main culture in 200 ml NaG-medium with 100 g/l sucrose, incubated at 30 °C and 180 rpm for 48 h.

were chosen. After presenting the data of growth experiments during the exponential phase, data of growth, pH and selected metabolites of the 72 hour lasting fermentations will be provided. To get an overview about EPS production, EPS yields are presented in separate diagrams, before they were set in relation to all other metabolites measured for each single medium.

3.3.1 Influence of varying carbon sources on bacterial growth in the exponential phase

In order to detect an influence of additional sugars apart from sucrose on bacterial growth in the start phase, optical density of fermentations which were inoculated with very low cell counts was measured during the first 12 h.

In Figure 3.3, the change in optical density at 590 nm (OD_{590}) of fermentations with selected strains in six media with different sugar compositions during the first twelve hours is shown. At t = 0, all flasks were inoculated with OD_{590} between 0.033 and 0.05 apart from *N. chiangmaiensis*, whose OD_{590} lay between 0.28 and 0.31.

In fermentations with *G. albidus* (Figure 3.3b), OD_{590} was highest in medium F (Sucr + SoGlu, 2.05) and lowest in Medium A (Sucr, 0.66) after 12 h. The change in OD_{590} in media B-E during the first 12 h was quite similar, although it increased faster in medium B (Sucr+Man) after 7 h, resulting in the second highest OD value after 12 h (1.74). At this time point, OD_{590} in media E, D and C amounted 1.32, 1.16 and 1.04, respectively. During the first 8 h, OD_{590} in media A and F grew only slowly. After t_8, OD in medium F increased strongly and remained stable in medium A, remaining at 0.66 after 12 h of fermentation.

In opposite to *G. albidus*, the fermentations carried out with *G. frateurii* did not show big differences in turbidity during the first 12 h (Figure 3.3a). The increase of OD_{590} was quite similar, except that in medium F (Sucr+SoGlu) a slightly lower OD was measured. The resulting OD after 12 h was lower than in fermentations with *G. albidus* and ranged from 1.11 to 1.18 (medium B and D, respectively).

The starting OD_{590} of fermentations with *N. chiangmaiensis* was slightly higher com-

(a) *G. frateurii*

(b) *G. albidus*

(c) *N. chiangmaiensis*
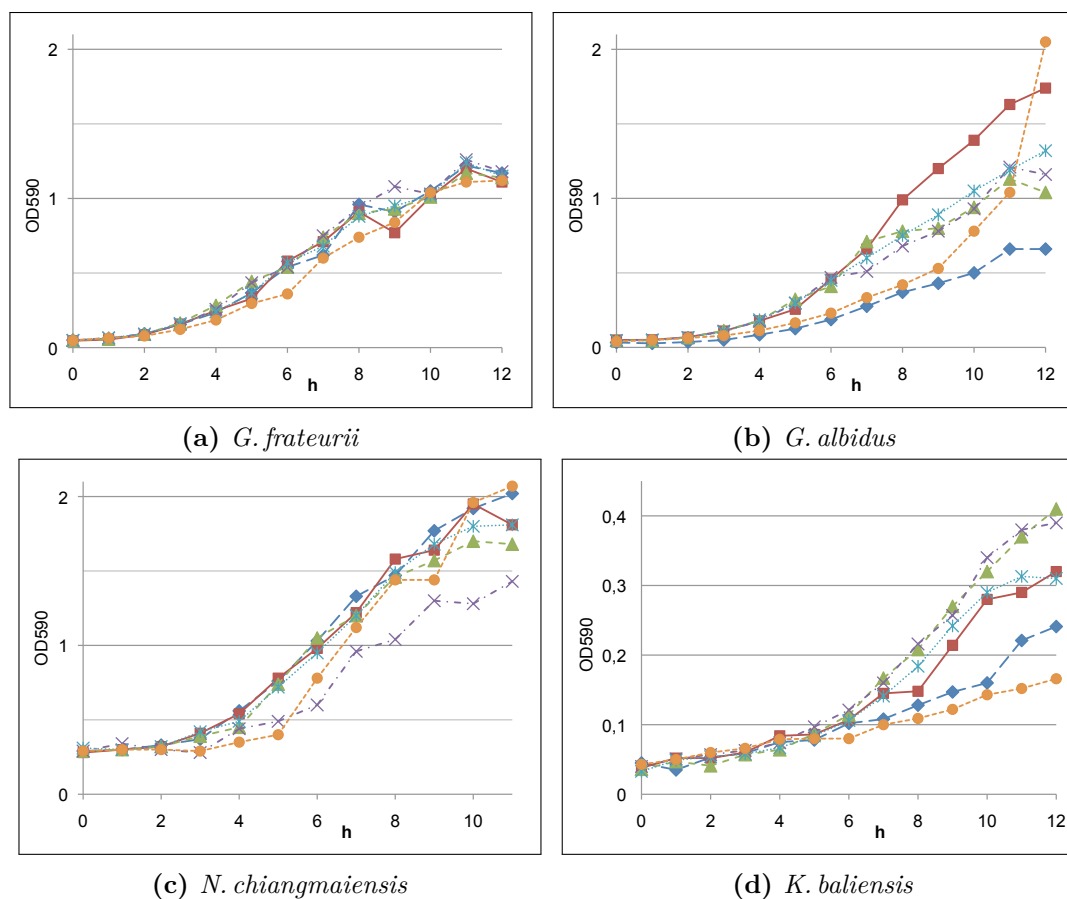
(d) *K. baliensis*

Figure 3.3 – OD_{590} of fermentations with different carbon sources with *G. albidus*, *G. frateurii*, *N. chiangmaiensis* and *K. baliensis*. ♦: Sucr (A); ■: Sucr + Man (B); ▲: Sucr + Glc (C); ×: Sucr + Frc (D); ∗: Sucr + Mal (E); •: Sucr + SoGlu (F).

pared to the other strains (Figure 3.3c). After a short adaption phase, OD_{590} of medium A (Sucr), B (Sucr + Man), C (Sucr + Glu) and E (Sucr + Mal) increased almost similarly between 3 and 8 h, before it differentiated to 2.02, 1.81, 1.68 and 1.81 in the end of fermentation, respectively. The exponential phase in medium F (Sucr + SoGlu) started about two hours later than in the other media, but in the end of fermentation, OD_{590} lay at 2.07. The increase in OD_{590} of medium D progressed slowly, resulting in the lowest final value of 1.43 after 11 h.

In fermentations with *K. baliensis*, OD_{590} increased only slowly and remained at a very low level after 12 h, compared to the other strains (Figure 3.3d). During the first 5 h, it stayed below 0.1 for all media. After 12 h, OD_{590} was highest in medium C (Glc) and D (Frc), laying at 0.40 ± 0.01, and second highest in medium B (Man) and E (Mal), where it amounted 0.32 and 0.31, respectively. In medium A and F, which contained only sucrose and sucrose with added sodium gluconate, OD_{590} remained at 0.24 and 0.17, respectively, and was therefore the lowest.

3.3.2 Growth, EPS and other metabolites of *G. frateurii* in media with varying carbon sources

G. frateurii was identified as a good and robust EPS-producer, but showed very low EPS formation in GfK medium which contained glucose as additional carbon source. Therefore in these experiments, the influence of additional carbon sources on growth, EPS formation and other metabolites of *G. frateurii* should be tested by eliminating a possible impact of other nutrient components.

In both fermentations, cell counts of *G. frateurii* developed similarly to each other. After 24 h, they reached $2.8 \cdot 10^8 \pm 3.5 \cdot 10^7$ CFU/ml in medium B (Sucr + Man), followed by medium E (Sucr + Mal), with $2.1 \cdot 10^8 \pm 4.2 \cdot 10^6$ CFU/ml). The lowest cell count was found in medium D (Sucr + Frc), laying at $7.8 \cdot 10^7 \pm 5.7 \cdot 10^6$ CFU/ml. In medium C and F, maximum growth was reached after 30 h and amounted $1.9 \cdot 10^8 \pm 2.1 \cdot 10^7$ CFU/ml and $2.4 \cdot 10^8 \pm 1.6 \cdot 10^7$ CFU/ml, respectively. After the maximum, cell counts remained stable in all media until the end of fermentation. In the second trial, peak cell counts of about $2.6 \cdot 10^8$ CFU/ml were again reached after 24 h in medium A, B, C, D and F, whereas in medium E, the maximum was reached after 48 h and lay at $6.9 \cdot 10^8 \pm 4.2 \cdot 10^7$ CFU/ml, respectively. In medium A, B and F, a local minimum was observed between 24 and 48 h, followed by a second local maximum. In medium C and D, cell counts decreased steadily until the end of fermentation. However, in both fermentations the variations in cell counts were negligible after 24 h.

In the first fermentation, pH in media A, B, C and D dropped rapidly from 6.05 ± 0.1 to 3.01 ± 0.08 within the first 24 h, followed by a further decrease to 2.75 ± 0.06 until the end of fermentation. During the same time, pH of media E and F reached 4.6 and 3.5 and further dropped to 3.4 and 2.8 after 72 h, respectively. In the second trial, pH of media A, B, C, D and E fell below 3.4 within the first 24 h and further dropped to 2.9 or lower thereafter. In medium F, the drop of pH was again slower than in the other media and still amounted 3.2 after 48 h, whereas after 72 h, it ended at 2.84.

In both replicates, the production of EPS started after 6 h of incubation (Figures 3.4a

and 3.4b). In the first trial (Figure 3.4a), most EPS was produced in medium A (Sucr) after 24 h (92.1 ± 18.8 mM Frc equ/l), followed by medium F (Sucr + SoGlu) after 72 h (90.2 ± 29.4 mM Frc equ/l). In the other media, maximum EPS yields were found after 30 h (medium B, C, D) and 72 h (medium E), laying between 51.6 ± 5.5 mM Frc equ/l (B) and 73.5 ± 36.4 mM Frc equ/l (C). After reaching a peak, EPS yields decreased slightly or remained stable in most media except E and F, where the fermentation was finished at maximum EPS level. At the time of maximum EPS yields, between 34 % (medium C) and 90 % (F) of the initial sucrose was consumed. In medium A, where the EPS peak occured quite early, about 68 % of start sucrose had been degraded at this time point. In the second fermentation (Figure 3.4b), maximum EPS yields were again recovered from medium A and F, whereas in medium F, the EPS peak was reached after 48 h (91.0 ± 0.8 mM Frc equ/l) and after 30 h in medium A (83.5 ± 3.5 mM Frc equ/l). In the other four media, EPS yields increased to around 50 mM Frc equ/l within 24 h, while the lowest overall production was measured in medium E (Sucr + Mal) after 48 h and lay around 48.9 ± 1.6 mM Frc equ/l. Again the highest and lowest sucrose consumption at the time of peak EPS level were found in medium F (Sucr + SoGlu) and C (Sucr + Glc) and lay at about 78 % and 33 %, respectively.

(a) 1st fermentation   **(b)** 2nd fermentation
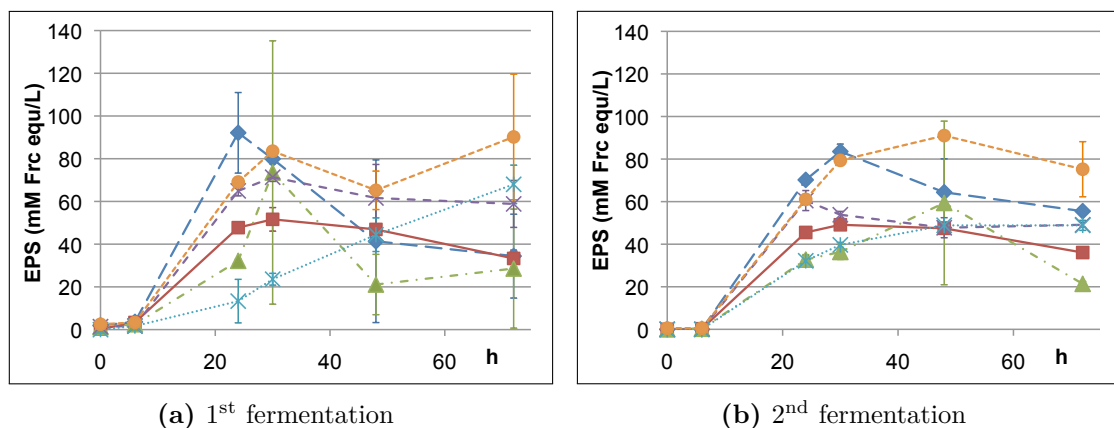
Figure 3.4 – EPS production of *G. frateurii* depending on different sugar compositions in two independent fermentations. ◆: Sucr (A); ■: Sucr + Man (B); ▲: Sucr + Glc (C); ×: Sucr + Frc (D); ✳: Sucr + Mal (E); ●: Sucr + SoGlu (F). Average and standard deviation of two independent measurements given.

During both trials in medium A (Sucr)(Figure 3.5), more than 60 % of sucrose was degraded between t_6 and t_{24}. After t_{24}, sucrose degradation almost stopped, so that in the end of fermentation, about 25 % and 23 % sucrose were left in the experiments. During the time of main sucrose degradation, EPS were produced until levels of 92.1 ± 47.7 and 83.4 ± 49.1 mM Frc equ/l after 24 and 30 h in fermentation I and II, respectively. After passing the peak, about 63 % (I) and 34 % (II) EPS of the peak concentration was degraded until the end of fermentation. In both experiments, glucose and fructose were present at low levels from the beginning on. However, fructose was formed additionally

during the fermentation and reached maximum levels after 24 h. In fermentation I, it increased from 57.3 to 74.1 mM/l and in fermentation II, from 47.1 to 102.9 mM/l. This was followed by a partly degradation to about 39 (I) and 37 % (II) of this maximal value. The glucose curves are similar to the fructose ones, but always lower. In the first trial, glucose yield changed from 29.7 (t_0) over 51.0 (t_{24}) to 12.2 mM/l (t_{72}), whereas in the second experiment, it started at 19.2 mM/l (t_0), increased to 68.6 mM/l (t_{24}) and then went down to 8.9 mM/l after 72 h. Small amounts of mannitol were detected in the end of fermentation I (4.1 mM/l) and at t_{24} and t_{30} of fermentation II (3.7 and 3.8 mM/l, respectively). Acetic acid was also found in low concentrations starting at around 1 mM/l (t_0) and increasing to about 9 mM/l after 72 h in both fermentations. During the first 24 h, 67.5 and 59.5 mM/l gluconic acid was formed in fermentation I and II, respectively, which was mostly degraded to 13 % and 11 % of peak level in the remaining fermentation time.

During both experiments in the mannitol-containing medium (B), about half of the initial sucrose was degraded, with the most rapid decrease occurring between t_6 and t_{24}. Highest EPS levels were found at t_{30}, laying at 51.6 ± 5.5 mM Frc equ/l in trial I and 49.1 ± 1.2 mM Frc equ/l in trial II. In both replicates, low start glucose concentrations were present which were degraded to about one half throughout the fermentation. Fructose levels started at 57.3 (I) and 44.7 mM/l (II), increased to 72.4 (I) and 79.2 mM/l (II) after 24 h and then decreased to 21.3 (I) and 37.6 mM/l (II) at t_{72}. During the first fermentation, about 58 % of start mannitol concentration was degraded by *G. frateurii*, whereas in the second trial, more than 90 % mannitol was consumed. Final acetic acid contents lay between 10 and 9 mM/l, whereas it was mainly produced during the last 24 to 42 h of fermentation. Gluconic acid maxima of 18 and 19 mM/l were reached after 24 and 30 h, respectively, followed by degradation to 1.2 - 2.8 mM/l at t_{72} in trial I and trial II.

In the glucose-containing medium (C), the degradation of sugars was similar in both experiments (Figure 3.5). During the first 24 h, about 25 % of the initial glucose was consumed. Sucrose consumption was weak and almost stopped after 30 h, where about 35 % of start sucrose had been degraded. EPS formation was rather low in both trials, with maxima of 73.6 ± 61.6 mM Frc equ/l at t_{30} (fermentation I) and 59.4 ± 38.5 mM Frc equ/l at t_{48} (fermentation II). Fructose was degraded almost linearly, resulting in levels between 74.3 mM/l (t_0) and 26.3 mM/l (t_{72}) in the first fermentation and 64.3 mM/l (t_0) and 49.5 mM/l (t_{72}) in the second fermentation. Starting at t_{24}, low amount of mannitol were found in both trials with final concentrations of about 10.9 mM/l. Like in medium A and B, acetic acid levels increased from about 1 to 9 and 10 mM/l throughout the fermentation. Maxima in gluconic acid were reached after 30 h and 24 h in trial I and trial II. They range between 22.2 – 29.2 mM/l and were followed by partly degradation to about 4 mM/l in both experiments.

In both fermentations in fructose-containing medium (D), the main sucrose degradation (more than 50 %) was noticed between t_6 and t_{24}, followed by only slight degradation until up to 45.6 mM/l (trial I) and 72.1 mM/l (trial II) at t_{72} . In the first fermentation, the EPS maximum was detected after 30 h and lay at 71.3 mM Frc equ/l, followed by a slight degradation to 58.8 mM Frc equ/l at t_{72}. In the second fermentation, the maxi-

mum EPS concentration was reached after 24 h and amounted only 60.5 mM Frc equ/l, whereas EPS were partly degraded to 49.1 mM Frc equ/l in the end of fermentation. In the beginning and end of both fermentations, fructose levels lay quite similar between 40 and 60 mM/l, whereas peaks of 88.2 and 56.0 mM/l were measured after 24 h and 6 h in fermentation I and II, respectively. In the first experiment, a glucose concentration of 42.8 mM/l has been detected after 24 h, which decreased to 19.6 mM/l at t_{72}. In the second fermentation, the glucose level was 27.3 ± 1.3 mM/l throughout the whole time. After 24 h, small amounts of mannitol were found in both replicates, reaching 7.9 mM/l at maximum. Acetic acid contents increased from 1.4 to about 9.4 ± 0.5 mM/l throughout both trials. Peaks in gluconic acid were recognized after 24 and 30 h in fermentation I and II and lay at 46.0 and 19.4 mM/l, respectively. Until the end of fermentation, gluconic acid levels decreased to 22.3 and 3.3 mM/l, respectively.

In the maltose-containing medium (E), the sucrose concentration decreased steadily to about 65 % and 54 % of the start level, while EPS yield were constantly rising throughout the whole fermentation to maxima of 68.0 ± 9.0 and 48.9 ± 2.4 mM Frc equ/l (fermentation I and II, respectively). Glucose was present at levels between 32.9 mM/l and 10.5 mM/l throughout both fermentations, with (local) minima after 30 h. Only traces of fructose were found in fermentation I, whereas in fermentation II, a peak of 77.4 mM/l fructose was found after 6 h, which was degraded to 61.3 mM/l at t_{72}. Very low levels of mannitol were found in the first experiment between t_{30} and t_{72}. Maltose levels decreased by about 35 % in fermentation I and 28 % in fermentation II. Acetic acid was formed during the fermentation and final levels amounted about 9 mM/l. Gluconic acid peaks amounted 7.4 and 20.2 mM/l and were detected after 30 and 24 h in fermentation I and II, respectively.

During both fermentations in the sodium-gluconate containing medium (F), almost all of the sucrose was utilized by *G. frateurii* with the most rapid decrease between t_6 and t_{24}. At the same time, EPS production started, whereas peak EPS levels were determined at t_{72} in the first trial and at t_{48} in the second trial, amounting 90.2 ± 29.4 and 91.0 ± 0.4 mM Frc equ/l, respectively. Fructose and glucose were present at stable concentrations throughout both fermentations with start levels of 53.8 mM/l and 26.5 mM/l in the first and 32.5 mM/l and 18.4 mM/l in the second fermentation, respectively. Only between t_{24} and t_{30}, both sugar levels increased about twofold. Between t_{30} and t_{72}, low levels of mannitol could be measured. Acetic acid yields increased throughout the whole fermentation to final concentrations of 7.5 and 8.3 mM/l in fermentation I and II. Start levels of gluconic acid lay at 34.4 and 28.4 mM/l. At t_{48}, peaks of 89.7 and 74.3 mM/l were measured in trial I and II, respectively, followed by degradation relating to the start level (I) or lower (II).
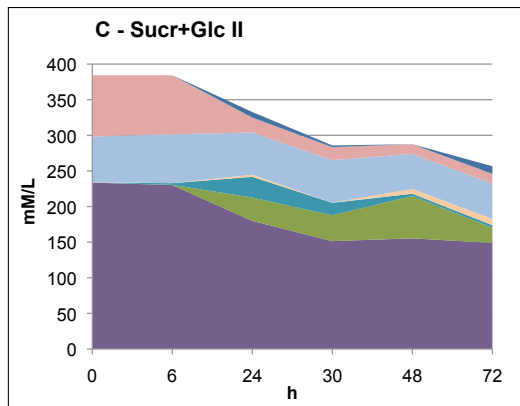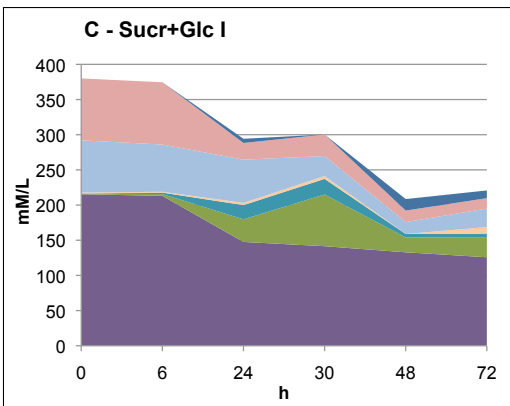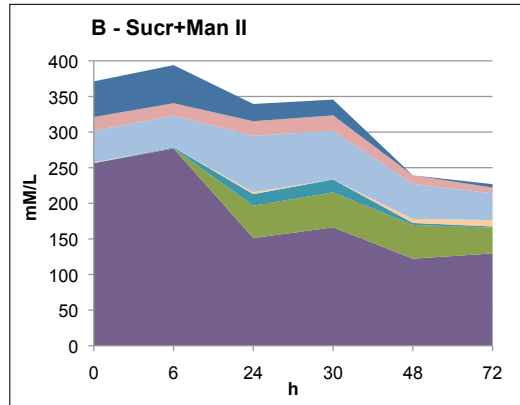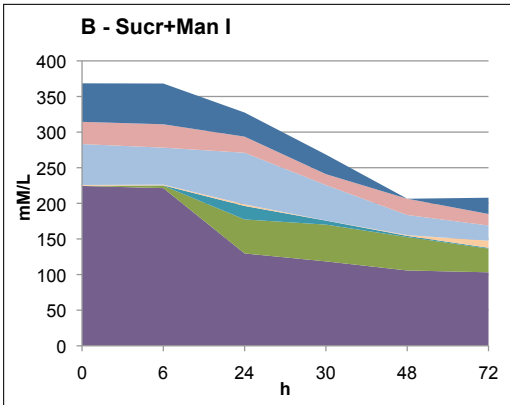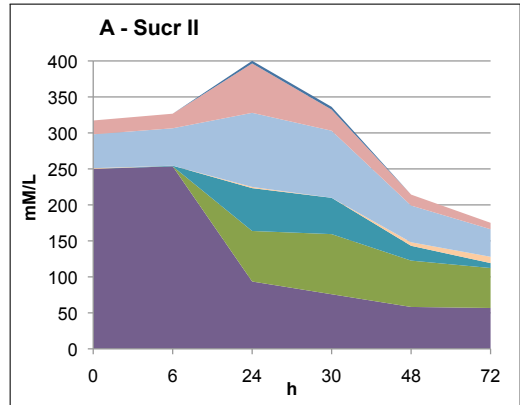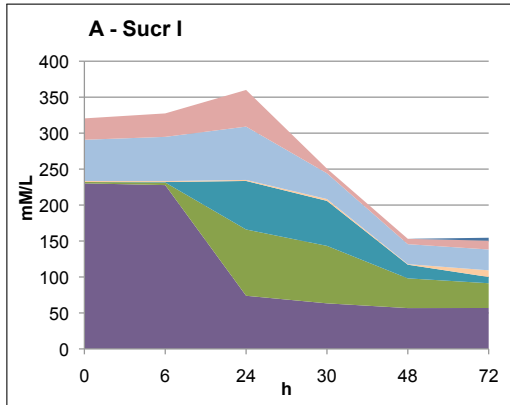
Figure 3.5 – *G. frateurii*: Sugar utilization and production of metabolites depending on different sugar compositions during two independent fermentations (I + II). Determined parameters shown as stacked areas. ■: Maltose; ■: Mannitol; ■: EPS; ■: Glucose; ■: Fructose; ■: Acetic acid; ■: Gluconic acid; ■: Sucrose.

3.3.3 Growth, EPS and other metabolites of *G. albidus* in media with varying carbon sources

Also *G. albidus* was identified as a good and robust EPS-producer, but showed very low EPS formation in GfK medium which contained glucose as additional carbon source. Therefore the influence of additional carbon sources on growth, EPS formation and other metabolites of *G. albidus* should be tested in these experiments by eliminating a possible impact of other nutrient components.

In the first fermentation, cell counts of *G. albidus* were quite similar in the six media. The highest number of cells was found in medium E (Sucr + Mal) at t_{24} ($1.2 \cdot 10^9 \pm 3.3 \cdot 10^8$ CFU/ml), followed by the mannitol-containing medium (B) ($6.6 \cdot 10^8 \pm 0.0$ CFU/ml). Lowest cell count after 24 h showed medium A (Sucr) ($6.2 \cdot 10^7 \pm 6.9 \cdot 10^6$ CFU/ml). While cell counts were decreasing after the maximum at t_{24} in media B $-$ E, it reached its maximum at t_{48} in medium F ($3.9 \cdot 10^8 \pm 1.3 \cdot 10^7$ CFU/ml). In all media apart from medium A, cell numbers decreased until the end of fermentation. The lowest final cell count was found in medium E ($1.7 \cdot 10^7 \pm 5.7 \cdot 10^5$ CFU/ml). In the second experiment, the maximum cell count was also reached in medium B after 24 h ($6.4 \cdot 10^8 \pm 2.8 \cdot 10^7$ CFU/ml). Again, the only other medium where maximum growth was found at t_{30} instead of t_{24}, was medium A ($5.6 \cdot 10^8 \pm 5.1 \cdot 10^7$ CFU/ml) and again cell numbers decreased until the end of experiment. In both duplicates, the highest cell number after 72 h was found in medium F, laying at ($1.8 \cdot 10^8 \pm 5.7 \cdot 10^6$ CFU/ml) and ($1.0 \cdot 10^8 \pm 1.3 \cdot 10^7$ CFU/ml) in trial one and two, respectively.

The pH values laid around 6.0 in the beginning of each fermentation. In the first experiment, pH dropped rapidly and reached pH 4.0 and 4.1 after 6 h in medium C and D (Sucr + Glu and Sucr + Fru). After 24 h, it had fallen to 3.0 in medium B, C, D and E and remained at this level until the end of the trial. In medium A and F (Sucr and Sucr + SoGlu), pH decreased steadily over the whole fermentation from pH 6.1 to pH 3.1. In fermentation 2, pH value behaved similarly in medium A $-$ E. It decreased to 3.1 ± 0.3 within 24 h and remained stable thereafter (pH at t_{72}: 2.7 ± 0.1). The pH of medium F (Sucr + SoGlu) decreased slower and stayed on a slightly higher level (2.9) after 72 h.

In both fermentations, EPS production started after 6 h of incubation (Figures 3.6a and 3.6b). In the first trial, the highest EPS levels were reached in medium B after 24 h (83.0 mM Frc equ/L) and in medium D after 30 h (68.8 mM Frc equ/l), whereas lowest levels lay below 27 mM Frc equ/l and were recovered from medium A and C. The third highest EPS concentration was found in medium E after 48 h (65.2 mM Frc equ/l) and medium F (44.7 mM Frc equ/l), whereas in medium E, EPS production started after 30 h of incubation. In the second experiment, highest EPS yields were recovered after 48 h of fermentation in medium F (96.3 mM Frc equ/l) and A (77.2 mM Frc equ/l), followed by B and E. Like in the first experiment, the lowest EPS production took place in the glucose-containing medium (C), where the maximum lay at 19.4 mM Frc equ/l. In all cultures except this one, EPS were partly degraded until the end of fermentation. Until EPS peaks were reached, 31.8 % (C) to 92.1 % (F) of initial sucrose has been consumed in the second experiment, whereas the conversion rate of initial sucrose to EPS lay between

8.8 % (C) and 41.1 % (F).

(a) 1$^{\text{st}}$ fermentation
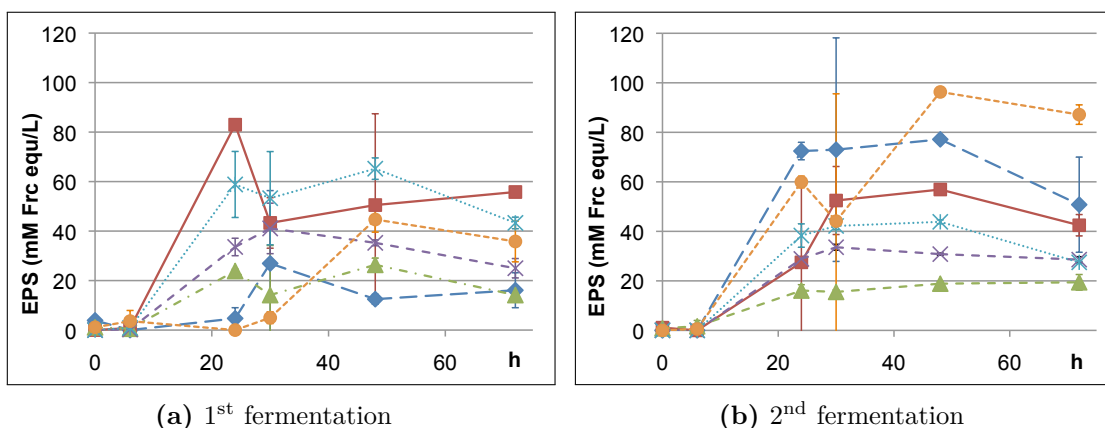
(b) 2$^{\text{nd}}$ fermentation

Figure 3.6 – EPS production of *G. albidus* depending on different sugar compositions in two independent fermentations. ♦: Sucr (A); ■: Sucr + Man (B); ▲: Sucr + Glc (C); ×: Sucr + Frc (D); ∗: Sucr + Mal (E); •: Sucr + SoGlu (F). Average and standard deviation of two independent measurements given.

Regarding the conversion of sugars, nearly no sucrose degradation could be observed in medium A (Sucrose only) during the first fermentation (Figure 3.7 top left). Only traces of EPS were produced, starting at t_{30}. After 72 h, 84 % of initial sucrose was still present. In the second experiment, sucrose degradation started after 6 h and reached 12 % of its initial level after 72 h (Figure 3.7 top right), whereas EPS production was related to the decrease in sucrose. Between 24 and 48 h, 74 ± 2.6 mM Frc equ/l EPS was detected. During the last 24 h of fermentation, EPS decreased to 50.6 mM Frc equ/l, accompanied by a slight increase in fructose to 57.9 mM/l, which had first been found after 6 h of fermentation (42.8 mM/l). Low glucose levels were determined during the whole fermentation, varying between 14.7 mM/l (0 h) and 23.9 mM/l (24 h). Acid production started after 24 h, whereas the gluconic acid concentration constantly increased to 65.3 mM/l in the end of fermentation. Acetic acid showed a peak after 48 h (110.4 mM/l), followed by a strong degradation to 7.5 mM/l when the experiment was stopped.

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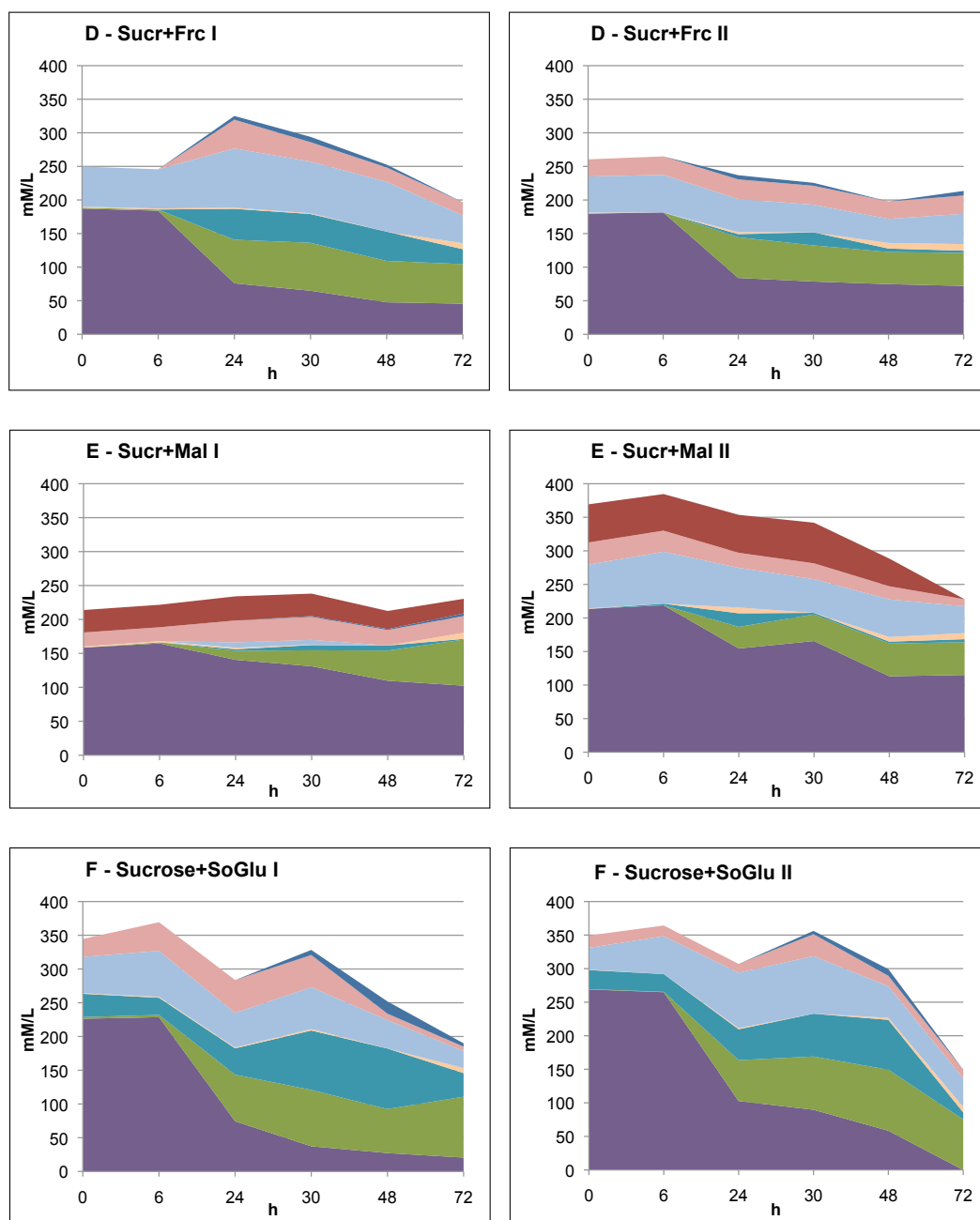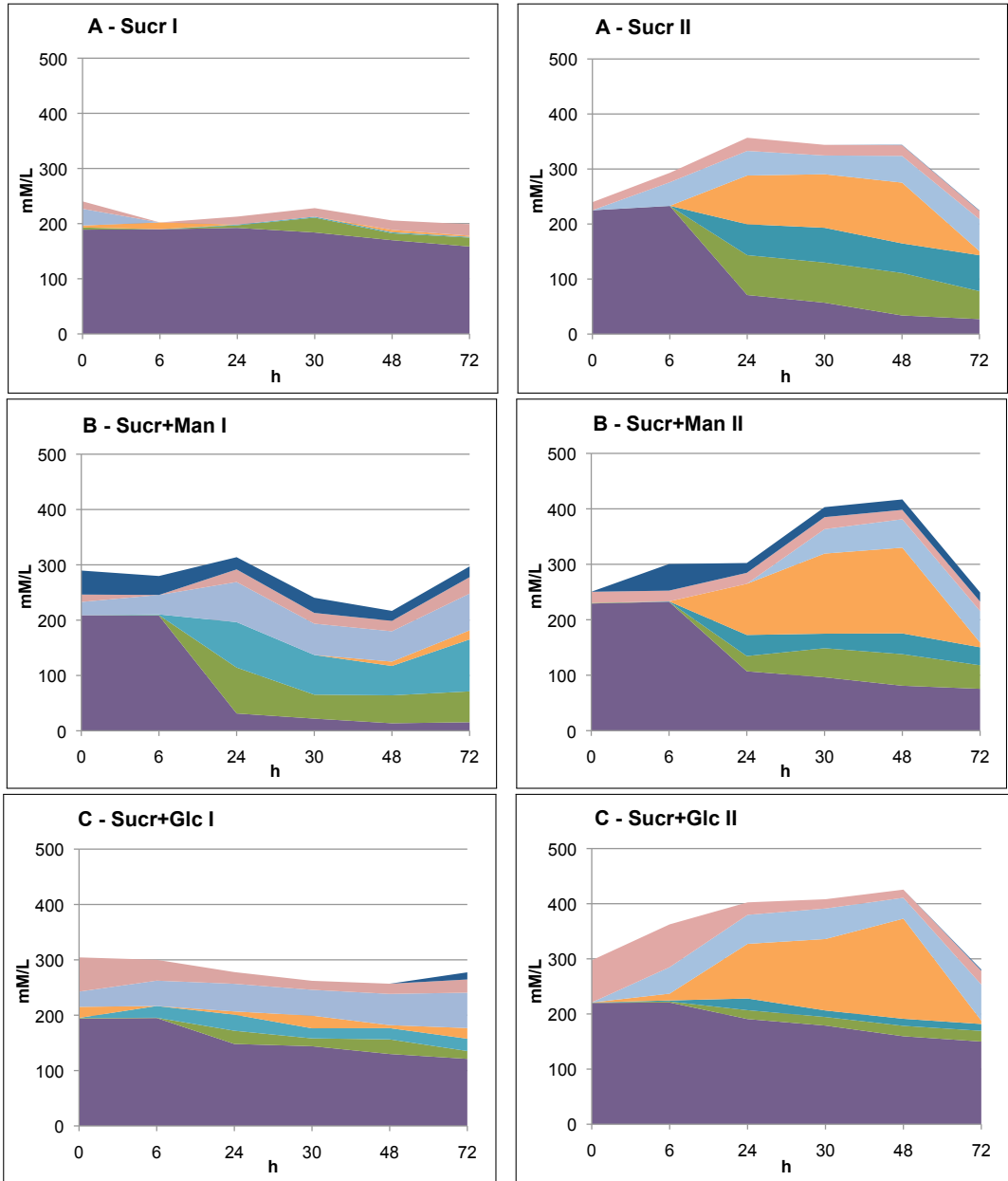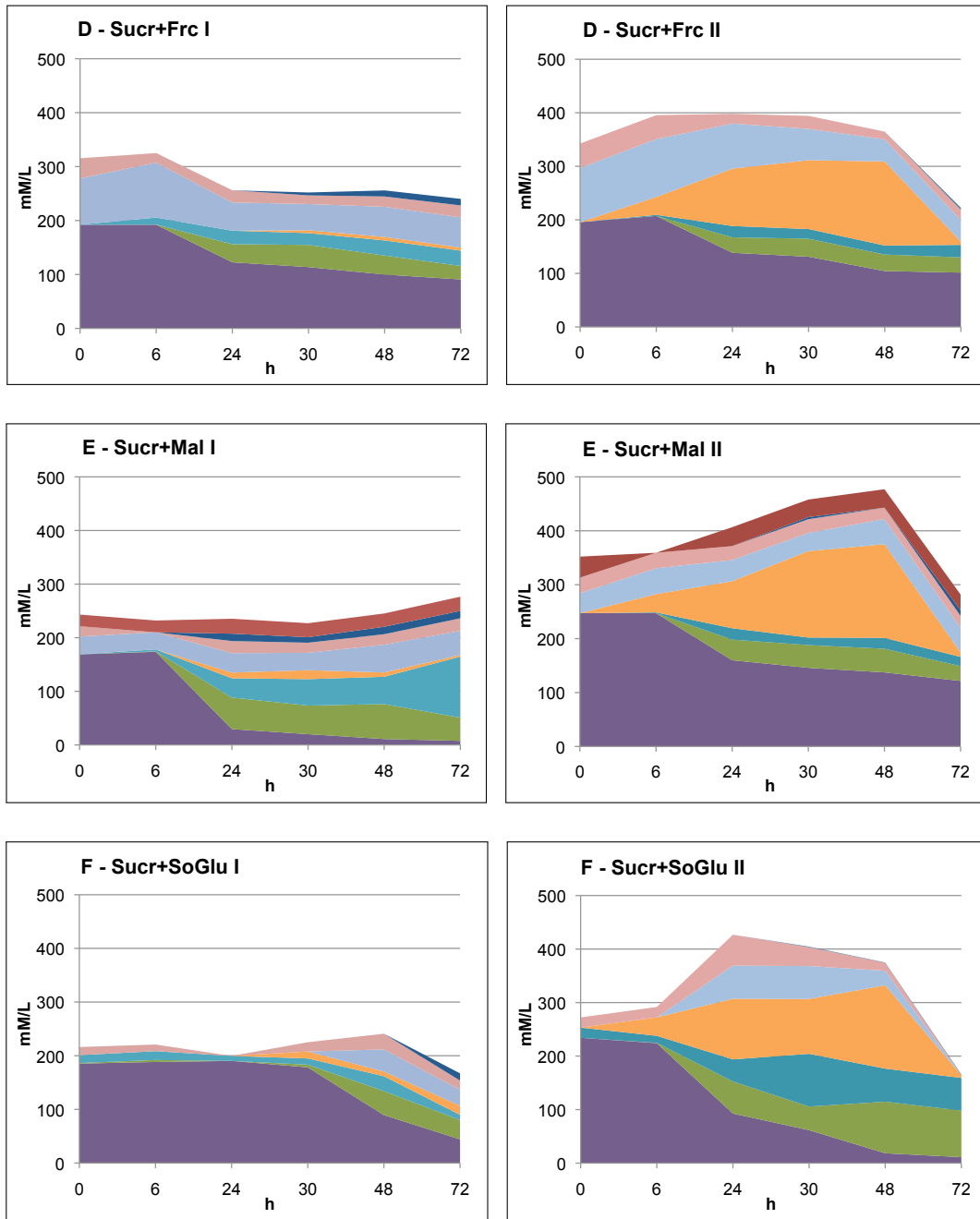**Figure 3.7** – *G. albidus*: Sugar utilization and production of metabolites depending on different sugar compositions during two independent fermentations (I + II). Determined metabolites shown as stacked areas. ■: Maltose; ■: Mannitol; ■: EPS; ■: Glucose; ■: Fructose; ■: Acetic acid; ■: Gluconic acid; ■: Sucrose.

During the first fermentation in medium B (Sucr + Man)(Figure 3.7 second top left),

sucrose has been degraded to 10.5 % within 30 h. Low but slightly increasing levels of glucose (13.4 − 29.8 mM/l between 0 − 72 h) and about two to three times higher fructose levels which were also increasing (23.9 − 66.5 mM/l between 0 − 72 h) could be measured. The added mannitol was partly degraded to 44.6 %, related to the original dosage, after 72 h. EPS reached a peak level of 83 ± 0.4 mM Frc equ/l after 24 h and was degraded to 55.8 ± 0.4 mM Frc equ/l after 72 h. Low acetic acid concentrations were first detected after 48 h (7.8 mM/l) and increased to 16.4 mM/l. Gluconic acid formation started after 6 h (1.3 mM/l) and reached 93.9 mM/l in the end of experiment. In the second duplicate of medium B, sucrose level decreased slower and ended at a higher level in the end of fermentation, laying at 75.5 mM/l. The maximum EPS level was found at t_{48} (56.9 ± 2.0 mM Frc equ/l) and was followed by a slight decrease to 42.5 ± 4.3 mM Frc equ/l in the end of trial. Similar to the first fermentation, low concentrations of glucose (19.2 ± 1.9 mM/l) and increasing fructose levels between t_{30} (44.1 mM/l) and t_{72} (56.9 mM/l) were detected. Mannitol was degraded during the fermentation to about one third of the original dosage. In opposite to the first trial, high acetic acid concentrations were remarked between 24 h (44.1 mM/l) and 48 h (154.5 mM/l), which were degraded to 8.5 mM/l after 72 h. Gluconic acid increased between 6 h and 24 h to 38.1 mM/l and remained at an average level of 32.2 mM/l thereafter.

In both fermentations of the glucose-enriched medium (C)(Figure 3.7), sucrose was degraded slowly and lay at 62.6 and 68.2 % of the original level in the end of fermentation. The final glucose levels lay at 38.6 % (I) and 32.6 % (II) related to the beginning, whereas gluconic acid contents increased during the first day to 29.1 mM/l and 21.1 mM/l in the first and second replicate, respectively, followed by a slight decrease. In the first fermentation, the EPS peak occured after 24 h and lay at 23.9 mM Frc equ/l, whereas fructose levels ranged from 27.5 mM/l in the beginning to 64.0 mM/l in the end of fermentation. Acetic acid concentration showed a peak after 30 h which lay at 22.7 mM/l and decreased slightly to the end of fermentation. In the second fermentation, EPS maximum was detected after 72 h (19.4 mM Frc equ/l). After 6 h, 48.1 mM/l fructose was determined, which slightly increased to 65.4 mM/l after 72 h. Acetic acid was first detected after 6 h and its yield increased during the first two days up to 181.7 mM/l. After 72 h, low levels of mannitol were found in both fermentations, amounting 12.9 mM/l (I) and 2.5 mM/l (II).

Also in medium D, which contained initial fructose, sucrose consumption was low for *G. albidus*, as within 3 days, only about half of initial sucrose was degraded in both fermentations with the most rapid decrease happening between t_6 and t_{24} (Figure 3.7). In both duplicates, glucose levels fell from about 40 mM/l to 20 mM/l within 72 h. At the same time, 34.6 % and 60 % of initial fructose was consumed in fermentations I and II, whereas the EPS peaks lay at 30 h and amounted 41.1 and 33.6 mM Frc equ/ml. After this time point, a partial degradation of EPS occurred during both fermentations. In fermentation I and II, gluconic acid level increased steadily after 6 h to 28.5 and 23.27 mM/l in the end of fermentation. A difference was perceived in acetic acid production, which occurred only in amounts below 7 mM/l in fermentation I and reached 157 mM/l after 48 h in fermentation II, followed by a strong degradation to 6.1 mM/l. In the first experiment mannitol was measured between 30 and 72 h, ranging from 5.3

to 12.1 mM/l, whereas in the second experiment, it was measured only after 48 and 72 h in much lower amounts.

In the first fermentation in maltose-containing medium (E), the start sucrose content was consumed almost completely until 72 h (Figure 3.7). EPS level increased up to 48 h and reached 65.2 ± 4.3 mM Frc equ/l. Fructose amounted $32.0 - 36.2$ mM/l within the first 30 h and increased to a final concentration of 45.1 mM/l after 72 h. Gluconic acid increased linearly to 113.8 mM/l after 72 h. Maximum values of acetic acid were measured after 30 h and amounted 16.7 mM/l. During the second experiment, only 50.8 % sucrose were degraded.

An EPS peak was found after 48 h and lay at 43.9 mM Frc equ/l, whereas the fructose level remained relatively stable at around 41.8 mM/l. In contrast to the first fermentation, gluconic acid reached a concentration of only 20.17 mM/l after 48 h and acetic acid concentration increased to 173.7 mM/l after 48 h, followed by a strong decrease. In both fermentations, glucose was present in low concentrations at around 20 mM/l throughout the experiment. Maltose levels remained constant in both duplicates, corresponding to start concentration. Low concentrations of mannitol were detected in fermentation I starting from t_{24} and in fermentation II starting from t_{30}.

In the first trial in sodium-gluconate containing medium (F), sucrose remained constant for the first 30 h, before its concentration was decreased to 23.9 % of initial concentration (Figure 3.7). In this replicate, only low EPS yields were recovered which reached a peak after 48 h (44.7 mM Frc equ/l). Glucose levels lay around 18.2 ± 6.5 mM/l during the whole fermentation with a peak of 29.3 mM/l after 48 h. Fructose was detected after 48 and 72 h of fermentation and amounted 41.2 and 30.1 mM/l, respectively. Only low acid concentrations were measured, with a peak of gluconic acid after 48 h (27.8 mM/l) and acetic acid after 72 h (16.8 mM/l). After 72 h, 13.4 mM/l mannitol was detected in the sample.

In the second trial in medium F, sucrose was degraded exponentially, until 4.7 % of initial sucrose was left after 72 h. Relatively high EPS levels were reached with a maximum of 96.3 mM/l after 48 h, followed by a slight decrease. Glucose concentration increased between 24 h from 19.0 to 57.6 mM/l and was then completely degraded until the end of fermentation, whereas fructose was first discovered after 24 h and also completely degraded in the following fermentation time. Very low levels of mannitol (1 mM/l in average) were found in samples taken at t_{30}, t_{48} and t_{72}. Gluconic acid reached a maximum after 30 h which lay at 98.5 mM/l, whereas the maximum in acetic acid was detected after 48 h and lay at 155.6 mM/l. Acids were partly (gluconic acid) or completely (acetic acid) degraded after reaching the peak.

3.3.4 Growth, EPS and other metabolites of *N. chiangmaiensis* in media with varying carbon sources

In the preceding trials, *N. chiangmaiensis* was shown to be a good and robust EPS-producer, but EPS formation in GfK medium which contained glucose as additional carbon source was very low. Therefore in these experiments, the influence of additional carbon sources on growth, EPS formation and other metabolites of this strain should be

tested by eliminating a possible impact of other nutrient components.

Although *N. chiangmaiensis* showed good growth in all media, a few differences were observed. In fermentation I, maximum cell counts were measured in all media after 30 h, apart from medium D (Sucr + Frc). In medium A and B (Sucr and Sucr + Man), they exceeded 10^9 CFU/ml, whereas in medium F, $9.2 \cdot 10^8 \pm 1.7 \cdot 10^7$ CFU/ml, in medium E, $7.4 \cdot 10^8 \pm 2.8 \cdot 10^7$ CFU/ml and in medium C, $5.9 \cdot 10^8 \pm 7.1 \cdot 10^7$ CFU/ml were reached. Cells in medium D grew for 48 h, at which time they reached almost $5 \cdot 10^8$ CFU/ml. After the maximum, cell numbers decreased. In the second fermentation, cell counts increased faster and peaks were reached already after 24 h in medium A $(6.8 \cdot 10^8 \pm 5.7 \cdot 10^7$ CFU/ml), B $(6.3 \cdot 10^8 \pm 2.1 \cdot 10^8$ CFU/ml) and F $(9.8 \cdot 10^8 \pm 1.1 \cdot 10^8$ CFU/ml). In medium B, a second maximum in cell count was measured at t_{48} and lay at $(6.6 \cdot 10^8 \pm 0.0$ CFU/ml). In the other three media, cell counts increased until t_{30} up to levels between $5.2 \cdot 10^8$ CFU/ml (C) and $(7.0 \cdot 10^8$ CFU/ml (E). In medium C and D, cell number decreased until t_{48} and remained stable thereafter. In medium A, B, E and F, cell counts remained almost at peak level for the rest of fermentation.

The pH values were quite similar in both fermentation series with *N. chiangmaiensis*. After a rapid decrease during the first 24 h where pH values fell from 6.1 ± 0.1 to 3.6 ± 0.3, they dropped slowly to an average end level of 2.9 ± 0.1 in the first fermentation and 2.4 ± 0.2 in the second one. In medium C (Sucr + Glc), pH was always slightly lower than in the other media and ended in fermentation II at the lowest overall value of 2.1. The pH of medium F (Sucr + SoGlu) tended to be slightly higher than the others, followed by medium D (Sucr + Frc) after t_{24}.

High levels of EPS laying around 100 mM Frc equ/l were found in medium A, B and D after 48 h in the first fermentation (Figure 3.8a). In medium F, the same EPS level was reached already after 30 h. In medium C and E, EPS levels increased during the whole fermentation and reached 70.8 ± 3.5 and 89.1 ± 1.2 mM Frc equ/l at t_{72}. The EPS level could be maintained in medium F until the experiment was finished, whereas a slight decrease was observed in medium A, B and D. At maximum EPS levels, 8 %, 12 %, 5 %, 16 %, 9 %, 23 % of original sucrose was still present in media A, B, C, D, E, F, respectively. In medium F, sucrose was completely degraded at t_{72}, whereas in the other media, sucrose levels at t_{72} lay between 4 and 9 % of the original level. In the second experiment, the highest EPS yield was reached in medium F (Sucr + SoGlu) after 48 h and amounted 121.6 ± 59.7 mM Frc equ/l (Figure 3.8b). In medium A, B, C and E, EPS levels increased throughout the whole fermentation and lay at 76.3 ± 5.1, 93.2 ± 7.1, 73.3 ± 13.3 and 88.8 ± 3.1 mM Frc equ/l at t_{72}. Only in medium D (Sucr + Frc), the EPS peak was reached at t_{48}, too, and amounted 91.3 ± 6.3 mM Frc equ/l. In medium A and F, a slight increase followed the maximum EPS levels. At the time of EPS maxima, about 23 %, 23 %, 15 %, 16 %, 14 % and 4 % of initial sucrose was still present in media A, B, C, D, E, F, respectively.

In medium A (Sucr), about 96 % of initial sucrose was degraded throughout the first fermentation, starting at t_6 (3.9). In the second fermentation, 23 % of initial sucrose was left in the end. This coincided with higher EPS yields in the first trial. In both replicates, an increasing fructose content starting at about 27 mM/l at t_0 and ending at

(a) 1$^{\text{st}}$ fermentation   **(b)** 2$^{\text{nd}}$ fermentation
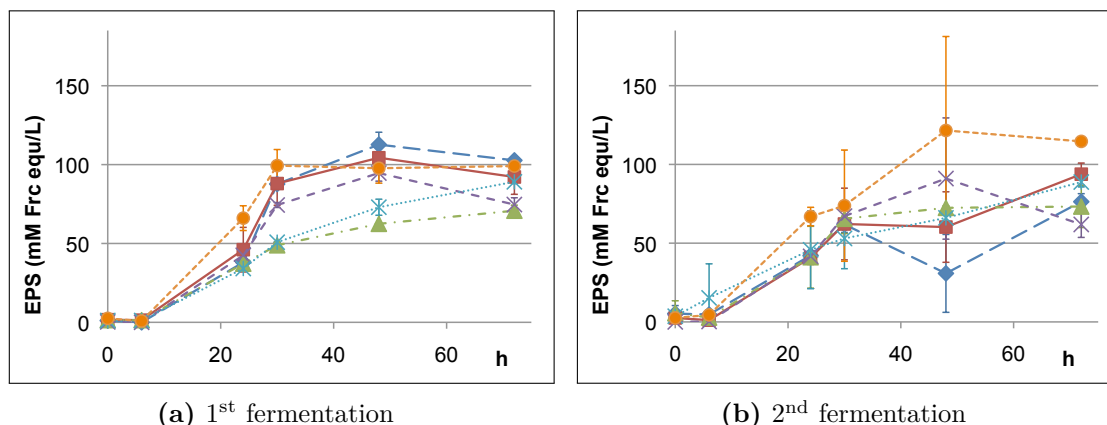
Figure 3.8 – EPS production of *N. chiangmaiensis* depending on different sugar compositions in two independent fermentations. ♦: Sucr (A); ■: Sucr + Man (B); ▲: Sucr + Glc (C); ×: Sucr + Frc (D); ∗: Sucr + Mal (E); •: Sucr + SoGlu (F). Average and standard deviation of two independent measurements given.

72.6 (I) and 79.7 mM/l (II) was measured, whereas levels remained constant during the first 6 h. Glucose levels started at around 11.7 mM/l and ended at 5.6 (I) and 11.7 mM/l (II) after 72 h, whereas peaks of about 87.4 mM/l were observed after 48 and 30 h in fermentation I and II, respectively. Between t_{24} and t_{72}, increasing mannitol yields were observed, which lay at 25.6 (I) and 31.9 mM/l (II) after 72 h. Acetic acid yields lay below 6.6 mM/l in both fermentations. Also gluconic acid was formed throughout the trials with peaks of 50.2 (I) and 20.1 mM/l (II) at t_{48}, followed by a degradation of around 80 - 90 %.

In the mannitol-containing medium (B), *N. chiangmaiensis* degraded about 95 % and 77 % of initial sucrose during the first and second fermentation, respectively, whereas sucrose consumption started at t_6. In trial I, EPS maximum lay at 104.4 ± 0.8 mM Frc equ/l (t_{48}). In the second duplicate, EPS levels increased throughout the whole fermentation up to 93.8 ± 7.1 mM Frc equ/l. Again a parallel increase of fructose was observed, reaching from 26.5 (I) and 36.1 mM/l (II) at t_0 to 53.4 (I) and 82.4 mM/l (II) at t_{72} with a peak of 87.3 mM/l occuring after 30 h in fermentation I. Glucose levels between 14.4 and 11.7 mM/l in the beginning and end of both replicates were found, whereas peaks of 52.2 (I) and 38.4 mM/l (II) were detected after 24 and 30 h, respectively. Mannitol content decreased from 43.8 (I) and 47.2 mM/l (II) to 24.2 (I) and 3.5 mM/l (II) within the first 24 (I) and 30 h (II), followed by an increase up to 40.9 (I) and 73.2 mM/l (II) in the end of fermentation. Up to 6.9 mM/l of acetic acid was measured throughout both trials. Gluconic acid levels increased during the first 48 h in the first experiment and amounted 56.1 mM/l. In the second experiment, the gluconic acid peak lay at 25.0 mM/l and was reached after 30 h. Following the peak, gluconic acid was degraded by about 90 % in both replicates.
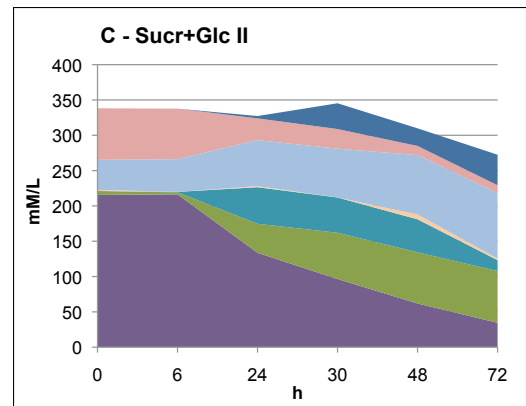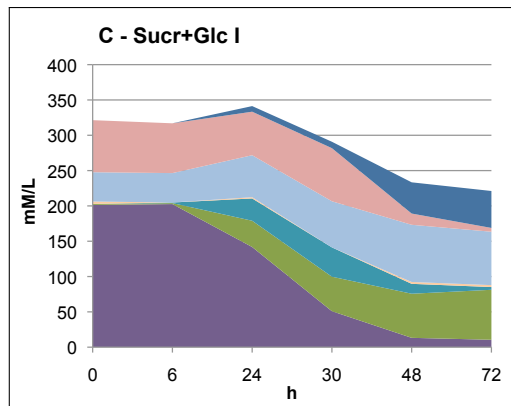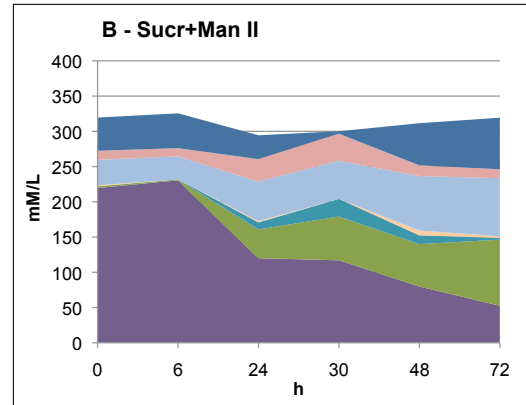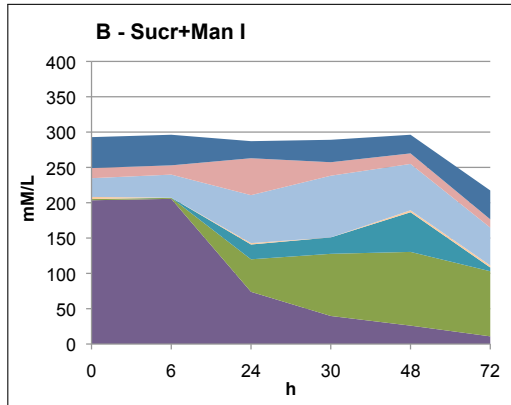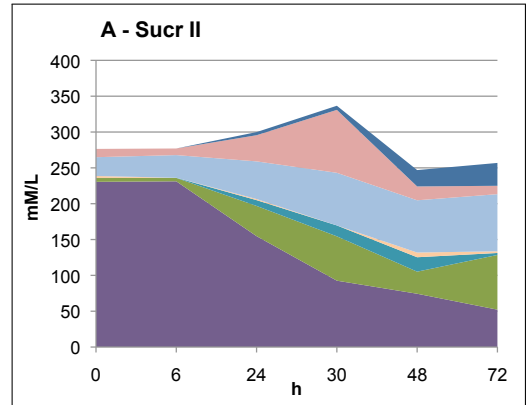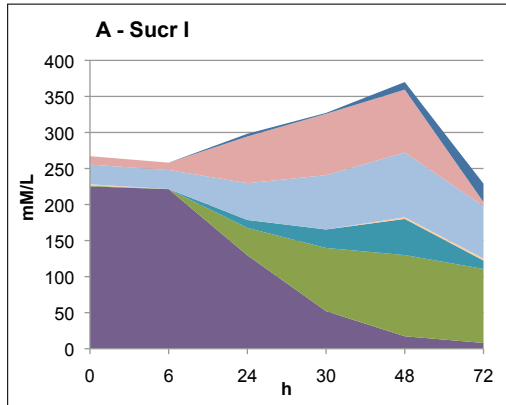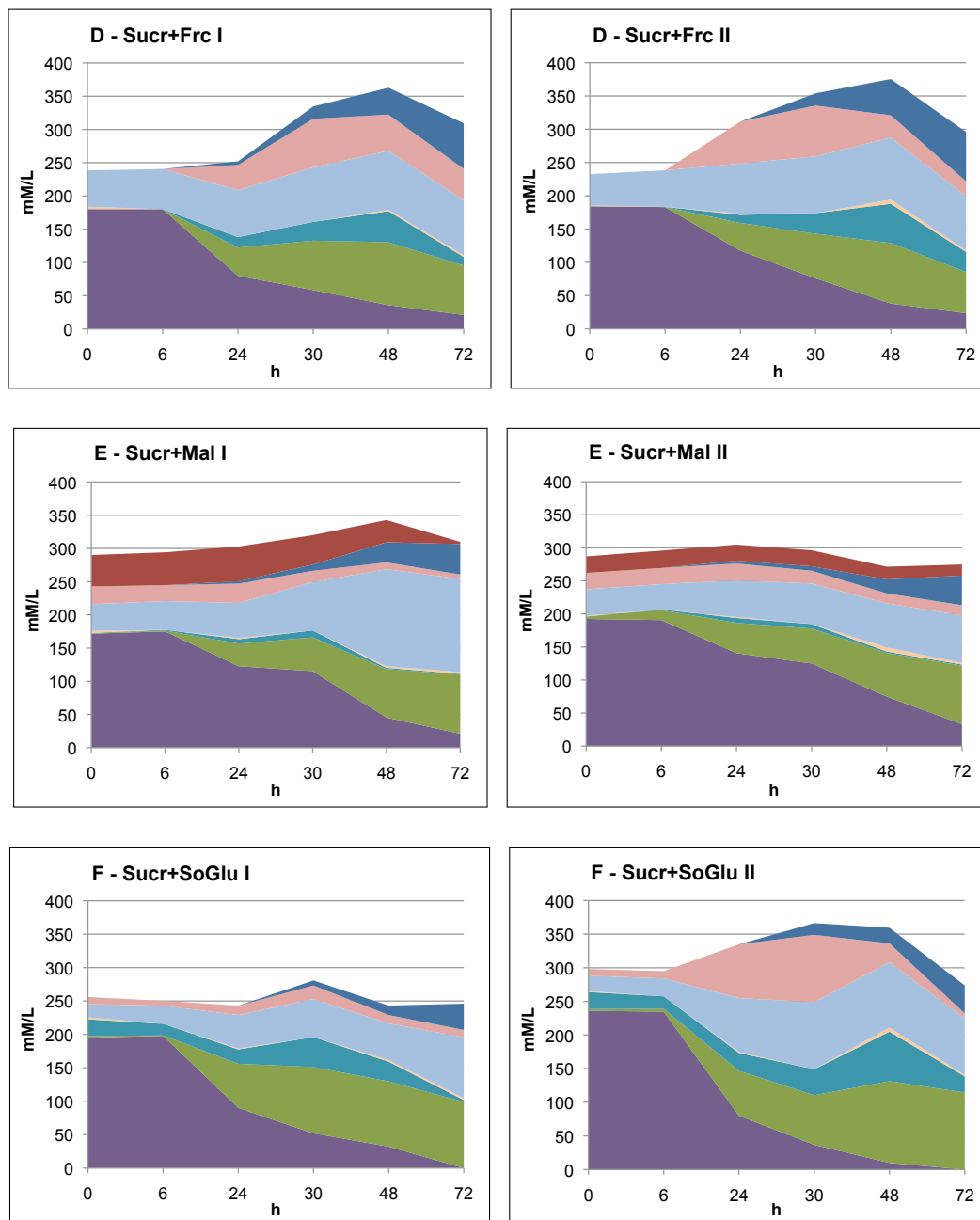
Figure 3.9 – *N. chiangmaiensis*: Sugar utilization and production of metabolites depending on different sugar compositions during two independent fermentations (I + II). Determined metabolites shown as stacked areas. ■: Maltose; ■: Mannitol; ■: EPS; ■: Glucose; ■: Fructose; ■: Acetic acid; ■: Gluconic acid; ■: Sucrose.

Also in medium C (Sucr + Glc), metabolic activity started after a lag-phase of 6 h

(3.9). Sucrose was consumed to about 95 % in the first and 85 % in the second fermentation. The sucrose decrease was accompanied by an increase in EPS yield, reaching the maxima of 70.8 ± 3.5 (I) and 73.3 ± 13.3 mM Frc equ/l (II) at t_{72}. In both replicates, a constant fructose increase was detected in the medium, which started at around 42 mM/l and reached 75.6 mM/l in the first and 92.6 mM/l in the second experiment. At the same time, a steady decrease in glucose was detected in both fermentations, ending up in the almost complete consumption of 93 and 85 % of start glucose in fermentation I and II, respectively. Mannitol was first found after 24 h at low levels which continuously increased to 52.3 (I) and 43.2 mM/l (II) at t_{72}. Acetic acid concentrations below 6.7 mM/l were observed during the whole experiment. Gluconic acid levels increased during the first 30 h in fermentation I and 24 h in fermentation II up to 41.5 and 51.8 mM/l, respectively. After reaching the peak values, gluconic acid was degraded to 9 and 30 % of peak levels during the remaining time.

In medium D (Sucr + Frc), about 88 % of initial sucrose was degraded throughout both fermentations after a lag-phase of 6 h without remarkable metabolic activity. At the same time, EPS yields increased and resulted in peak levels of 94.6 ± 5.1 and 91.0 ± 38.5 mM Frc equ/ml in fermentation I and II after 48 h, respectively. During the following 24 h, about 21 and 32 % of EPS were degraded. Despite high start concentrations, fructose levels increased during the first two days of fermentation from 54.6 to 88.8 mM/l in the first experiment and from 46.9 to 93.1 mM/l in the second experiment, followed by a slight degradation. Glucose was first determined after 24 h and reached maximum levels between 73.1 (I) and 76.4 mM/l (II) at t_{30}. Until t_{72}, glucose yields decreased to 47.0 mM/l in the first and 22.4 mM/l in the second fermentation. Mannitol was first detected after 24 (I) and 30 h (II). Its amount increased throughout the experiment to 68.5 and 74.2 mM/l in trial I and II, respectively. During the whole incubation time, acetic acid concentrations below 6.5 mM/l were recognized. Increasing gluconic acid levels with peaks of 46.7 (I) and 58.9 mM/l (II) at t_{48}, followed by 73 % – 49 % degradation, were detected.

In the maltose-containing medium (E), significant changes were recognized after 6 h. Sucrose was constantly degraded to about 15 % of initial dosage in both duplicates, while EPS contents rose steadily to final level of about 89 mM Frc equ/l (t_{72})(3.9). A start level of about 40 mM/l fructose was measured, which increased to 140.4 mM/l during the first and 71.9 mM/l during the second fermentation. Low glucose concentrations of about 26.8 (I) and 25.0 mM/l (II) were detected from the beginning on, whereas it was partly degraded in both trials starting after 24 h. Degradation of maltose started after 30 h of incubation. In the first fermentation, 7 % of start maltose was present after 72 h, whereas in the second fermentation, only 33 % of start maltose have been consumed. Mannitol was first detected after 24 h and increased continuously to levels around 45.4 mM/l until the end of both experiments. Low levels of acetic acid which never exceeded 6.5 mM/l where recognized during the duration of both trials. Gluconic acid content increased during the first 30 h in trial I and 24 h in trial II, reaching 10.5 and 6.8 mM/l, respectively. Until the end of fermentations, the major part of gluconic acid was degraded and remaining values lay between 1.1 and 1.5 mM/l.

In both replicates of the sodium-gluconate containing medium (F), sucrose was com-

pletely degraded between 6 and 72 h. In the first fermentation, the EPS level reached a peak of 99.4 ± 10.2 mM Frc equ/l at t_{30}. In the second experiment, EPS peak was detected after 48 h and lay at 121.6 ± 59.7 mM Frc equ/l, whereas maximum EPS levels were maintained until the end. During both experiments, fructose contents increased. In the beginning, fructose levels lay at 19.7 (I) and 22.9 mM/l (II) and amounted 91.2 (I) and 83.8 mM/l (II) after 72 h, whereas a peak of 99.8 mM/l occured after 30 h in fermentation II. At the start and end point of the first fermentation, about 10.8 mM/l glucose were measured and a glucose peak of 20.3 mM/l occured after 30 h. In the second fermentation, start and end glucose concentrations lay in the same range, but glucose level strongly increased between 6 and 30 h to a peak of 100.1 mM/l. A continuous increase in mannitol was found between t_{30} and t_{72} in both experiments, reaching 39.2 (I) and 41.6 mM/l (II) at t_{72}. Throughout both fermentations, low levels of acetic acid which never exceeded 6.1 mM/l were found. Gluconic acid levels started at about 25.3 mM/l and increased to 44.8 and 73.6 mM/l after 30 and 48 h in fermentation I and II, respectively, before it was partly degraded until the end of experiments.

3.3.5 Growth, EPS and other metabolites of *K. baliensis* in media with varying carbon sources

In precedent fermentations, *K. baliensis* was identified as the best and most robust EPS-producer. However, also this strain showed very low EPS formation in GfK medium which contained glucose as additional carbon source. Therefore in these experiments, the influence of further carbon sources in addition to sucrose on growth, EPS formation and other metabolites should be tested by eliminating the impact of other nutrient components.

In fermentation I with *K. baliensis*, the highest cell number was found in the sodium-gluconate containing medium (F) after 30 h ($1.3 \cdot 10^9 \pm 1.4 \cdot 10^7$ CFU/ml), followed by the glucose-enriched medium (C) after 24 h ($1.1 \cdot 10^9 \pm 1.7 \cdot 10^8$ CFU/ml) and the medium without additional carbon source (A), where also $1.1 \cdot 10^9$ CFU/ml were counted after 24 and 30 h. In the other media, maximum cell counts lay between about $6.5 \cdot 10^8$ CFU/ml (D) and $8.5 \cdot 10^8$ CFU/ml (B) at t_{24}, whereas in medium D, the lowest peak cell count was detected after 30 h of incubation. After reaching the maximum, cell counts decreased, apart from medium A, where it remained at maximum level for about 6 h. In the end of fermentation, cell counts had dropped to $1.6 - 4.3 \cdot 10^7$ CFU/ml. Only in medium F, about $4.5 \cdot 10^8 \pm 2.8 \cdot 10^7$ CFU/ml were left. In fermentation II, maximum cell counts were counted after 30 h in all media except A, where the maximum was arrived after 48 h of incubation and lay at $1.5 \cdot 10^9 \pm 1.1 \cdot 10^8$ CFU/ml. The second highest cell count occured in medium B (Sucr + Man) and amounted $1.6 \cdot 10^9 \pm 2.3 \cdot 10^8$ CFU/ml, followed by medium E with $9.9 \cdot 10^8 \pm 4.2 \cdot 10^7$ CFU/ml. In the other media, $6.2 \cdot 10^8$ CFU/ml were reached at minimum. After the peak level, cell numbers decreased to values between $3.3 \cdot 10^6$ CFU/ml (C) and $2.9 \cdot 10^7$ CFU/ml (A) after 72 h.

In the first fermentation, pH values of the six media fermented with *K. baliensis* dropped to values between 2.9 (B, E), 3.0 (A), 3.1 (C), 3.5 (D) and 3.9 (F) between t_6 and t_{30}. During the rest of fermentation, pH remained stable or increased slightly,

apart from medium D and F, where a further decrease to 3.2 (D) and 3.5 (F) was recognized. Final pH values of the other media amounted 3.1 ± 0.1. In the second duplicate, the decrease in pH value started after 6 h in medium B (Sucr + Man) and C (Sucr + Glc), after 24 h in medium D (Sucr + Frc) and E (Sucr + Mal) and after 30 h in medium A (Sucr) and F (Sucr + SoGlu). In medium B, C, D and E, pH values dropped to 3.5, 3.2, 3.5 and 3.9 at t_{30}, respectively, whereas in medium A, this level was found after t_{48}. Then, pH levels were maintained until the end of experiment. The pH of the sodium-gluconate containing medium (A) did not drop that far. It lay at 4.4 after 48 h and 4.3 after 72 h of fermentation.

High EPS levels were reached after 48 h in both duplicates of medium A, B, D, E and after 72 h in both trials in medium C and the first fermentation in medium F (Figures 3.10a and 3.10b). After 48 h, EPS yields ranged from 96.3 to 106.0 mM Frc equ/l in medium A, B, D and E. In the glucose-containing medium (C), the maximum EPS value was 96.0 mM Frc equ/l in the first and 90.0 mM Frc equ/l in the second trial after 72 h. In fermentation I with medium F (Sucr + SoGlu), 109.6 mM Frc equ/l were quantified at t_{72}, whereas the maximum level amounted 47.2 mM Frc equ/l in the second duplicate at the same time point. 24 h after the peak, $10-17\%$ of maximum EPS content were degraded in medium E (Sucr + Mal) and B (Sucr + Man), whereas in medium A (Sucr) and D (Sucr + Frc), still $99-100\%$ of peak EPS yield were found. When reaching the maximum EPS concentration, only $9.1 \pm 2.2\%$ of initial sucrose was left in all media apart from the second fermentation in medium F. Here, only 22 % of initial sucrose had been consumed until this time point.

(a) 1$^{\text{st}}$ fermentation   **(b)** 2$^{\text{nd}}$ fermentation
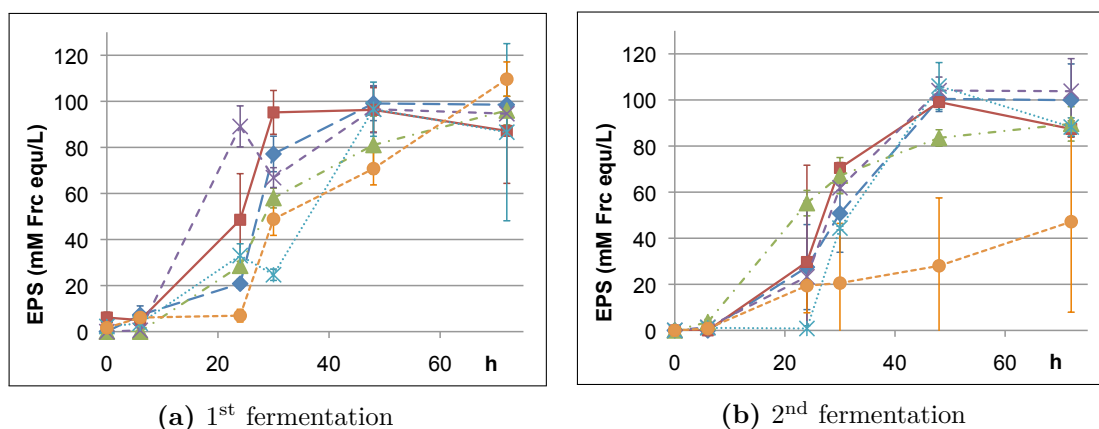
Figure 3.10 – EPS production of *K. baliensis* depending on different sugar compositions in two independent fermentations. ◆: Sucr (A); ■: Sucr + Man (B); ▲: Sucr + Glc (C); ×: Sucr + Frc (D); ✳: Sucr + Mal (E); ●: Sucr + SoGlu (F). Average and standard deviation of two independent measurements given.

In medium A (Sucr), sucrose concentration decreased steadily after a short lag-phase of about 6 h, until only 11 % (I) and 9 % (II) of initial sucrose were left at the time of maximum EPS content at t_{48} (Figure 3.11). In the first trial, glucose and fructose levels

increased steadily from 13.4 mM/l (t_0) to 45.3 mM/l (t_{72}) and from 31.6 mM/l (t_0) to 103.1 mM/l (t_{72}), respectively. In fermentation II, in contrast, both sugars were first detected after 48 h (Frc) and 30 h (Glc) in amounts of 82.9 mM/l (Frc) and 20.6 mM/l (Glc), changing to 65.8 mM/l and 62.0 mM/l in the end of fermentation. Low levels of mannitol were found in both fermentations starting from t_{30} (I) and t_{48} (II) which increased up to 16.9 mM/l (I) and 11.4 mM/l (II) at t_{72}. During both replicates, traces of acetic acid were detected. Gluconic acid concentrations increased between t_0 and t_{30} (I), respectively t_{48} (II) up to 29.0 mM/l (I) and 50.7 mM/l (II), followed by degradation between 46 % (I) and 12 % (II) until the end of the trial.

In both experiments in mannitol-containing medium (B), the most part of sucrose was degraded between t_6 and t_{48}, whereas only 13 % (I) and 8 % (II) of initial sucrose were left. This finishing point coincided with the maximum EPS level. In the first trial, fructose and glucose were detected from the beginning on in levels of 38.5 mM/l and 16.0 mM/l, respectively, and increased steadily to 167.4 mM/l (Frc) and 59.6 mM/l (Glc) in the end of fermentation. In the second fermentation, both sugars were first detected after 24 h in concentrations of 53.5 mM/l (Frc) and 27.0 mM/l (Glc), whereas at t_{72}, 124.6 mM/l fructose and 63.0 mM/l glucose were determined. Initial mannitol was degraded by 62 % (I) to 72 % (II) between 6 and 24 h of fermentation. This was followed by an increase in mannitol up to 30.4 (I) and 19.0 mM/l (II) at t_{72}, which related to 61 % (I) and 40 % (II) of start concentration. During both fermentations, traces of acetic acid which were never exceeding 1.9 mM/l were detected. Gluconic acid formation started after t_6 and increased until t_{48}, where 36.5 mM/l were found in fermentation I and 52.6 mM/l in fermentation II. During the following 24 h, gluconic acid was degraded by 49 % (I) and 35 % (II).

In both fermentations using the glucose-containing medium (C), sucrose was cleaved steadily starting after t_6. After 72 h of incubation, only 7 % (I) and 12 % (II) of initial sucrose was left. The sucrose degradation was accompanied by a steady increase in EPS, reaching 96.0 ± 6.3 (I) and 89.6 ± 7.5 mM Frc equ/l (II) in the end of fermentations. Between 6 h and 30 h, initial glucose levels were decreased from 66.5 mM/l to 32.3 mM/l in the first trial and from 66.0 to 32.1 mM/l in the second trial. This was followed by an increase in glucose level until the end of fermentation which reached 85.1 mM/l and 83.0 mM/l in the first and second experiment, respectively. In fermentation I, fructose was detected only at t_{30} and t_{48} in high levels of 103.29 mM/l and 133.7 mmM/L, respectively. In the second fermentation, fructose levels changing from 50.6 mmM/l to 64.4 mM/l were detected between t_{30} and t_{72}. In the same period of time, mannitol concentrations which ranged from 9.9 mM/l to 12.9 mM/l were determined in the first fermentation and from 5.1 mM/l to 7.2 mM/l in the second fermentation. Throughout both duplicates, traces of acetic acid never exceeding 1.1 mM/l were quantified in both trials. Gluconic acid production started after 6 h and increased up to 29.6 mM/l at t_{72} (I) or up to 35.1 mM/l at t_{48} (II), followed by degradation to two third of peak value.
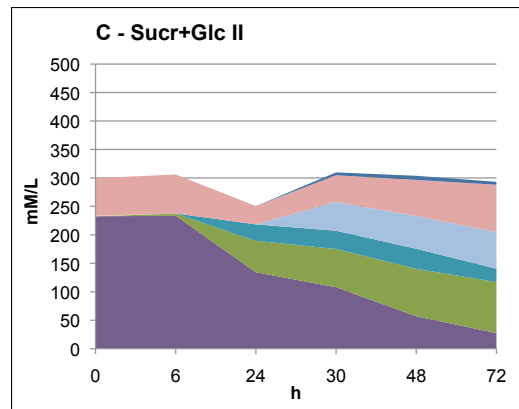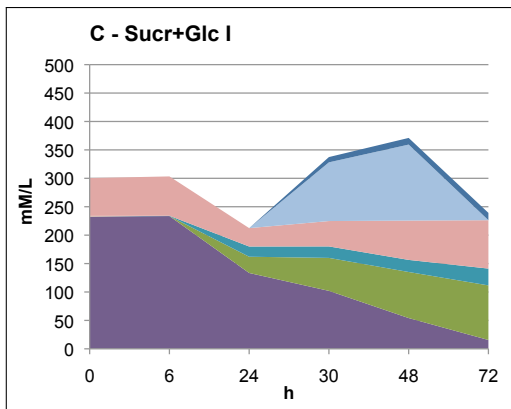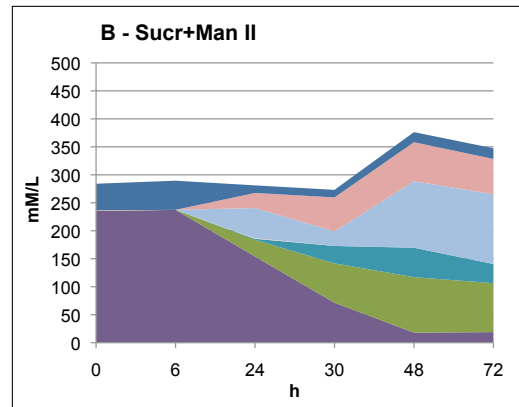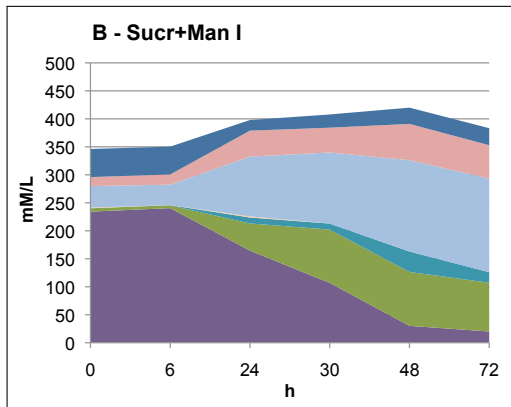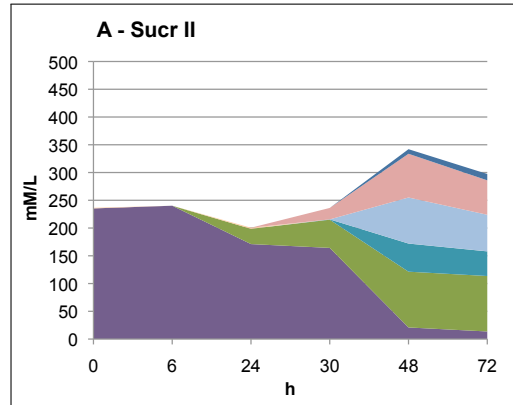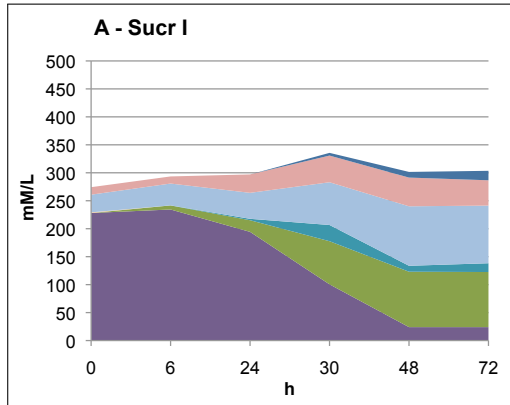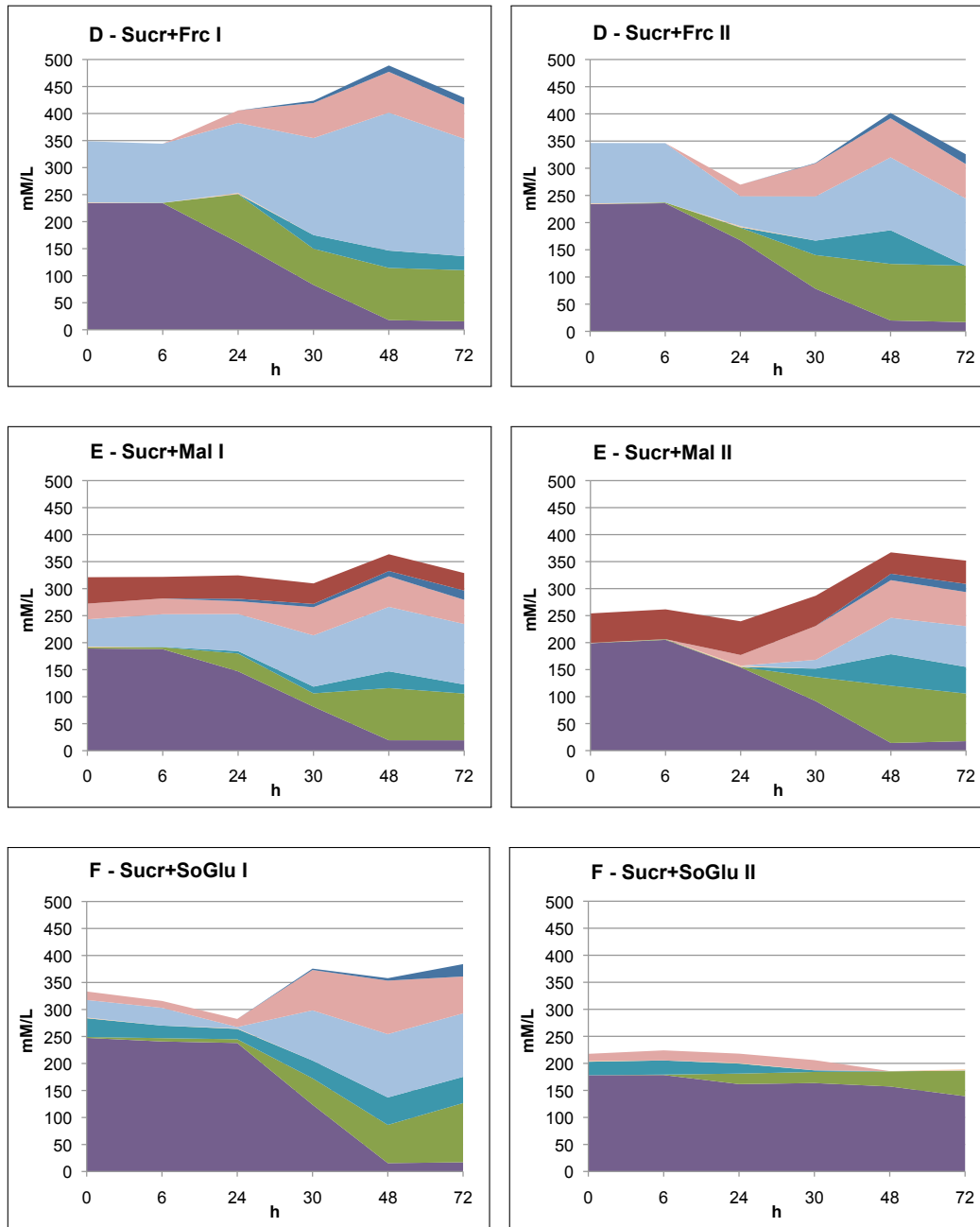
Figure 3.11 – *K. baliensis*: Sugar utilization and production of metabolites depending on different sugar compositions during two independent fermentations (I + II). Determined metabolites shown as stacked areas. ■: Maltose; ■: Mannitol; ■: EPS; ■: Glucose; ■: Fructose; ■: Acetic acid; ■: Gluconic acid; ■: Sucrose.

In both experiments using the fructose-enriched medium (D), sucrose was consumed

by 93 % of the initial dosage within 72 h of incubation. Maximum EPS values laying at 96.6 (I) and 104.0 mM Frc equ/l (II) were reached after 48 h, where 92 % (I) and 91 % (II) of sucrose was used up. Peak EPS levels were maintained until the end of fermentation. In the first experiment, fructose levels started to increase after t_{24}, reached a peak value of 255.1 mM/l at t_{48} and lay at 217.2 mM/l after 72 h. In the second replicate, fructose contents decreased to 55.2 mM/l between 6 and 24 h of incubation, followed by an increase to 123.5 mM/l at t_{72}. In both fermentations, glucose was detectable for the first time after 24 h in levels of around 22 mM/l and increased up to about 63.2 mM/l in the end of trials. Starting from t_{30}, low but increasing mannitol levels were detected which reached 12.9 mM/l (I) and 18.0 mM/l (II) at t_{72}. Acetic acid concentrations lay below 2 mM/l throughout both experiments. Gluconic acid levels increased between t_{24} and t_{48} up to 32.1 mM/l (I) and 61.2 mM/l (II), and were then partly degraded in trial I and completely degraded in trial II. In the maltose-containing medium (E), sucrose consumption started after t_6 and was degraded by 91 % until the end of fermentation. After 6 h in the first and 24 h in the second trial, main EPS production started and peak EPS levels laying at 96.6 mM Frc equ/l (I) and 106.0 mM Frc equ/l (II) were reached after 48 h, followed by a 10 % (I) and 17 % (II) degradation. In the first experiment, fructose amounted 50.4 mM/l at t_0. After 24 h, a strong increase started which led to a peak level of 119.3 mM/l at t_{48}, followed by a slight decrease. In the second trial, fructose was detected for the first time at t_{30} (16.4 mM/l) and increased up to 75.3 mM/l after 72 h. The glucose content was stable in the first duplicate until t_{24} (27.3 ± 3.1 mM/l) and showed a slight increase until the end of fermentation (44.8 mM/l). In the second trial, glucose was detected for the first time at t_{24} (19.8 mM/l) and increased to 63.1 mM/l during the rest of the experiment. Mannitol was detected since t_{24} (I) and t_{48} (II) in small amounts which increased to 17.1 mM/l (I) and 15.2 mM/l (II) until t_{72}. Throughout the whole fermentation time, only 34 % (I) and 21 % (II) of initial maltose were consumed, starting immediately in the first fermentation and after 30 h in the second one. Acetic acid was quantified only in traces of 2.1 mM/l or lower throughout both replicates. Gluconic acid levels increased between 6 h (24 h) and 48 h to 31.0 mM/l (58.4 mM/l) in fermentation I (II), followed by 46 % (16 %) degradation.

In fermentation I in the medium with sodium-gluconate (F), sucrose utilization started after t_{24} and was finished after 48 h, where only 6 % of initial sucrose was left. This was accompanied by an increasing EPS yield with a maximum at 109.6 mM Frc equ/l after 72 h. The increase of fructose and glucose started at the same time. Peak fructose and glucose levels of 117.5 mM/l (t_{72}) and 99.0 mM/l (t_{48}), respectively, were detected. The glucose maximum was followed by a slight decrease by 31 %. Between 30 h and 72 h, mannitol levels increased from 2.5 mM/l to 22.9 mM/l, whereas acetic acid always lay below 1.2 mM/l. Gluconic acid increased from a start level of 34.8 mM/l to 50.9 mM/l (t_{48}) and was then degraded by 4 %. In the second fermentation in medium F, only 22 % of sucrose was consumed by the bacteria. No gluconic acid was produced and initial gluconic acid levels were reduced by 98 % throughout the fermentation. Start glucose was degraded during the fermentation and no fructose, mannitol or maltose was detected. Acetic acid always lay below 2 mM/l. Beginning after 6 h of incubation, EPS was produced, resulting in a relatively low peak level of 47.2 mM Frc equ/l at t_{72}.

3.3.6 Influence of varying carbon sources on pH values during fermentations with selected strains

As EPS formation depends on the activity of the corresponding FTF which is in turn influenced by pH value, the dependence of pH value on strain and carbon source during fermentations was monitored. The pH values of all fermentations started at pH 6 and dropped to a level of about 3 during the first 48 h. Then, they mainly remained at this level, whereby some differences have been recognized by means of statistical data analysis. In Table 3.1, the final model for ANOVA of pH values is shown. All estimates refer to the intercept which includes *G. albidus* in medium A at time t_0. The model is to be used as follows: The pH value of a certain strain in a certain medium at a certain time is obtained by adding up the estimated levels of intercept, strain, medium and time:

$$\text{pH} = intercept + strain + medium + time \tag{3.1}$$

where the relevant numbers are listed in table 3.1. For example, the pH value of *N. chiangmaiensis* in medium D at t_{48} is predicted by:

$$\text{pH} = 5.98007 + 0.12403 + (-0.13479) + (-3.19604) \tag{3.2}$$

The asterisks in the last column highlight significant p-values of 0.05 or smaller. Obviously, pH values of *K. baliensis* are significantly higher than of the other strains, whereas pH of both *G. frateurii* and *N. chiangmaiensis* differ not significantly from the intercept, which refers to *G. albidus*.

Furthermore, pH of medium C is always significantly lower than of all other media, and pH of medium B is lower than of all other media apart from medium C. In medium F, pH is a significantly higher than the pH represented by intercept.

Besides this, also the drop of pH with time can be seen in the table.

As the effects of strain, medium and time are added up, pH value of *K. baliensis* in medium F, for example, was even higher compared to the other media and the other isolates. On the other hand, pH in medium C was always lower compared to the other media for all strains, but in fermentations with *K. baliensis*, it was not as low as pH for the other isolates due to the influence of the strain.

3.4 Sourdough fermentations

Based on the results obtained from section 2.3, the four strains *G. frateurii*, *G. albidus*, *N. chiangmaiensis* and *K. baliensis* were selected for the fermentations in sourdough. When being exposed to daylight for a few days, all strains showed a pink colony morphology, which is why they were easily distinguishable from the accompanying microbiota growing on Spicher agar. In the beginning of fermentations, dry matter of EPS and recovery rate from sourdoughs were determined to enable a conclusion from measured to real EPS values. After testing growth on various flours under different oxygen conditions, fermentation experiments were divided in gluten-containing and gluten-free

Table 3.1 – Coefficients of the linear model investigating pH values of fermentations with *G. albidus*, *G. frateurii* (*Gf*), *N. chiangmaiensis* (*Nc*) and *K. baliensis* (*Kb*) in media A – F between $0 - 72$ h. Intercept includes *G. albidus*, medium A and t_0. Significant differences are indicated by asterisks using the common coding for *p*-values: smaller than 0.001 ***; between 0.001 and 0.01: **; between 0.01 and 0.05: *

		Estimate	Std. Error	t value	PR($>$|t|)	
Intercept		5.98007	0.10537	56.753	$<$2e-16	***
Strain	*Gf*	0.10569	0.07965	1.327	0.185631	
	Kb	0.62889	0.07965	7.895	7.01e-14	***
	Nc	0.12403	0.07965	1.557	0.120598	
Medium	B	-0.20042	0.09755	-2.054	0.040883	*
	C	-0.37000	0.09755	-3.793	0.000183	***
	D	-0.13479	0.09755	-1.382	0.168184	
	E	-0.10604	0.09755	-1.087	0.277989	
	F	0.25042	0.09755	2.567	0.010791	*
Time (h)	6	-0.22958	0.09755	-2.353	0.019309	*
	24	-2.31833	0.09755	-23.765	$<$2e-16	***
	30	-2.73042	0.09755	-27.989	$<$2e-16	***
	48	-3.09521	0.09755	-31.728	$<$2e-16	***
	72	-3.19604	0.09755	-32.762	$<$2e-16	***

flours. Although every flour constituent apart from gluten can have an effect on bacterial metabolism, this division was useful for practical applications.

3.4.1 Total titrable acids and total titrable bases of selected flours

To reveal differences in the effect of organi acids which are formed during fermentations on pH value and therefore on enzyme activity and bacterial metabolism, the total titrable acids (TTA) and total titrable bases (TTB) were tested. TTA and TTB of gluten containing and gluten-free doughs are shown in Figure 3.12. Both values were highest for spelt dough and amounted about 4.8 (TTA) and 3.7 (TTB), whereas this was the greatest deviation between both parameters. Lowest TTA was found in whole wheat (2.0), CO (2.1) and browntop millet dough (2.1), followed by wheat (2.3). The TTA of oat, rye and rice dough lay in the middle at 2.7, 3.5 and 3.8, respectively. Concerning TTB, lowest values were measured in corn (1.7), whole wheat (2.5), wheat (2.6) and millet II dough (2.9). Again, oat, rye and rice lay in the middle and TTB values amounted 3.2, 3.1 and 3.6.

| **(a)** gluten containing flours | **(b)** gluten-free flours |
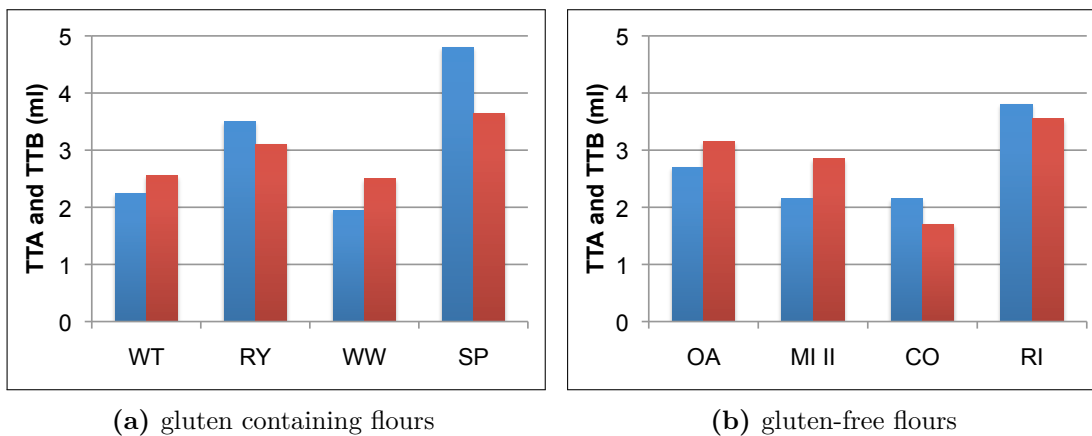
Figure 3.12 – Total titrable acid (■, left column) and total titrable base (■, right column) of doughs produced from different flours (DY=500) as indicator for buffer capacity. WT: Wheat; RY: Rye; WW: Whole wheat; SP: Spelt; OA: Oat; MI II: Browntop millet; CO: Corn; RI: Rice.

3.4.2 Dry matter of EPS and recovery rate from doughs

Differences in dry matter of EPS and their recovery rate from doughs can influence data interpretation, which is why these parameters were tested before performing sourdough fermentations. The dry matter of isolated EPS from *G. frateurii*, *G. albidus*, *K. baliensis* and *N. chiangmaiensis* was constantly high and lay at $99.65 \pm 0.01\%$.

The recovery rate of EPS from different strains added to doughs made of wheat (WT), rye (RY), browntop millet (MI II), spelt (SP), rice (RI), corn (CO) and oat (OA) flour is shown in Figure 3.13a. Overall, it was quite low and varied between 41.53 % (EPS

from *N. chiangmaiensis* in oat dough) and 74.57 % (EPS from *K. baliensis* in rice dough). Differences between flours were minor compared to the differences found between the strains. The average recovery rate of *G. frateurii*, *G. albidus*, *K. baliensis* and *N. chiangmaiensis* EPS from all flours tested lay at 68.53 ± 2.30 %, 54.14 ± 1.16 %, 66.08 ± 3.35 % and 44.59 ± 3.62 %, respectively. Recovery rate was not included into the calculation of EPS from sourdoughs or statistical analysis, but was considered during the discussion of results.

(a) Recovery rate   (b) Influence of ethanol ratio
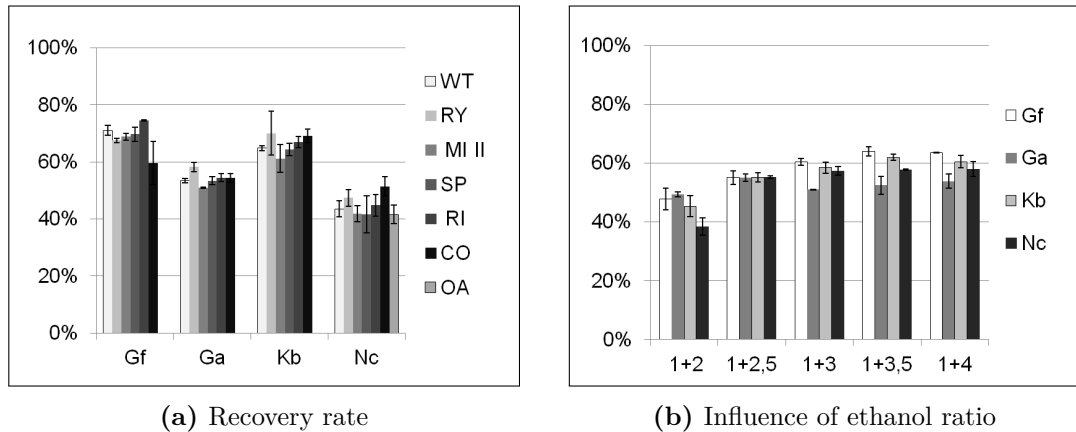
Figure 3.13 – Recovery rate of isolated EPS from *G. frateurii* (Gf), *G. albidus* (Ga), *K. baliensis* (Kb) and *N. chiangmaiensis* (Nc) from wheat (WT), rye (RY), browntop millet (MI II), spelt (SP), rice (RI), corn (CO) and oat (OA) dough and influence of ethanol ratio during precipitation in WT dough [dough extract:ethanol ratio]. Average and standard deviation of $2-4$ replicates shown.

3.4.3 Growth of selected strains on various flours and under different oxygen conditions

In these experiments, the ability of selected strains to grow in aerobic and anaerobic sourdoughs was tested. Among the gluten containing flours, the strains *G. albidus*, *G. frateurii*, *K. baliensis* and *N. chiangmaiensis* remained in aerobic fermentations with wheat, rye and spelt flour. Concerning the gluten-free flours, aerobic fermentations with golden millet, rice and corn flour were successful. As the only strain, *N. chiangmaiensis* could grow in aerobic oat sourdough, whereas none of the strains was able to become dominant in buckwheat dough. Under anaerobic conditions, none of the tested strains was successful, because they either could not increase their cell count within the fermentation time and/or contaminants reached more than 10 % of total cell count. The addition of mannitol in fermentations with *G. frateurii* and *G. albidus* did not show clear effects on growth or on the ability of strains to become dominant.

3.4.4 Optimization of EPS and other metabolites in aerobic gluten containing doughs with sucrose

After the strains were assessed for their ability to grow on various cereal substrates in section 3.4.3, the formation of EPS and other metabolites should be examined to identify most suited strains and flours for EPS production. Therefore, different sucrose dosages were tested for each strain-dough combination and yields of EPS, sucrose, acetic acid, gluconic acid and pH value were monitored in gluten-containing doughs.

K. baliensis

In aerobic fermentations of gluten containing flours with sucrose supplementation, *K. baliensis* has produced high EPS yields. The maximum concentrations were found in spelt dough with 100 and 75 g/l sucrose after 48 h and lay at around 273.8 ± 147.5 and 218.0 ± 74.2 mM Frc equ/kg flour, which relates to 49.3 ± 26.6 and 39.3 ± 13.4 g EPS/kg flour, respectively. EPS yields between 32.0 and 35.5 g/kg flour were found after 24 h in spelt (75 and 100 g/l sucrose) and whole wheat dough (100 g/l sucrose) and after 48 h in wheat (50 g/l sucrose) and rye dough (100 g/l sucrose).

During the whole fermentation, sucrose was degraded, whereby final sucrose levels were always related to the original sucrose dosage. At the time points of maximum EPS yields, 79.5 ± 12.9 (spelt, 100 g/l sucrose), 54.7 ± 8.6 (spelt, 75 g/l sucrose) and 38.2 ± 8.7 g/kg flour (wheat, 50 g/l sucrose) were measured.

Furthermore, gluconic acid and acetic acid were produced throughout the experiments. At 100 g/l sucrose addition and 24 h of fermentation, gluconic acid levels reached maxima of up to 164.9 ± 80.8 (whole wheat), 147.8 ± 119.2 (rye) and 117.2 ± 21.2 mM/kg flour (spelt), followed by a decrease. In opposite to this, acetic acid concentrations increased in most fermentations during the whole time. In sucrose-enriched experiments, maximum values amounted 145.1 ± 23.8 (spelt, 50 g/l sucrose, 48 h), 124.2 ± 27.6 (rye, 100 g/l sucrose, 48 h) and about 100 mM/kg flour (whole wheat, 50, 75 and 100 g/l sucrose, 24, 48 and 48 h, respectively).

With sucrose addition, pH values dropped to levels of about 3 within 24 h and either remained at this level or were followed by a slight increase thereafter. Lowest pH values were found in wheat doughs with 75 and 100 g/l sucrose addition and lay at 2.6 after 24 h.

Mathematical modelling of data obtained from fermentations with *K. baliensis* was performed based on the equation $v = \beta_0 + \beta_1 t + \beta_2 s + \beta_3 t^2 + \beta_4 s^2 + \beta_5 s \cdot t$. Whenever $u = y$, the model is linear or $u = lny$, the model is labelled as logarithmic in the following. This way, dependencies of the chosen parameters on the variables fermentation time and initial sucrose dosage were revealed. Significant dependencies for gluten containing flours are depicted in Figure 3.14. Models were either logarithmic or linear, relating to the parameter and the chosen flour. A table with the chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ can be found in the appendix. For example, the relation between EPS, time and sucrose dosage in wheat, spelt and rye dough was logarithmic, whereas it was linear for whole wheat.
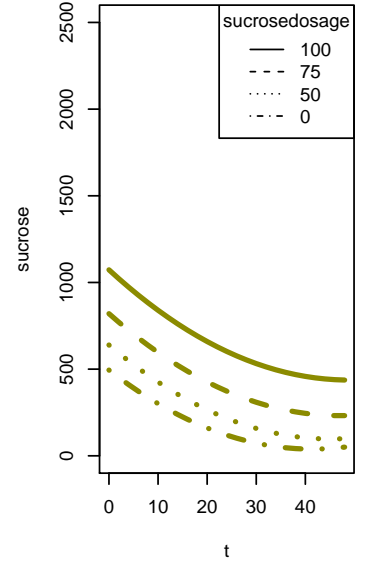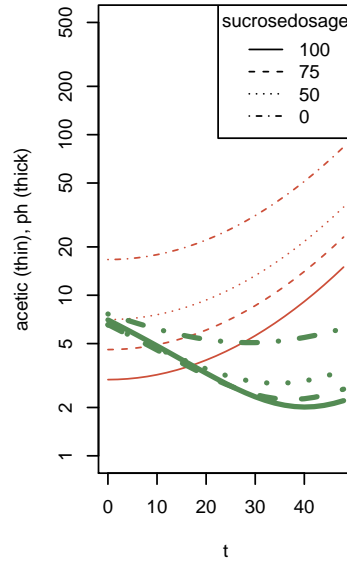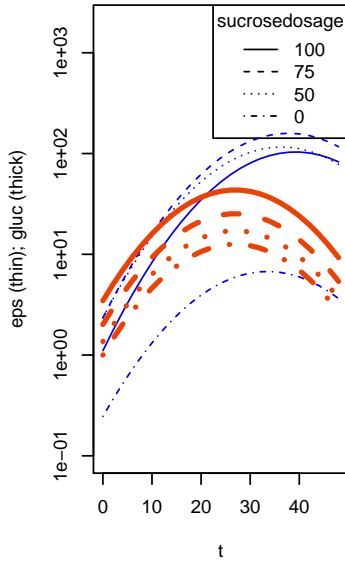
K. baliensis (WT)

K. baliensis (WW)
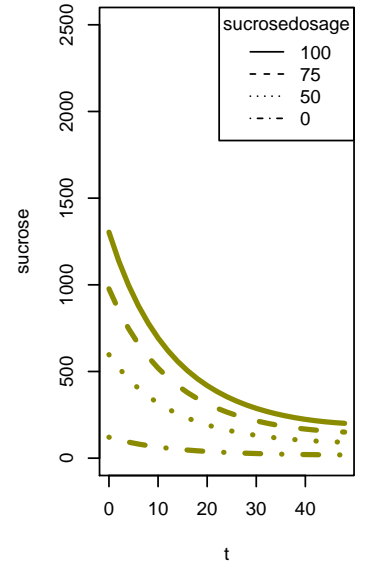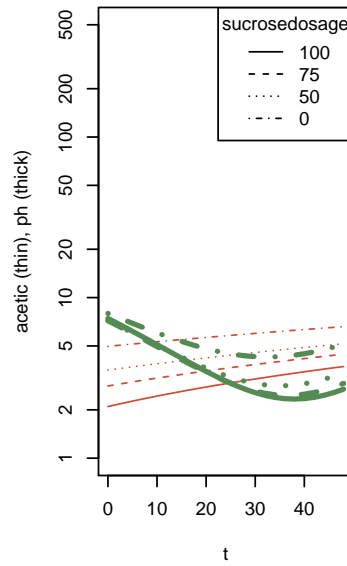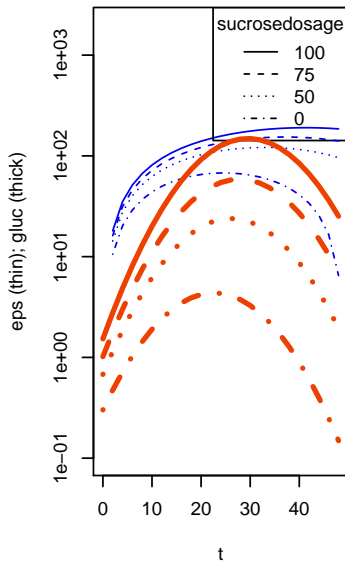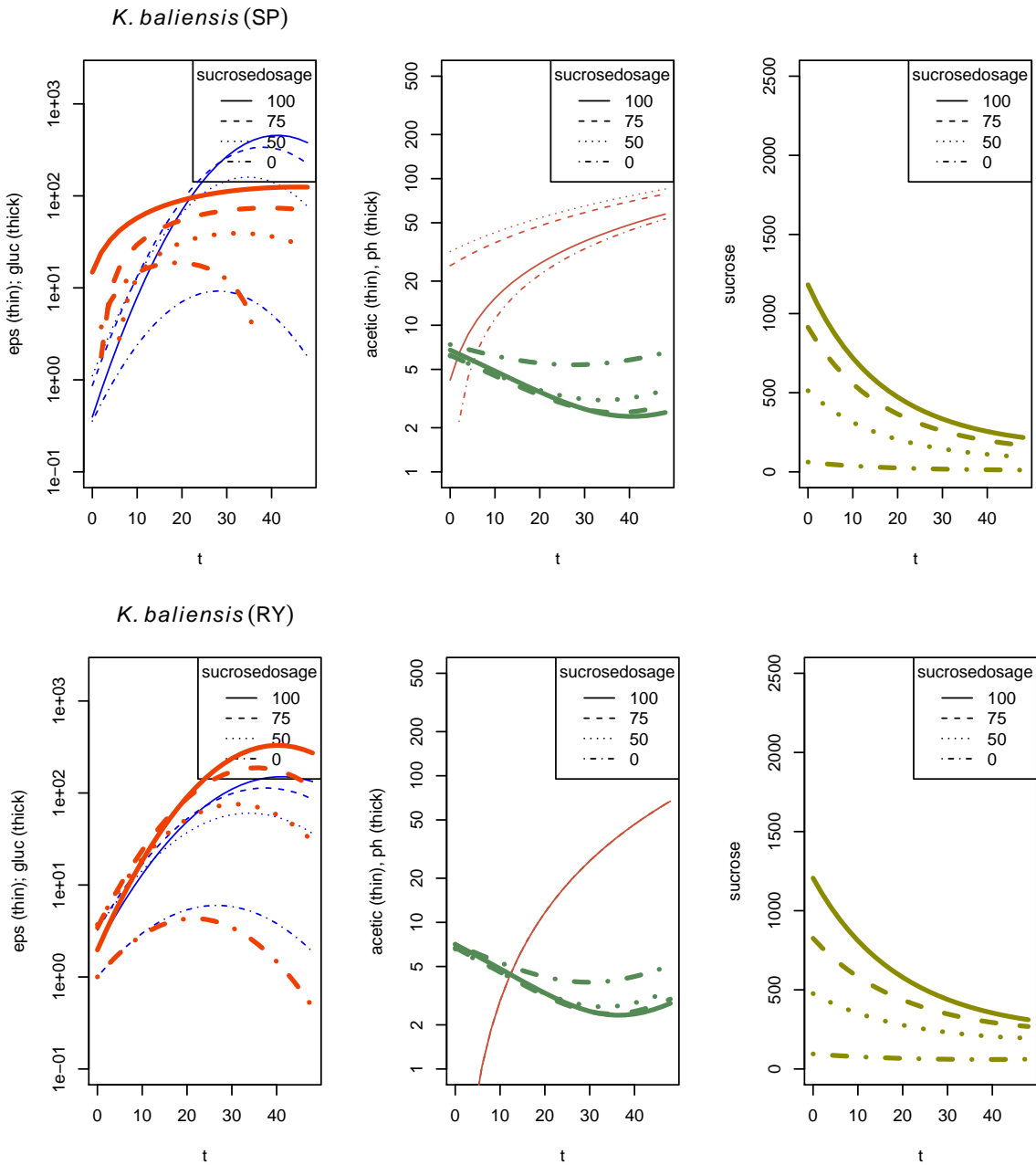
Figure 3.14 – *K. baliensis*: Graphical summary of the time-sucrose-dependency of selected metabolites during fermentations in gluten containing doughs. WT: Wheat; WW: Whole wheat; SP: Spelt; RY: Rye. Left: EPS (■), gluconic acid (■); Middle: pH value (■), acetic acid (■); Right: remaining sucrose level (■).

Calculated EPS maxima lay at around 35 h of fermentation for all flours (visual estimation), followed by slight degradation. Higher start sucrose dosages lead to higher EPS yields. The model for sucrose degradation showed a logarithmic relation to time and initial sucrose dosage for all gluten containing flours except wheat, where it was linear. The most effective sucrose degradation was found for whole wheat and spelt flour. For gluconic acid, a logarithmic model was chosen for wheat, whole wheat, and rye and a linear one for spelt. The maxima for gluconic acid production lay between 25 and 30 h for wheat and whole wheat, at about 35 h for rye and in the end of fermentation for spelt dough. Also here, a higher initial sucrose dosage was positively correlated to higher gluconic acid levels. The models show that after the maximum, gluconic acid was partly degraded in most gluten containing flours.

This was contrary to acetic acid, which reached maximum values mostly in the end of fermentations and whose yields were rather negatively correlated to high initial sucrose dosages. The model for acetic acid was logarithmic for wheat and whole wheat flour and linear for spelt and rye. In spelt dough, initial sucrose dosage did not make a big difference and in rye, a relation between time and acetic acid was found only for doughs with a start sucrose content of 100 g/l. The most suited model for pH value was linear for all doughs and revealed that higher sucrose dosages led to lower pH values, whereas a minimum pH between 35 and 45 h was predicted for all doughs, followed by a slight increase.

Depending on different target values in terms of desired EPS, acid and remaining sugar amounts, selected flours can fulfill different needs. In terms of maximum EPS yields, spelt flour in combination with a high sucrose dosage and harvesting after 48 h were most suited for fermentations with *K. baliensis*. In spelt doughs, sucrose consumption was most effective and acid contents lay at average levels compared to the other fermentations in gluten containing flours with *K. baliensis*.

N. chiangmaiensis

In fermentations with gluten containing flours and *N. chiangmaiensis*, the highest EPS yields were detected in spelt and whole wheat flour, followed by rye and wheat. The maxima for each flour lay at 157.7 ± 78.8 mM Frc equ/kg in whole wheat (100 g/l sucrose, 48 h), 142.2 ± 44.6 mM Frc equ/kg in spelt (100 g/l sucrose, 24 h), 105.4 ± 72.0 mM Frc equ/kg in rye (100 g/l sucrose, 48 h) and 96.6 ± 129.2 mM Frc equ/kg in wheat dough (75 g/l sucrose, 48 h), which related to 28.4 ± 14.2 g/kg (whole wheat), 25.6 ± 8.0 g/kg (spelt), 19.0 ± 13.0 g/kg (rye) and 17.4 ± 23.3 g/kg (wheat). The most effective flour for EPS formation appeared to be spelt, as it was the only flour where all three sucrose dosages reached reliable EPS yields between 118 and 142 mM Frc equ/kg, relating to 21.4 and 25.6 g EPS/kg. Following the mathematical model obtained from the data, the relation between EPS formation, sucrose dosage and time was logarithmic for all gluten containing flours (Figure 3.15). The maximum EPS contents was predicted for 30 h of fermentation in wheat dough, about 35 h fermentation in spelt dough, and in the end of fermentation for whole wheat and rye dough. Sucrose dosage seemed to have a greater influence on EPS content in whole wheat, spelt and rye dough than in wheat dough,

where differences between doughs with start sucrose contents between 50 and $100\,\text{g/l}$ were only low.

The flour with the lowest final sucrose level was spelt flour, because after $48\,\text{h}$, remaining sucrose amounted $17.2 \pm 29.8\,\text{g/kg}$ flour ($50\,\text{g/l}$ sucrose), $53.6 \pm 13.9\,\text{g/kg}$ flour ($75\,\text{g/l}$ srucose) and $82.2 \pm 14.3\,\text{g/kg}$ flour ($100\,\text{g/l}$ sucrose). In spelt dough with $50\,\text{g/l}$ start sucrose content, the degradation amounted more than $90\,\%$. In the other flours with $50\,\text{g/l}$ start sucrose, final levels lay at $35.3 \pm 23.7\,\text{g/kg}$ flour in wheat, $84.8 \pm 41.1\,\text{g/kg}$ flour in rye and $46.2 \pm 26.6\,\text{g/kg}$ flour in whole wheat dough. However, under fermentation conditions where maximum EPS levels have been reached, the sucrose contents were quite high and lay at 251.7 ± 194.8 in whole wheat ($100\,\text{g/l}$ sucrose) and $115.7 \pm 54.4\,\text{g/kg}$ flour in spelt dough ($100\,\text{g/l}$ sucrose). Mathematical modelling revealed a logarithmic relation between time, sucrose dosage and final sucrose content (Figure 3.15). Sucrose degradation was more efficient in spelt than in wheat dough, followed by whole wheat and rye dough.

The production of gluconic acid increased with higher sucrose dosage and was maximum in spelt dough, followed by rye dough. In spelt dough with $100\,\text{g/l}$ sucrose, $249.6 \pm 15.2\,\text{mM/kg}$ gluconic acid were found. This was more than twice as much as the maximum detected in rye dough with $100\,\text{g/l}$ sucrose, which lay at $119.2 \pm 152.2\,\text{mM/kg}$ flour. In wheat and whole wheat doughs, only 61.1 ± 33.5 and $12.6 \pm 5.5\,\text{mM/kg}$ gluconic acid were reached at maximum. The maxima were, according to the graphs, after one day of fermentation and were followed by a strong degradation. Mathematical modelling confirmed the dependency of gluconic acid on sucrose dosage and time, with peaks after about $24\,\text{h}$. The chosen models were logarithmic for wheat, whole wheat and spelt flour and linear for rye flour.

In opposite to this, the mathematical model for acetic acid was linear for wheat and logarithmic for the other gluten containing flours. Furthermore, although sucrose dosage had a significant influence on acetic acid production, it changed from a negative correlation in wheat, whole wheat and spelt dough to a positive correlation in rye dough. All in all, acetic acid contents were relatively low and never reached more than $30\,\text{mM/kg}$ in fermentations with sucrose addition, apart from whole wheat with $50\,\text{g/l}$ sucrose, where $37.5 \pm 26.5\,\text{mM/kg}$ of acetic acid were detected after $48\,\text{h}$ of fermentation.

Within the first $24\,\text{h}$ of fermentation, pH values dropped from $6-7$ to levels below 3.0 in all fermentations with sucrose addition except whole wheat with $50\,\text{g/l}$ sucrose (pH $= 3.12$). The pH then either remained at this level or showed a slight increase. In doughs without sucrose addition, it never dropped below 4.18 (rye, $24\,\text{h}$), but showed the same course apart from whole wheat, where a more even decrease in pH value was observed. The data, and thus the mathematical model clearly show that a higher sucrose dosage led to lower pH values, whereas the more suited models were linear for all gluten containing flours. Following the modelled graphs, the lowest points of pH value should lay at about $35\,\text{h}$.

All in all, spelt flour was the most successful gluten-containing flour in terms of a high and reliable EPS production with *N. chiangmaiensis*, whereby highest levels were detected after 24 and $48\,\text{h}$, depending on sucrose dosage. Furthermore, in spelt doughs the lowest remaining sucrose and acetic acid and average gluconic acid levels were reached.

N. chiangmaiensis (WT)
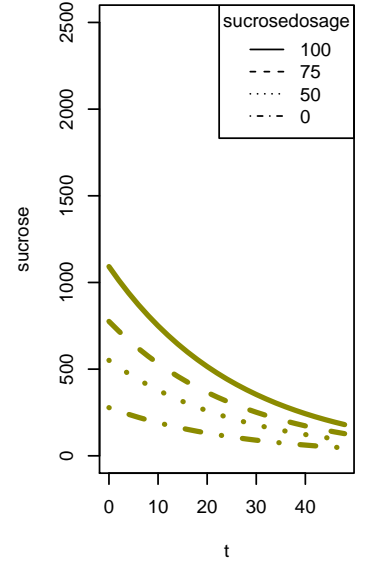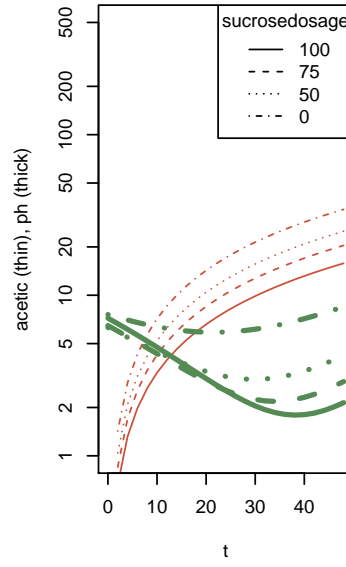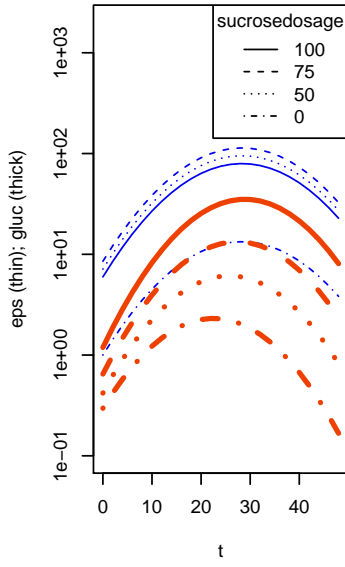
N. chiangmaiensis (WW)
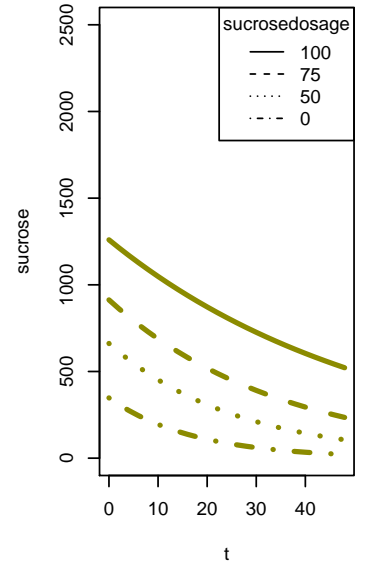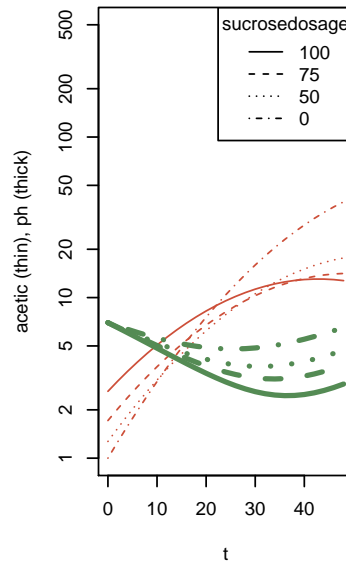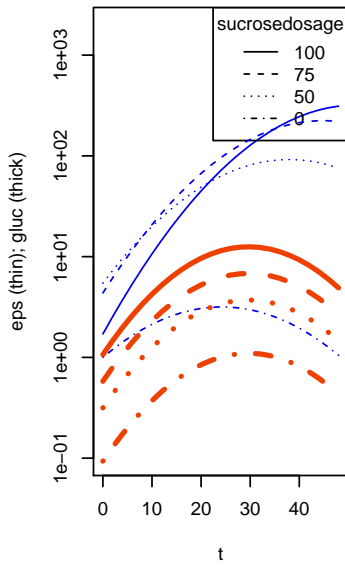
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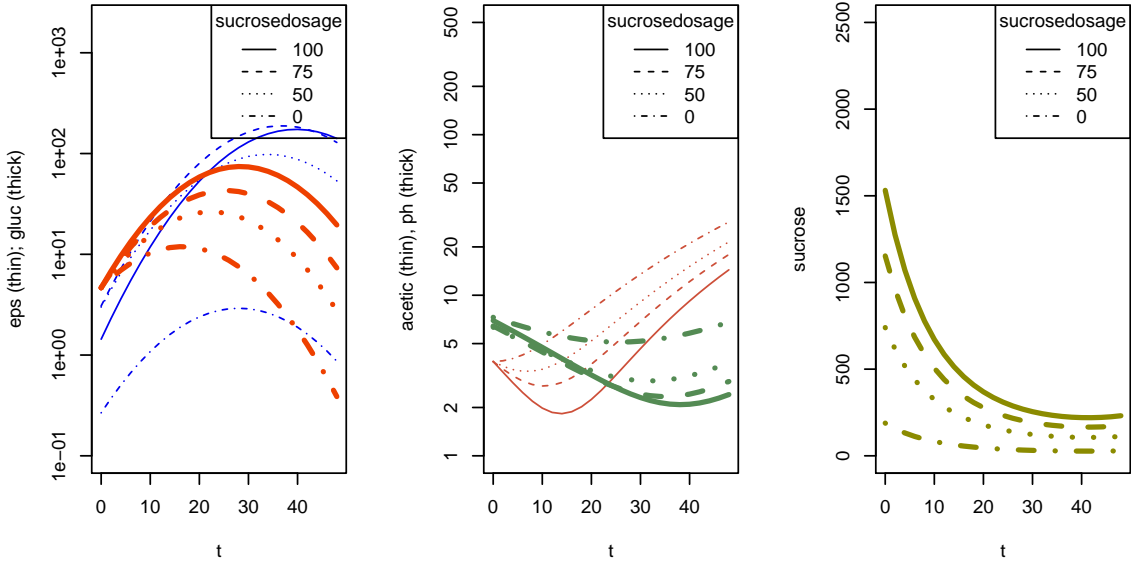
N. chiangmaiensis (RY)
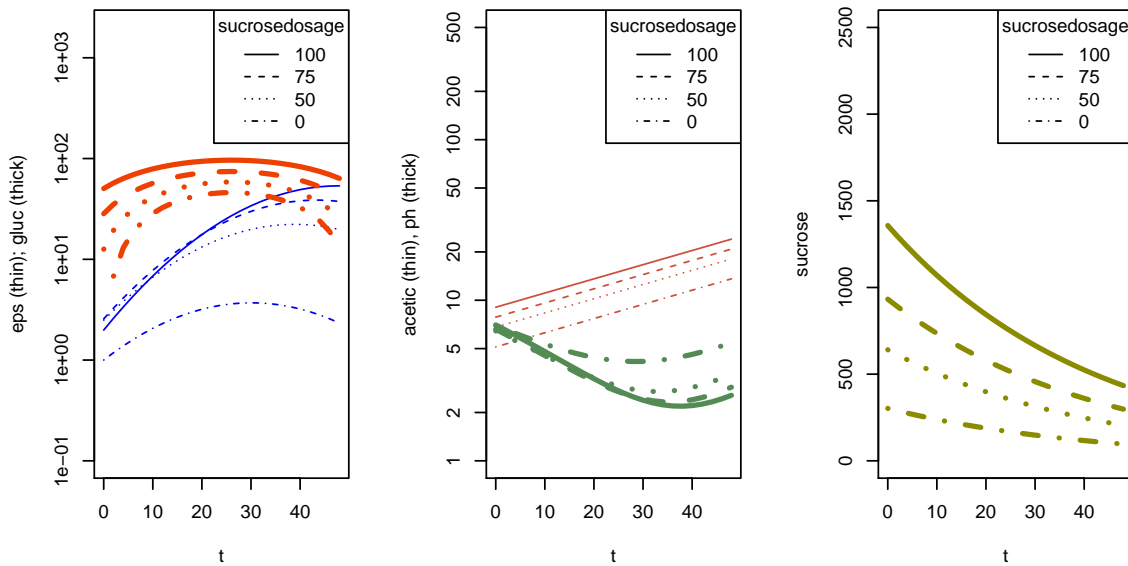
Figure 3.15 – *N. chiangmaiensis*: Graphical summary of the time-sucrose-dependency of selected metabolites during fermentations in gluten containing doughs. WT: Wheat; WW: Whole wheat; SP: Spelt; RY: Rye. Left: EPS (■), gluconic acid (■); Middle: pH value (■), acetic acid (■); Right: remaining sucrose level (■).

G. albidus

Similar to *K. baliensis* and *N. chiangmaiensis*, *G. albidus* has reached the highest EPS levels in spelt dough (Figure 3.16). However, maximum yields were lower than of the previously described strains and lay at 91.7 ± 16.9 (50 g/l sucrose, 48 h) and 89.2 ± 10.9 mM Frc equ/kg (75 g/l sucrose, 48 h), which relates to 16.5 ± 3.1 and 16.1 ± 2.0 g/kg, respectively. In wheat and rye flour, the maximum EPS levels amounted 49.1 ± 20.1 and 68.1 ± 37.8 mM Frc equ/kg and were found after 24 h in fermentations with 100 g/l sucrose. As the other two strains, EPS were partly degraded after reaching the maximum.

EPS formation was related to a degradation of sucrose. After 48 h in spelt flour with 50 and 75 g/l sucrose, sucrose levels lay at 61.5 ± 26.6 and 79.1 ± 59.1 g/kg, relating to 47 % and 45 % degradation of start sucrose. The sucrose levels correspond to the maximum in rye and wheat dough after 24 h and lay at 179.1 ± 53.3 and 162.7 ± 87.8 g/kg flour, which means about 27 % sucrose degradation. In flours with lower sucrose dosage, a higher consumption percentage occurred in rye and spelt dough, whereas in wheat dough, no such differences were detected.

The highest levels of acetic acid were found in the same doughs with maximum EPS yields. In spelt dough with 50 and 75 g/l sucrose, 120.2 ± 18.6 and 126.7 ± 24.1 mM/kg acetic acid were detected. In rye and wheat dough, EPS maxima were not directly related to maximum acetic acid levels, whereas acetic acid yields were still high: they lay at 93.2 ± 47.4 mM/kg in rye and 82.6 ± 34.7 mM/kg in wheat dough at peak EPS levels.

The levels of gluconic acids related to peak EPS yields lay at 57.0 ± 17.4 (spelt, 50 g/l sucrose, 48 h), 71.6 ± 46.1 (spelt, 75 g/l sucrose, 58 h), 78.8 ± 50.7 (wheat, 100 g/l sucrose, 24 h) and 25.4 ± 7.4 mM/kg flour (rye, 100 g/l sucrose, 24 h). The maximum gluconic acid level of all fermentations was 121.5 ± 112.4 mM/kg and was found in wheat dough with 100 g/l sucrose after 48 h. In this dough, gluconic acid levels significantly increased with higher sucrose dosage, whereas this was not the case for spelt and rye dough (Figure 3.16). Overall, yields of both organic acids produced in fermentations with *G. albidus* constantly increased during the fermentations while no degradation was detected.

In fermentations with sucrose, pH values fell to around 3.0 or lower within 24 h of incubation, followed by a slight further decrease in most trials. If no sucrose was present in the experiment, pH levels remained between 3 and 4. The highest pH values were found in spelt dough, where they remained between 2.8 and 3.0 during the whole fermentation. In rye dough, they fell to levels around 2.7 during 48 h and in wheat dough, they even reached 2.5 in doughs with 75 and 100 g/l sucrose.

Statistical analysis of data and mathematical modelling revealed that in some fermentations, time and sucrose dosage had no significant influence on the variables. Graphs which are based on significant relations are shown in Figure 3.16. The most suited mathematical models for EPS, gluconic acid, acetic acid and sucrose in wheat, spelt and rye flour were logarithmic, apart from EPS in spelt flour. In wheat flour, a higher sucrose dosage led to a higher EPS content, whereas the opposite was the case for spelt flour. In rye flour, no influence of sucrosedosage on EPS content was predicted by the model. Furthermore, acetic and gluconic acid formation were not clearly related to sucrose dosage

Figure 3.16 – Graphical summary of the time-sucrose-dependency of selected metabolites during fermentations in gluten containing doughs with *G. albidus*. WT: Wheat; SP: Spelt; RY: Rye. Left: EPS (■), gluconic acid (■); Middle: pH value (■), acetic acid (■); Right: remaining sucrose level (■).

except for wheat, but rather to time of fermentation, whereas sucrose dosage was positively correlated to remaining sucrose levels in wheat and rye flour. In spelt dough, no clear relation of sucrose dosage to sucrose level could be found. The linear model for pH values was significant for all three gluten containing flours, whereby a higher sucrose dosage led to lower pH values.

As EPS levels were quite low in fermentations with *G. albidus*, this strain is rather interesting if high gluconic acid levels and average acetic acid levels are required. In this case, wheat dough with high sucrose dosage seems to be most suited, leading to acetic acid yields compareable to *G. frateurii* and gluconic acid levels above 100 mM/kg flour after 48 h.

G. frateurii

In fermentations with *G. frateurii*, maximum EPS yields were again reached in spelt dough, followed by wheat and rye dough (Figure 3.17). They were slightly higher than in fermentations with *G. albidus* and lay at 125.1 ± 22.6 (spelt, 100 g/l sucrose, 48 h), 100.9 ± 91.7 (spelt, 50 g/l sucrose, 48 h) and 94.9 ± 35.2 mM Frc equ/kg (wheat, 75 g/l sucrose, 48 h). In rye flour, the maximum EPS level lay at 69.8 ± 17.6 mM Frc equ/kg (100 g/l sucrose, 48 h). Thus the only dough in which more than 20 g/kg EPS have been detected was spelt dough with 100 g/l sucrose. As maximum EPS yields were recovered in the end of fermentations, it cannot be said whether degradation of EPS took place after reaching the peak.

Levels of remaining sucrose were the highest, compared to the levels of other strains. In the fermentation where the maximum EPS amount has been reached (spelt, 100 g/l sucrose, 48 h), 305.0 ± 65.3 g/kg sucrose were measured. This relates to only 36 % sucrose degradation. In the fermentation with the second highest EPS level (spelt, 50 g/l sucrose, 48 h), however, only 120.6 ± 59.1 g/kg flour remained at the point of maximum EPS level, relating to 58 % sucrose consumption. The other two EPS maxima were both accompanied by sucrose levels above 292 g/kg. Overall, a higher percent consumption of sucrose occured in flours with lower start sucrose. This was most obvious for wheat dough, in which 40 % sucrose has been used at 50 g/l start sucrose content and only 21 % sucrose at 100 g/l start sucrose content.

Compared to the other three strains, *G. frateurii* has formed the lowest amounts of gluconic acid. Gluconic acid levels never exceeded 20 mM/kg flour and were always higher after 24 h than after 48 h of fermentation. At the points of maximum EPS formation, gluconic acid always lay below 10 mM/kg flour.

The levels of acetic acid, however, were about as high as in fermentations with *G. albidus* and *K. baliensis*. The highest amount of acetic acid was detected in rye flour, where between 105.6 ± 46.3 and 129.9 ± 45.4 mM/kg flour were found after 48 h. At maximum EPS formation, acetic acid amounted 89.7 ± 31.3 (spelt, 100 g/l sucrose, 48 h), 108.9 ± 57.8 (spelt, 50 g/l sucrose, 48 h) and 100.3 ± 32.6 mM/kg flour (wheat, 75 g/l sucrose, 48 h). Maximum acetic acid levels were most often recovered in the end of fermentations, which is why it cannot be said whether acetic acid has been further degraded in these experiments.

Figure 3.17 – Graphical summary of the time-sucrose-dependency of selected metabolites during fermentations in gluten containing doughs with *G. frateurii*. WT: Wheat; SP: Spelt; RY: Rye. Left: EPS (■), gluconic acid (■); Middle: pH value (■), acetic acid (■); Right: remaining sucrose level (■).

Despite the acetic acid levels in fermentations with added sucrose, pH values did not decrease to the same extent compared to the other three strains. In wheat dough, they fell to about 3.8 within 24 h and remained at about this level. In rye dough, they fell to $2.9 - 3.0$ within 24 h, followed by a slight decrease to $2.8 - 2.9$. This was compareable to spelt dough, whereas spelt was the only flour, where a pH below 2.8 has been measured (100 g/l sucrose, 48 h).

Statistical analysis and mathematical modelling revealed that the influence of the variables sucrose dosage and time on the chosen parameters was not always significant for *G. frateurii* (Figure 3.17). The chosen model for EPS was logarithmic for wheat and spelt dough and linear for rye dough. In opposite to time, which caused an increase up to about 35 h, followed by a slight decrease, sucrose had no significant influence within the tested dosages. Also for gluconic and acetic acid, the influence of sucrose dosage was not significant, contrary to time. The models show that acetic acid increases throughout the whole fermentation in wheat and rye dough, but shows a peak after about 35 h in spelt dough, followed by partial degradation. The model for gluconic acid shows a constant value for wheat, a peak in the middle of fermentation followed by partial degradation for spelt, and a clear dependency from sucrose dosage for rye dough. It can further be seen that higher sucrose dosages reliably lead to a lower pH value and were further related to higher final sucrose levels.

The most promising flour in terms of EPS optimization with *G. frateurii* was spelt flour (100 g/l sucrose, 48 h), although this strain was not among the two best EPS producers in this study. EPS maxima were accompanied by high levels of remaining sucrose, low gluconic acid and high acetic acid levels, which were similar to *G. albidus*.

3.4.5 Optimization of EPS and other metabolites in aerobic gluten-free doughs with sucrose

After the strains were assessed for their ability to grow on various gluten-free cereal substrates in section 3.4.3, the formation of EPS and other metabolites should be examined to identify most suited strains and flours for EPS production. Therefore, different sucrose dosages were tested for each strain-dough combination and yields of EPS, sucrose, acetic acid, gluconic acid and pH value were monitored also in gluten-free doughs. Concerning the selection of gluten-free flours, all strains were tested in millet dough. Although two different millet flours were used for fermentations A, B (golden millet) and F (wild browntop millet), no significant differences were found between these doughs. In addition, *N. chiangmaiensis* was tested in oat dough, because it was the only strain which could grow there and become dominant in the previous trial series. Both *Gluconobacter* strains were furthermore tested in corn and rice doughs.

G. albidus

In gluten-free flours, *G. albidus* behaved similar to gluten containing flours and showed low EPS-formation (Figure 3.18). The highest EPS-yields were recovered from millet flour after 48 h (50 and 75 g/l sucrose) and 24 h (100 g/l sucrose) and lay around 70 mM

Frc equ/kg, relating to $12-13$ g/kg flour. In rice and corn flour, only around 7 and 4 g EPS per kg flour were detected, respectively.

Sucrose degradation was highest in millet dough, where after 48 h, $85-212$ g/kg sucrose was left ($50-100$ g/l sucrose). This was followed by rice dough with a final sucrose content of $106-222$ g/kg and corn dough with $142-323$ g/kg final sucrose ($50-100$ g/l start sucrose, respectively).

In contrast to the low EPS formation, high amounts of organic acids were formed by *G. albidus*. The maximum gluconic acid level was reached in rice flour with 100 g/l sucrose and lay at 97.6 ± 41.9 mM/kg, followed by millet flour (same sucrose dosage) with 57.6 ± 14.0 mM/kg. In corn flour and in flours with lower sucrose dosages or at earlier harvesting points, gluconic acid levels never exceeded 42.3 mM/kg.

The level of acetic acid was highest in millet flour after 24 h, where it lay between 108 and 110 mM/kg (75 and 100 g/l sucrose), followed by a decrease to 88.6 and 93.8 mM/kg after 48 h (respectively). The second highest levels of acetic acid were recovered from rice dough at the same time point and lay between 76.6 and 82.9 mM/kg (50 and 75 g/l sucrose, respectively). Also here, a partly degradation during the next 24 h was noticed. In corn flour, not more than 40.9 mM/kg acetic acid were detected.

The behaviour of pH value was similar to gluten containing doughs and dropped within 24 h to $2.9-3.0$ in rice and to $2.7-2.9$ in millet dough, both with sucrose. In corn dough with sucrose, they lay at around 4.0 at the same time point. During the following time, a further drop was observed, leading to $2.6-2.8$ in millet, $2.8-2.9$ in rice and $3.2-3.6$ in corn dough with sucrose.

Statistical analysis of data and mathematical modelling showed that a logarithmic model is most suitable to describe the behaviour of EPS, gluconic acid, acetic acid and sucrose in rice and millet dough. In corn dough, differences were found between fermentations A, B on the one hand and F on the other hand in terms of EPS formation. In fermentation A and B, it depended on $t \cdot s$ and in fermentation F, it depended on t^2. Models for gluconic acid and sucrose were logarithmic and for acetic acid and pH value linear in doughs made form corn flour. A positive influence of sucrose dosage on EPS was found four corn dough, on gluconic acid for corn and rice dough and on acetic acid for millet dough (Figure 3.18). A negative correlation between sucrose dosage and pH was detected for all gluten-free flours. Furthermore, EPS content was negatively influenced by sucrose dosage in rice dough. Higher sucrose yields generally led to higher final sucrose concentrations.

All in all, the flour-sucrose-time combination which can be recommended the most for high EPS yields is millet flour, 50 g/l sucrose, 48 h. Accompanying gluconic acid, acetic acid and sucrose levels lay at around 40.2, 78.3 and 84.7 mM/kg flour, respectively.

G. frateurii

In fermentations with gluten-free flours, *G. frateurii* has reached higher concentrations of EPS than in fermentations with wheat, rye or spelt flour (Figure 3.19) The highest levels were recovered from rice and millet dough after 48 h and lay at 154.1 ± 25.2 (rice) and 148.9 ± 62.0 mM Frc equ/kg (millet) in flours with 100 g/l sucrose, followed by 147.0 ± 61.2
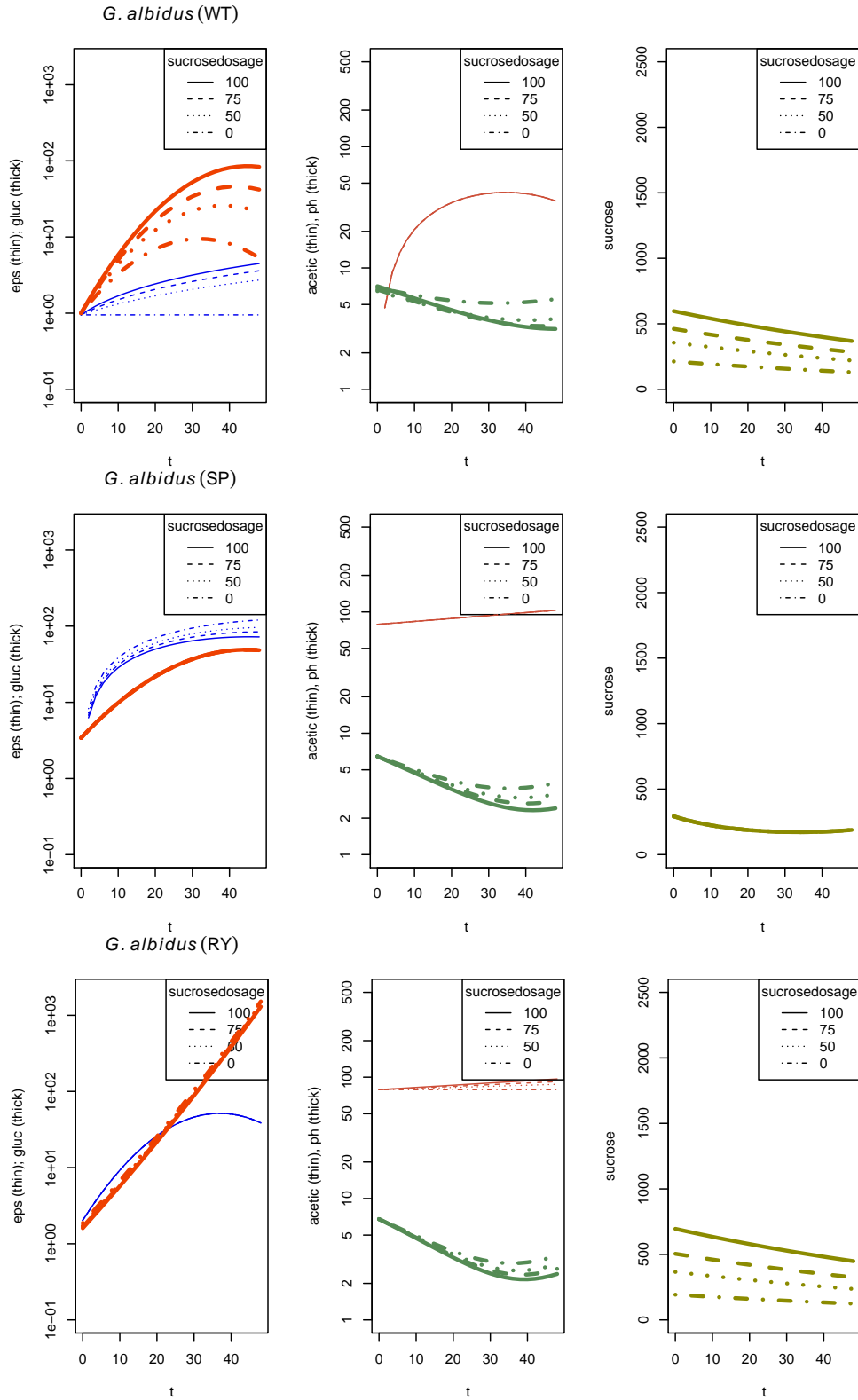
Figure 3.18 – Graphical summary of the time-sucrose-dependency of selected metabolites during fermentations in gluten-free doughs with *G. albidus*. MI: Millet; CO: Corn; RI: Rice. Left: EPS (■), gluconic acid (■); Middle: pH value (■), acetic acid (■); Right: remaining sucrose level (■).
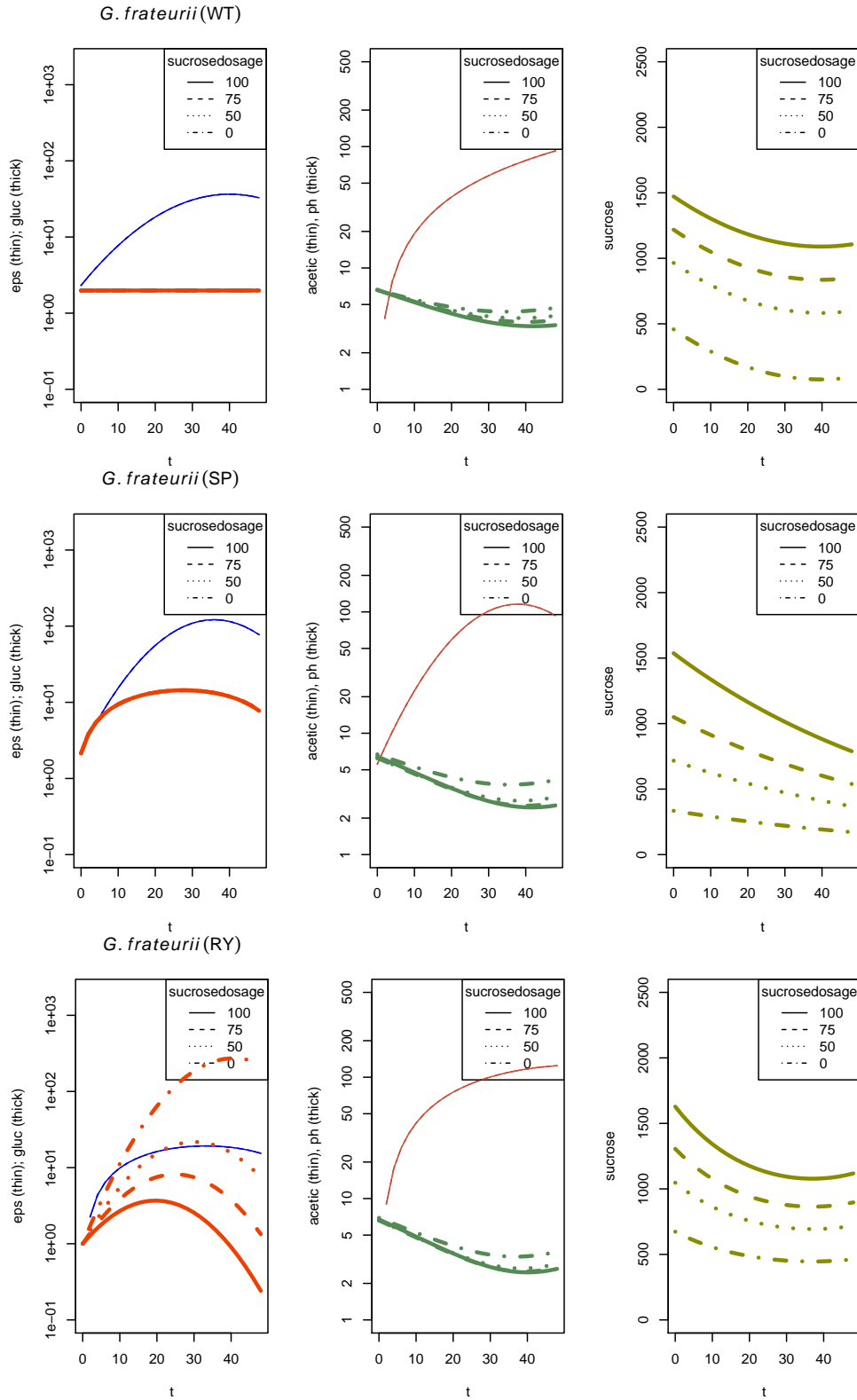
Figure 3.19 – Graphical summary of the time-sucrose-dependency of selected metabolites during fermentations in gluten-free doughs with *G. frateurii*. MI: Millet; CO: Corn; RI: Rice. Left: EPS (■), gluconic acid (■); Middle: pH value (■), acetic acid (■); Right: remaining sucrose level (■).

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(millet, $75 \, \text{g/l}$ sucrose) and $123.5 \pm 40.5 \, \text{mM}$ Frc equ/kg (corn, $100 \, \text{g/l}$ sucrose). This relates to EPS levels of more than $25 \, \text{g/kg}$ in rice and millet flour and of more than $22 \, \text{g/kg}$ in corn flour.

In contrast to high EPS formation, the consumption of sucrose was weak. At the time points of maximum and second maximum EPS yield, there was more than $300 \, \text{g/kg}$ sucrose left, relating to a $25 \, \%$ and $39 \, \%$ degradation of start sucrose in rice and millet dough, respectively. In millet dough with $75 \, \text{g/l}$ sucrose and corn dough with $100 \, \text{g/l}$ sucrose, degradation of sucrose amounted $48 \, \%$ and $54 \, \%$ of start level.

The formation of gluconic acid was very low in gluten-free fermentations with *G. frateurii*. At t_1, between 2.4 and $4.4 \, \text{mM/kg}$ was found. During the fermentations, the metabolite levels remained more or less constant, compared to the beginning. Only in rice dough with 50 and $100 \, \text{g/l}$ sucrose, a slight decrease to 4.9 ± 2.4 and $6.4 \pm 3.9 \, \text{mM/kg}$ were detected after $24 \, \text{h}$, followed by a decrease to below $3.3 \, \text{mM/kg}$.

In opposite to gluconic acid, acetic acid formation was quite high in millet and rice dough. Here, between 95.0 ± 21.8 and 80.3 ± 18.3 (millet dough, $50 - 100 \, \text{g/l}$ sucrose) and 92.5 ± 54.0 and $121.0 \pm 17.7 \, \text{mM/kg}$ (rice dough, $50 - 100 \, \text{g/l}$ sucrose) were found. In corn dough, not more than $18.1 \pm 8.7 \, \text{mM/kg}$ gluconic acid were found.

The pH value dropped from $6 - 7$ to about $3.1 - 3.8$ within $24 \, \text{h}$ in fermentations with sucrose. This was followed by a further slight drop in millet dough, a balance at the same level in corn dough and a slight increase in rice dough. In gluten-free doughs, the pH level of fermentations with *G. frateurii* never fell below 3.1.

Statistical data analysis and mathematical modelling revealed significant influences of time and sucrose dosage on the chosen parameters in most fermentations. The chosen models for EPS, gluconic acid formation and remaining sucrose were logarithmic in gluten-free doughs. In contrast, acetic acid and pH value were linearly influenced by time and sucrose dosage, apart from corn flour, where a logarithmic model was chosen for acetic acid in fermentations A and B. Significant graphs are depicted in Figure 3.19. It can be seen that a higher start sucrose level always led to higher final sucrose concentrations. Furthermore, higher start sucrose caused a lower pH value. Data of EPS, gluconic acid and acetic acid were significant only for $100 \, \text{g/l}$ sucrose in fermentations with corn and rice flour. In millet flour, a positive influence of sucrose on gluconic acid can be recognized. Concerning EPS and acetic acid, again only the data obtained from fermentations with $100 \, \text{g/l}$ sucrose were significant in terms of time.

In terms of a pure EPS optimization in gluten-free flours, rice and millet flour with high sucrose dosages and harvesting after $48 \, \text{h}$ can be recommended for *G. frateurii*. However, these yields were accompanied by high acetic acid and remaining sucrose levels and low gluconic acid formation.

K. baliensis

Among the gluten-free flours, *K. baliensis* was only tested in millet flour. After $48 \, \text{h}$, EPS yields between 140.0 ± 146.9 ($50 \, \text{g/l}$ sucrose) and $163.8 \pm 210.5 \, \text{mM}$ Frc equ/kg ($75 \, \text{g/l}$ sucrose) were recovered. This related to about $25.2 - 29.5 \, \text{g/kg}$ EPS.

Lowest final sucrose levels were found in doughs with lower start sucrose content,

in which a higher percent of start sucrose was consumed throughout the fermentation. Therefore in millet dough with 50 g/l sucrose, 57.2 ± 15.3 g/kg final sucrose were found, whereas in millet dough with 100 g/l sucrose, 192.7 ± 52.6 g/kg final sucrose were detected.

Gluconic acid formation was generally low with a peak in the middle of fermentation time. It never exceeded 25 mM/kg (100 g/l sucrose, 24 h).

The formation of acetic acid was generally low, too. Although in millet dough, acetic acid increased throughout the whole fermentation, the maximum value lay at 20.1 ± 26.2 mM/kg in dough with 75 g/l sucrose. In the other fermentations, 3.6 mM/kg acetic acid were never exceeded.

Despite the low acid levels, pH value dropped to below 2.8 within 24 h in fermentations with added sucrose, whereby higher sucrose levels lead to a lower pH.

Statistical data analysis and mathematical modelling showed that the best fitting model was logarithmic for EPS, gluconic acid and sucrose and linear for acetic acid and pH. Sucrose dosage had a clearly positive effect on gluconic acid and final sucrose, whereas the relation to pH was rather negative. Concerning EPS and acetic acid, only the time affected yields, whereby only data of fermentations with 100 g/l sucrose addition was significant. Referring to the mathematical model (Figure 3.20), EPS maximum was reached after about 30 h of fermentation and therefore lay before the maximum acetic acid concentration and slightly before the lowest pH value. Gluconic acid levels showed a linear increase in doughs with 75 g/l sucrose or more and decreased if less sucrose was present.

Among the tested strains, *K. baliensis* has reached highest EPS-yields in millet dough. The maximum level of about 29.5 g EPS/kg flour (75 g/l sucrose, 48 h) was related to 9.8 mM/kg flour gluconic acid, 20.1 mM/kg flour acetic acid and 114.3 g/kg remaining sucrose.

N. chiangmaiensis

In gluten-free fermentations with *N. chiangmaiensis*, high EPS levels were found in millet and in oat flour, whereby in millet flour, maximum yields were reached after 24 h and in oat flour after 48 h. They amounted 129.7 ± 61.9 and 124.6 ± 34.5 mM Frc equ/kg in millet four with 75 and 100 g/l sucrose, which relates to 23.4 ± 11.2 and 22.4 ± 6.2 g/kg, respectively. In oat flour, maximum levels were 166.0 ± 89.3 (50 g/l sucrose), 145.6 ± 70.2 (75 g/l sucrose) and 171.9 ± 136.3 mM/kg (100 g/l sucrose). This relates to about 29.9, 26.2 and 31.0 g/kg EPS, respectively.

In millet flour, sucrose levels corresponding to peak EPS values lay at 33.1 ± 57.3 (75 g/l sucrose) and 194.5 ± 55.3 g/kg (100 g/l sucrose). In oat flour, remaining sucrose amounted 63.2 ± 26.9 (50 g/l sucrose), 132.1 ± 27.2 (75 g/l sucrose) and 219.6 ± 31.4 g/kg (100 g/l sucrose). Therefore most effective sucrose consumption took place in millet dough, where 78 % of start sucrose was used up in the end of fermentation. In oat dough, between 74 % (50 g/l sucrose) and 50 % (100 g/l sucrose) of start sucrose was consumed throughout the fermentation.

Same as in doughs with gluten, the gluconic acid formation was weak in doughs without sucrose addition. In doughs with sucrose, gluconic acid levels of up to 63.5 (oat,

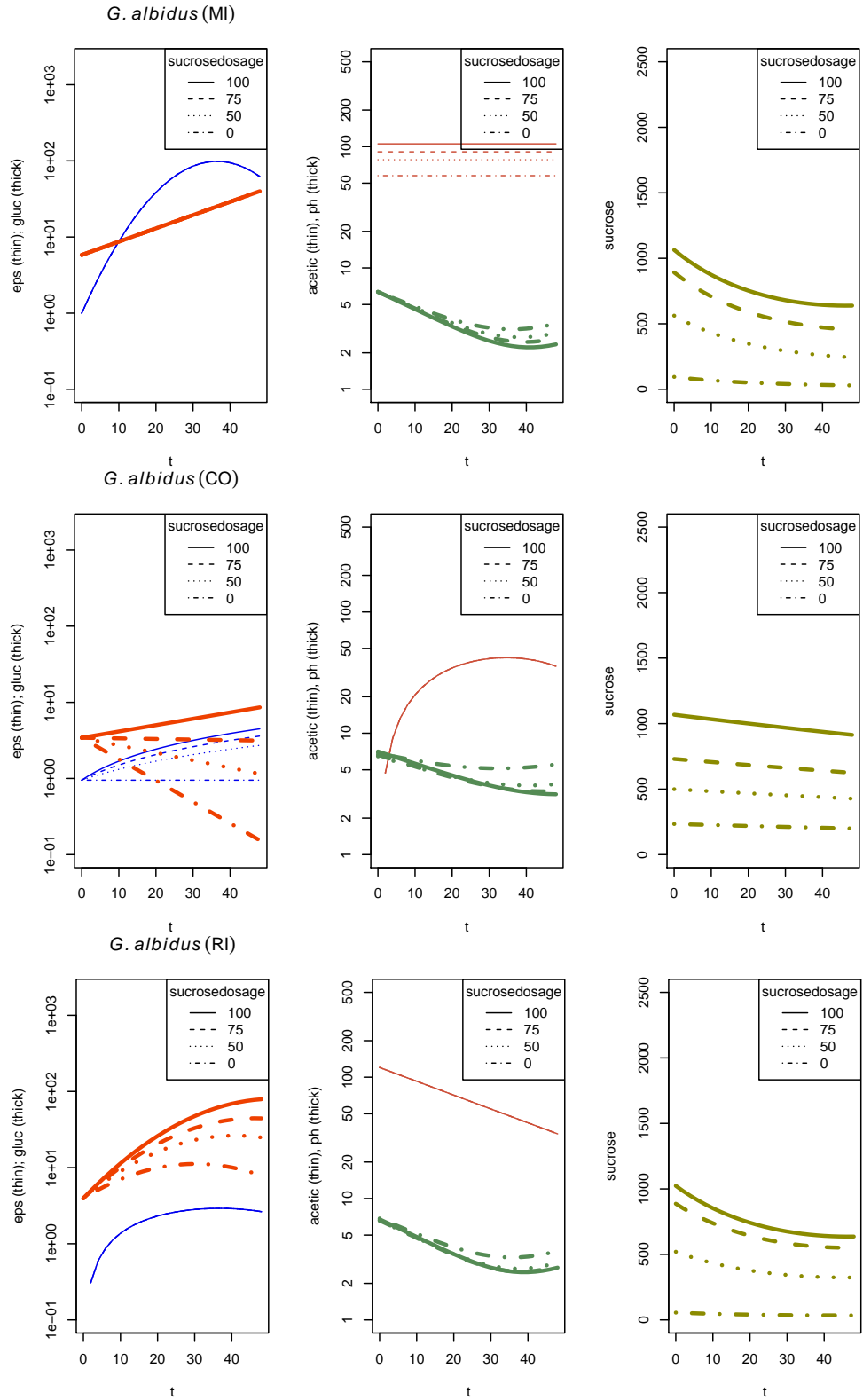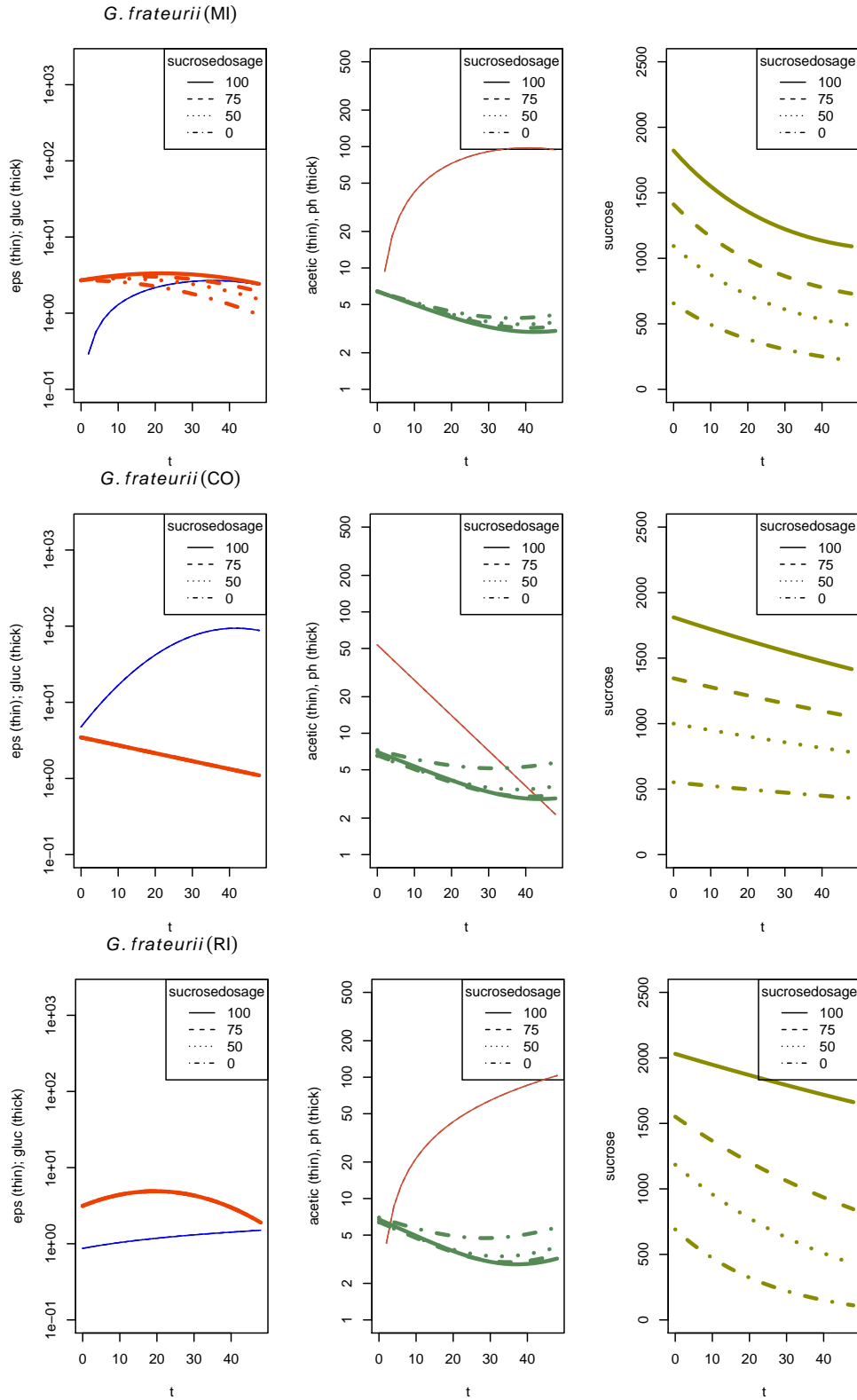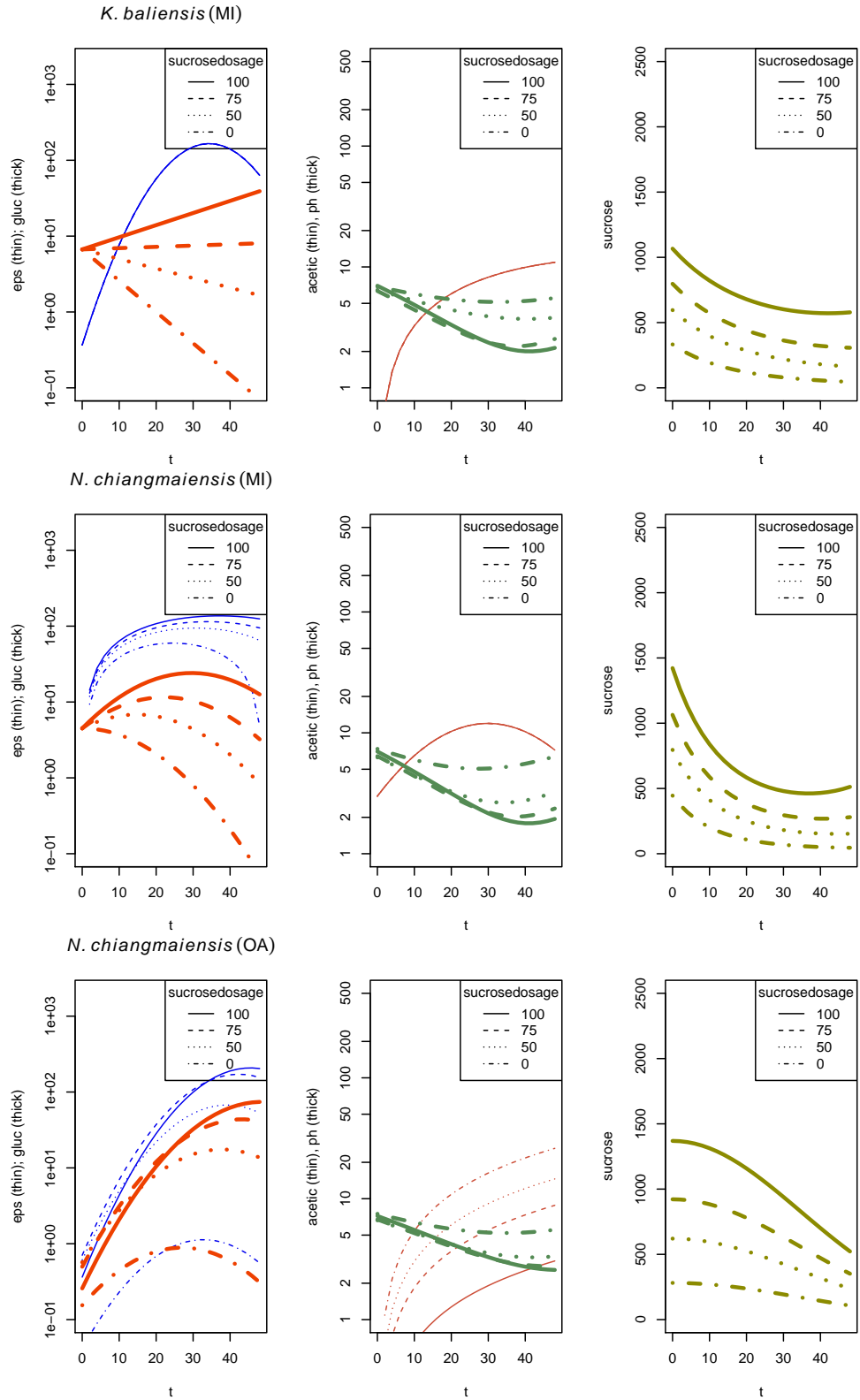**Figure 3.20** – Graphical summary of the time-sucrose-dependency of selected metabolites during fermentations in gluten-free doughs with *N. chiangmaiensis* and *K. baliensis.* MI: Millet; OA: Oat. Left: EPS (■), gluconic acid (■); Middle: pH value (■), acetic acid (■); Right: remaining sucrose level (■).

100 g/l sucrose, 48 h) and 85.9 (millet, 75 g/l sucrose, 24 h) were detected. After reaching the maximum, gluconic acid was partly degraded.

Acetic acid levels were quite low and therefore compareable to doughs with gluten-containing flours. In oat dough without added sucrose, about 28.6 mM/kg acetic acid was found in the end of fermentation. With sucrose addition, up to 13.6 ± 4.2 mM/kg acetic acid was found in oat dough in the end of fermentation. Maximum acetic acid levels in millet dough reached only 12.1 ± 3.5 mM/kg flour (100 g/l sucrose, 24 h), followed by a slight decrease.

Within 24 h, the pH value dropped to $3.4 - 3.9$ in oat doughs with sucrose and to $2.4 - 2.6$ in millet doughs with sucrose. In oat doughs, it was followed by a further decrease to $2.9 - 3.0$, whereas in millet doughs, pH remained at about the former level.

Statistical data analysis of data and mathematical modelling showed significances for all observed parameters in both flours. Chosen models were logarithmic for gluconic acid and sucrose and linear for pH value in both flours. In oat flour, the model for EPS was logarithmic, too, whereas it was linear for acetic acid. In millet flour, however, the model for EPS was linear and for acetic acid it was logarithmic. Graphs obtained from mathematical modelling are shown in Figure 3.20. One can see that start sucrose level positively influenced gluconic acid formation, EPS and final sucrose, with the most effective and faster sucrose usage in millet dough. For acetic acid and pH value, a negative correlation to higher start sucrose was found. As already recognized from the data, the maximum EPS level was reached earlier in millet dough than in oat dough. By graphical inspection of the statistical model, this results in approximately 30 h in millet and in around 45 h in oat dough. The same can be observed for gluconic acid and acetic acid. However, despite the slow metabolism, final EPS yields were clearly higher in oat than in millet dough.

As *N. chiangmaiensis* has reached higher EPS levels in oat dough, but after a longer time than in millet dough, where also reasonable EPS levels have been found, available fermentation time must be considered. However, *N. chiangmaiensis* was the only strain that could grow in oat dough. Here, the maximum level of 31 g EPS/kg flour was reached after 48 h (50 g/l sucrose) and related to 15.9 mM/kg flour gluconic acid, 13.6 mM/kg flour acetic acid and 63.2 g/kg sucrose.

3.4.6 Competitiveness of *G. frateurii* and *K. baliensis* upon backslopping in aerobic spelt dough

In practical applications of aerobic bacteria in sourdoughs, their competitiveness under realistic fermentation conditions is decisive to avoid permanent re-inoculation with pure cultures at high dosages. Therefore, two strains were tested for their ability to remain in aerobic spelt sourdoughs over several refreshments.

The results of the backslopping experiment in spelt dough enriched with sucrose (50 g/l) are shown in Figure 3.21. *G. frateurii* and *K. baliensis* were both detectable in the dough over seven refreshments, whereas one refreshment was performed every 24 h. From the starting point, which relates to the first dough inoculation using a preculture, to day 1 (cell count after 24 h), *G. frateurii* grew from $1.5 \cdot 10^8$ to $6.9 \cdot 10^8$ CFU/ml and

K. baliensis from $5.6 \cdot 10^7$ to $9.3 \cdot 10^8$ CFU/ml. As always 1/10 of sourdough was utilized for dough refreshment, the start cell count for the new dough amounted $6.9 \cdot 10^7$ CFU/ml for *G. frateurii* and $9.3 \cdot 10^7$ CFU/ml for *K. baliensis*. During the next 24 h, cell counts increased to $1.9 \cdot 10^8$ CFU/ml (*G. frateurii*) and $7.5 \cdot 10^8$ CFU/ml (*Kozakia*)(day 2). Although cell counts of the two strains decreased during the whole experiment compared to day 1, no contaminants were detected on the agar plates. When the experiment was stopped on day 8, still $2.0 \cdot 10^7$ CFU/ml of *G. frateurii* and $1.2 \cdot 10^8$ CFU/ml of *Kozakia* were counted.
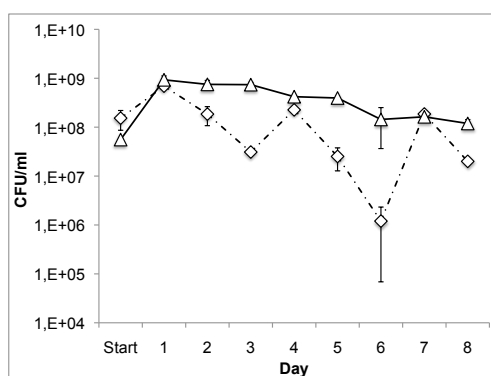
Figure 3.21 – Cell counts of backslopping experiment with *G. frateurii* ($\diamond$) and *K. baliensis* ($\triangle$) in spelt dough (DY = 500) with sucrose addition (50 g/l), using 1/10 of dough for daily refreshments. Start: cell count after the first dough inoculation. 1 to 8: cell count before dough refreshment.

3.5 Experiments with water kefir powder

As the range of applications for a food additive is determined by its properties, the overall and functional properties of WKP were tested in these experiments. Then, the potential of WKP to increase the quality of wheat breads was assessed by applying TPA-tests and sensory analyses of fresh and 48 h old breads.

3.5.1 Overall properties of WKP

The properties of the flour-like powder obtained from the procedure described in Section 2.7, which we termed WKP, were assessed macroscopically and also using a microscope to identify special characters and possible applications. WKP was homogeneous, white to cream-coloured and had a dry matter of $93.66 \pm 0.07\,\%$. It was stored in sealed plastic tins at room temperature for several months without changes. It was tasted internally and its aroma was described as mainly sour, starchy, acetic acid-like and fermented.

Adding WKP to aqueous solutions led to a decrease of pH value between 3.9 (1 % WKP) and 3.8 (2 % WKP).

Microscopic analysis of dry and wet WKP showed that it consisted of angular, sharp-edged particles whose angularity remained, but size increased during soaking. Furthermore, WKP became translucent when it was wet (Figures 3.22 and 3.23).
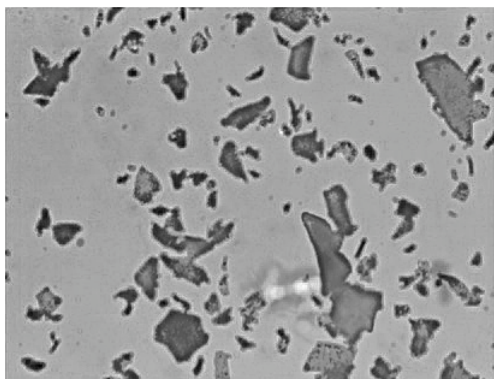
Figure 3.22 – Dry WKP, 40x.

Figure 3.23 – Wet WKP, 40x.

3.5.2 Functional properties of WKP

As it was assumed that WKP can act as a hydrocolloid and is therefore a valuable food ingredient, the functionality of the material was tested. The solubility of WKP was lower in organic than in inorganic solvents. It amounted $0.7 \pm 0.1\%$ for pure ethanol and $0.9 \pm 0.1\%$ for hexane. Considering inorganic solvents, most WKP was solved in $0.1\,M$ NaOH ($10.9 \pm 0.8\%$), followed by $0.1\,M$ HCl ($7.1 \pm 3.0\%$) and deionized water ($3.2 \pm 1.5\%$).

The water holding capacity of WKP showed, in opposite to starch, no temperature dependency in the range between room temperature and $75\,°C$: On both conditions, about nine times more water per WKP could be bound (w/w), as the water to WKP ratio was 9.19 ± 0.39 for room temperature and 8.69 ± 0.41 at $75\,°C$. Therewith the water holding capacity of WKP at room temperature lays between starch (0.82 ± 0.01) and xanthan (157.96 ± 21.13). Xanthan showed, like WKP, no temperature dependency up to $75\,°C$ (149.72 ± 10.16). In opposite to this, the WHC of starch increased as far as 5.35 ± 0.02 at $75\,°C$.

Watery WKP-suspensions were also subjected to freezing experiments, by which a freeze-thaw stability of $95.0 \pm 0.2\%$ for 8% (w/v) WKP, $96.8 \pm 0.4\%$ for 10% (w/v) WKP and $98.8 \pm 0.4\%$ for 12% (w/v) WKP concentration could be determined.

The fat binding capacity at room temperature amounted 1.29 ± 0.22 for WKP (w/w ratio of bound fat to sample), which was almost as high as soy protein (1.45 ± 0.03). The FBC of lecithin and gum arabic were both lower, laying at 0.84 for lecithin and 0.75 ± 0.03 for gum arabic.

For WKP, no gel-building activity could be determined, as it showed a thickening effect when being soaked in water, but did not develop any yield point during heating

or cooling.

Further, no emulsifying or foam-forming properties were found for WKP.

3.5.3 Quality of wheat breads with added WKP

The data obtained from bread evaluation are shown in Table 3.2, where WKP-enriched bread batches are compared to the corresponding standards prepared on the same day. Significant differences (bilateral homoscedastic t-test, $p < 0.05$) are indicated by asterisks. Crumb firmness relates to the maximum force which was required to compress the bread slice. Crust crispness was determined by the maximum force needed to break the standardized crust stripe (Section 2.9.3). As loaves were cut into cylinders and crust stripes before storage, bread height, weight and volume were determined for fresh breads only.

Fresh breads showed a significantly decreased crumb firmness for all levels of WKP addition, whereby the reduction amounted 15.1 % for 1 % WKP, 20.6 % for 2 % WKP and 16.0 % for 5 % WKP, compared to the relating standard group (Table 3.2). For 1 and 2 % WKP loaf weight and volume tended to increase, whereas for 5 % WKP, a significant reduction in maximum loaf height and volume was observed. Crust crispness of WKP-enriched breads tended to be lower than standard breads, although differences were not significant.

After 48 h, significantly decreased crumb firmness could be determined for all dosages of WKP addition, whereas this was accompanied by a slightly increased crust crispness for 1 % WKP and a decreased crust crispness for higher WKP dosages (Table 3.2).

3.5.4 Sensorial characters of WKP-enriched wheat breads

In this subsection, the results of an internal sensorial evaluation of baked breads are presented. The three different recipes were named as follows: St 10 relates to recipe E (Standard recipe with 10 g salt/kg flour), WKP 10 to recipe F (2 % WKP and 10 g salt/kg flour) and St 15 to recipe D (standard recipe with unaltered sodium content of 15 g/kg flour), whereas 10 g salt/kg flour mean 30 % sodium reduction. As breads were tasted by an untrained panel, the validity of data in this section is limited and should be assessed by further tastings with a trained panel.

Influence of WKP on sensorial characters of fresh breads and potential for sodium reduction

Panellists were asked to rate the three groups of fresh bread concerning smell, taste and overall acceptance. Regarding smell, the characters aromatic and sour were distinguished. The smell of standard breads with 30 % sodium reduction was perceived to be more aromatic than of WKP enriched breads (bilateral homoscedastic t test: $p = 0.006$)(Figure 3.24). Between standard breads with full sodium content and sodium reduced breads with and without WKP addition, no significant differences were detected. Concerning the parameter sour smell, all three bread groups reached a similar rating.

Table 3.2 – Effect of water kefir powder (WKP) addition on the quality of wheat breads compared to standard (St) breads. Mean values and standard deviation of 2−6 replicates shown. Significant differences between WKP-added and referring standard breads are indicated by asterisks (bilateral homoscedastic t test, $p < 0.05$).

h	Parameter tested	St 1%	WKP 1%	St 2%	WKP 2%	St 5%	WKP 5%
2	crumb firmness [N]	2.25 ± 0.25	1.91 ± 0.25*	2.96 ± 0.21	2.35 ± 0.28*	2.62 ± 0.16	2.20 ± 0.28*
	crust crispness [N]	7.46 ± 1.80	7.19 ± 2.01	8.75 ± 2.24	7.08 ± 1.42	8.42 ± 1.45	6.60 ± 1.59
	height [cm]	8.75 ± 0.19	8.70 ± 0.20	8.05 ± 0.13	8.09 ± 0.26	8.1 ± 0.27	7.63 ± 0.24*
	weight [g]	185.30 ± 1.31	186.46 ± 1.50	185.30 ± 1.05	186.09 ± 1.65	185.79 ± 1.23	184.51 ± 1.67
	volume [ml]	243.50 ± 2.12	252.00 ± 11.31	197.50 ± 6.36	227.00 ± 21.21	228.50 ± 2.12	192.50 ± 3.54*
48	crumb firmness [N]	7.16 ± 0.77	5.66 ± 0.41*	8.79 ± 0.74	5.94 ± 0.43*	7.61 ± 1.08	5.22 ± 0.72*
	crust crispness [N]	3.01 ± 0.47	3.24 ± 0.58	4.16 ± 0.69	3.31 ± 0.43*	3.65 ± 0.51	3.13 ± 0.64

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The characters for taste description of fresh breads included the attributes salty, sweet, sour, aromatic and bland. Applying a bilateral homoscedastic t test with $p < 0.05$, standard breads with 100% sodium dosage reached a significantly higher rating concerning salty taste than sodium-reduced breads with WKP ($p = 0.006$). Sweetness was valued similarly for all groups. Breads with WKP were rated less sour than breads without WKP, although differences were not significant concerning the full salt standard. The aromatic taste of WKP enriched breads was perceived to be significantly lower than of breads without WKP addition. These results were accompanied by a higher rated bland taste of WKP enriched breads towards standard breads with full salt content, without significant differences between breads with WKP and sodium reduced standard breads.

The question whether they would buy fresh, sodium-reduced breads with WKP addition was answered with "Yes" by 2 out of 11 panellists, whereas for standard breads without sodium reduction, the answer was "Yes" for 10 out of 11 panellists. Sodium-reduced breads without WKP addition would be bought by 6 out of 11 members of the sensorial panel (Figure 3.24).

Influence of WKP on aroma and staling of 48 h old breads

Breads which had been stored for 48 h were also evaluated by the untrained sensorial panel consisting of staff and students from the Institut für Technische Mikrobiologie, Technische Universität München and smell, taste and the decision to buy were assessed. Panellists were asked to rate the same characters as above (see Section 3.5.4), apart from the attributes musty smell and bland/sticky taste, which had been added to the questionnaire.

The smell of WKP enriched breads was rated significantly more sour than of standard breads with full sodium content ($p = 0.008$)(Figure 3.24). Between sodium reduced breads with and without WKP and between the sodium reduced and unreduced standard, differences were not significant.

Salty taste of the full salt standard was rated significantly higher than of sodium reduced breads with and without WKP addition ($p = 0.01$ for both groups).

The decision to buy WKP enriched, sodium reduced breads was answered with "Yes" by 6 out of 14 panellists. Only 2 panellists would buy sodium reduced breads without WKP addition. Concerning the full salt standard, 8 of the panellists stated that they would buy it.
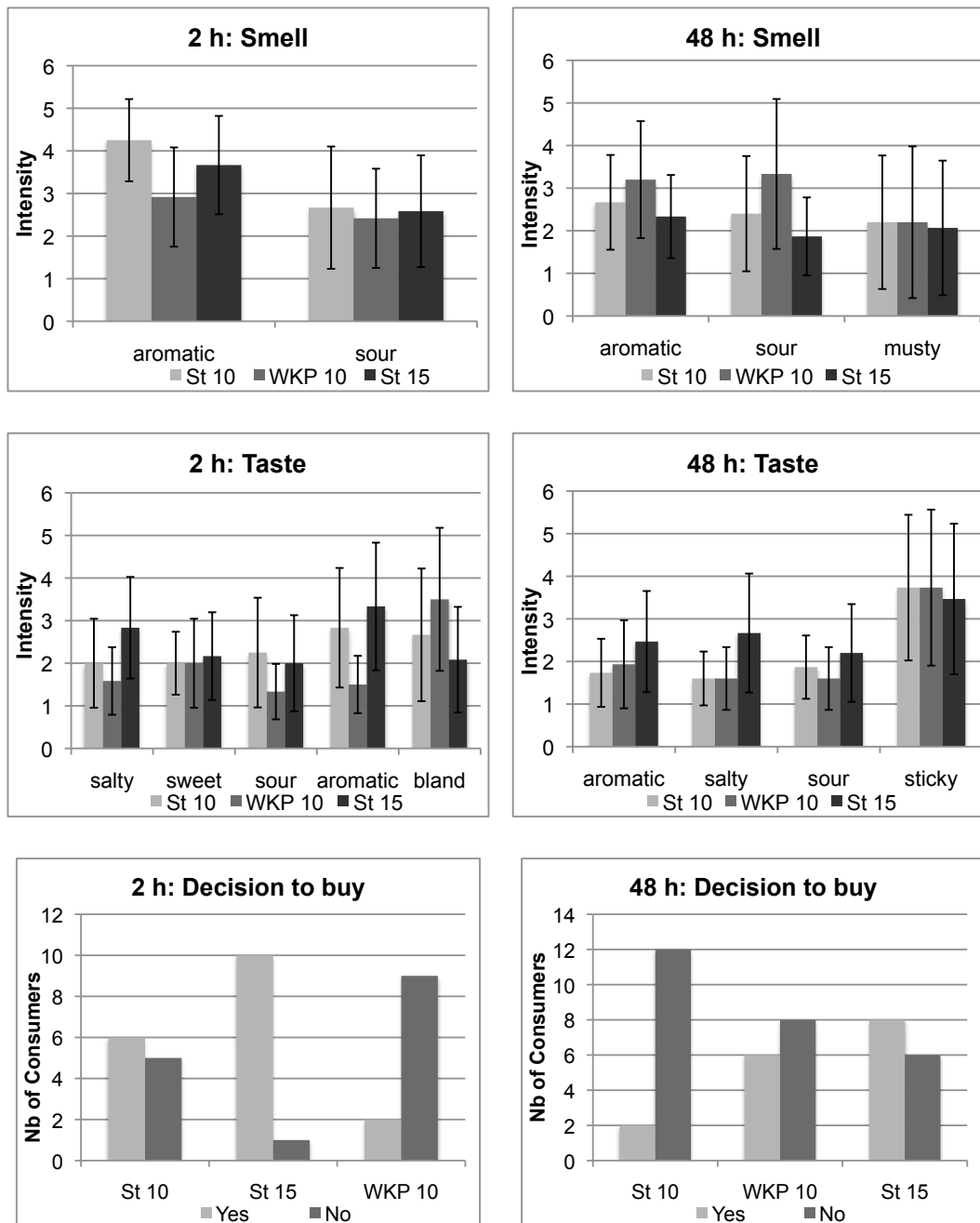
Figure 3.24 – Sensory evaluation of 2 h (left) and 48 h (right) old breads: Smell, taste and decision to buy were evaluated by 11 untrained panellists from the Institute of Technical Microbiology, Technische Universität München.

4 Discussion

The addition of hydrocolloids is a promising approach to achieve a quality improvement in breads ([Guarda et al., 2004]). In gluten containing breads, their application mainly focuses on the increase of volume and shelf-life ([Guarda et al., 2004]), whereas in gluten-free products, the texture, palatability and overall quality is targeted ([Lazaridou et al., 2007], [Galle et al., 2012]). In many applications, plant based hydrocolloids can be replaced by microbial exopolysaccharides. Depending on their composition, size and structure, they serve as viscosifying, stabilizing, emulsifying or gelling agents ([De Vuyst and Degeest, 1999]). Due to the increased demand for natural polymers for various industrial applications in the last 30 years, these bacterial exopolysaccharides (EPS) have emerged as new, industrially important polymeric materials ([Sutherland, 2002], [Kumar et al., 2007]). Recently, several AAB strains have been shown to produce very effective EPS with a positive influence on the quality and staling of wheat breads ([Jakob et al., 2012b]) As the isolation of EPS is a costly and time-consuming procedure and as these fructans have not been approved as food additives yet, the cultivation of AAB in sourdoughs and thereby the *in situ* production of EPS for baking applications is a promising approach for industrial applications. In this work, all the prerequisites to apply such aerobic bacteria in sourdoughs were tested. It was demonstrated that these strains were able to grow in gluten containing and gluten-free aerobic sourdough, whereby relevant amounts of fructans were produced *in situ*. By way of backslopping experiments, two of the strains were shown to establish in type I sourdough systems over several refreshments. Optimal conditions for bacterial growth and high EPS levels in laboratory media and in sourdoughs were identified, which can help to reduce fermentation time (and cost) in industrial applications. As an example for the application of novel EPS in baking, water kefir grains have been investigated thereafter. Water kefir is formed by a microbial consortium, which is embedded into jelly crystals. This work showed that a flour-like powder produced from the grains, which we termed WKP (water kefir powder), possesses hydrocolloid properties and is a promising material for food applications and beyond. Adding 1, 2 and 5 % WKP to wheat breads lead to decreased crumb firmness after 2 and 48 h and therefore increased freshness and retarded staling. No negative influence of WKP on smell and taste was found, whereas no potential for sodium reduction by using WKP could be proven.

4.1 Robustness of EPS formation in relation to different nutrient media

By selecting these seven media, the robustness of EPS formation in different habitats should be shown, i.e. in nutrient-rich, typical lactobacilli-media (Homohiochii and mMRS) and nutrient-poor *Gluconobacter* media (Mannitol and No. 5), in a medium with glucose as additional carbon source and further in a direct comparison between a nutrient-rich (GM+vitamins) and nutrient-poor (GM) medium. Thus the strains should be assessed towards their ability of EPS formation under different nutrient and therefore growth conditions and a general overview about the strains and their preferences for EPS formation should be obtained.

When comparing the results depicted in Figure 3.1, it can be seen that the tested strains show some similarities, but also differences concerning EPS-formation in the selected media.

The highest EPS amounts of all fermentations lay above 20 g/l and were found in experiments with *K. baliensis* (Spicher-medium, 72 h; SoG-medium, 54 h; mMRS-medium, 72 h)(Figure 3.1f) and *N. chiangmaiensis* (mMRS-medium, 72 h)(Figure 3.1e). The second highest EPS-concentrations ($15 - 20$ g/l) were reached by *G. frateurii* and *G. albidus* in SoG-medium after 48 and 54 h, respectively (Figures 3.1a and 3.1d). *G. cerinus* and *G. oxidans* did not produce more than 5 g/l EPS in any of the media tested. The lowest EPS levels of all strains were generally found in GfK-medium, which contained glucose as additional carbon source apart from sucrose. This is no indication for a repression of sucrose consumption by the monosaccharide glucose, because sucrose utilization started after 6 h, when still sufficient amounts of glucose were present. It is more likely that bacteria consumed glucose but produced FTF at the same time, which used sucrose for EPS formation. As glucose consumption lead to a lower pH value (Table 3.1), FTFs could not work as effective as in the other media and therefore less EPS have been formed. Similarly to EPS-production by lactobacilli, higher yields were obtained in complex media ([Notararigo et al., 2012]).

High EPS levels were either connected to the presence of sodium gluconate (*G. frateurii*, *G. albidus*, *K. baliensis*), different vitamins and sugars except glucose (*G. albidus*: mMRS; *K. baliensis*: Spicher, mMRS, *G. frateurii*: Spicher), vitamins (*G. albidus* and *K. baliensis*: GM+Vit) or mannitol as additional carbon source (*G. frateurii* and *K. baliensis*: GM, No. 5; *N. chiangmaiensis*: No. 5). In GM+Vit-medium, EPS yields were higher than in GM medium in fermentations with *G. albidus*, *N. chiangmaiensis* and *K. baliensis*. EPS contents in GM-medium were further outreached by No. 5-medium, which contained less mannitol (10 g/l instead of 25 g/l in GM), but therefore more yeast extract (15 g/l instead of 5 g/l yeast extract and 3 g/l peptone). In contrast to GM-medium, No 5. medium contained low amounts of magnesium sulfate (2.5 g/l) and glycerol (0.5 g/l). In experiments with *N. chiangmaiensis* and *K. baliensis*, EPS yields in No. 5 medium even outreached GM+Vit medium. This might be a hint that EPS formation of these two strains is more promoted by a higher protein content than by vitamin addition. In contrast to this, EPS formation of *G. albidus* was more affected by vitamin

addition (GM+Vit) instead of a higher protein content (No. 5), compared to pure GM medium (Figure 3.1d). In fermentations with *G. frateurii*, these differences were less pronounced and EPS formation was quite similar in the three media (GM, GM+Vit, No. 5)(Figure 3.1a). Here, the sodium gluconate-containing medium (SoG) has had the most promoting effect on EPS formation.

Also concerning the speed of EPS formation, a few differences have occurred: The slowliest EPS producer was *N. chiangmaiensis*, because EPS formation was still increasing after 3 days of fermentation (Figure 3.1e). In experiments with *K. baliensis*, EPS formation was slightly faster with peaks between 48 h and 72 h (Figure 3.1f), whereas peak EPS levels of fermentations with *G. frateurii* and *G. albidus* were reached between 24 and 48 h (Figures 3.1a and 3.1d). However, maximum levels of the latter two strains were lower than of *Kozakia* and *Neoasaia*.

Strains also behaved differently relating to the degradation of EPS after the maximum: EPS were either fully or partly degraded by *G. frateurii* and *G. albidus*. EPS degradation of *K. baliensis* was only weak and of *N. chiangmaiensis* it was not recognizable, as maximum yields had not been reached in the end of fermentations.

This shows that all of the chosen strains have to be seen individually and that they have different preferences towards nutrient media composition. So far, *K. baliensis* seems to be the most promising strain for EPS production, as it had reached levels of around 20 g/l in four out of seven media after a moderate incubation time and showed only low EPS degradation. As *G. oxidans* and *G. cerinus* have been identified as slow EPS-producers, they were excluded from further experiments.

4.2 Influence of carbon source on growth, metabolism and EPS formation

In the following, results of fermentations in laboratory media with varied carbon sources in addition to sucrose will be discussed, beginning with the growth experiments in the exponential phase. The results of these experiments are not significant, as they were not carried out in duplicate. Therefore trends and likely relations between carbon sources and strains supporting the further discussion can be derived from these data, but no reliable conclusions are possible. After this, results of 72 h lasting fermentations will be considered. As these experiments were carried out in duplicates, data are more reliable and may corroborate central trends found in the other experiments. Occuring differences between both fermentations will be discussed, too.

4.2.1 Effect of additional carbon sources on bacterial growth in the exponential phase

Measurements of OD_{590} during the first 12 hours of fermentations with different carbon source indicate an influence of sugar composition on growth, which varies between the tested strains (3.3). Although the optical density is a strain-specific parameter which is individually related to growth, the huge differences perceived here indicate a

higher growth rate of *G. albidus* and *N. chiangmaiensis* in comparison to *G. frateurii*. *K. baliensis* showed the lowest growth of all strains tested.

If only the fermentations of one bacterial strain are considered, *G. albidus* showed a large deviation in final OD_{590}, reaching from 1.0 to 2.1. Maximum growth was observed in medium B, which contained sucrose and mannitol (Figure 3.3b). One possible reason for this is the adaption of *G. albidus* metabolism to the preculture medium, which contained $10\,g/l$ mannitol, allowing *G. albidus* to grow faster than in the other media. To confirm this, the experiment should be repeated with a mannitol free preculture medium. The slowest growth was observed in medium A, which contained only sucrose. Here, the necessary cleavage of sucrose preceding the generation of energy from the resulting monosaccharides could have limited growth. In the media containing fructose (D) and glucose (C) in addition to sucrose, growth curves lay between the ones of medium A and B. Possibly, the dehydrogenases in the periplasmic membrane preferred the monosaccharides for the bacteria's fast gain of energy and subsequent growth towards the disaccharide sucrose. It is unclear, why growth in medium E (Sucr + Mal) was similar to the latter two media, as maltose was observed to not being significantly degraded in the long-term experiments by any of the tested strains. In medium F (Sucr + SoGlu), two stages of growth were observed: Within the first eight hours, growth rate resembled the one in medium A (Sucr), while for the next four hours, it increased and OD_{590} even exceeded the one in the mannitol medium. Here it seems as if some adaptions to the media have taken place during the fermentation. Possibly due to structural differences the adaption to sodium gluconate as carbon source required more time than to the other sugars. The higher buffer capacity caused by sodium gluconate addition and the accompanying higher pH value which was observed in the $72\,h$ lasting fermentations could have played a role in better growth in the end of experiment.

In fermentations with *G. frateurii*, added carbon sources seemed to have only a small influence on energy production and subsequent increase in cell count, as the OD_{590} after 12 hours lay between 1.1 and 1.2 in all fermentations (Figure 3.3a). Possibly, *G. frateurii* was growing at maximum rates even without the availability of monosaccharides in addition to the disaccharide sucrose, so that further sugars did not have any beneficial effect. Another explanation could be that growth was limited by other nutrients, i. e. vitamins or minerals, so that it was not accelerated by additional carbon sources. This might also be the reason for the lower overall growth compared to *N. chiangmaiensis* and *G. albidus*. However, *Gluconobacter* are commonly incapable of rapid growth even in a complete medium ([Macauley et al., 2001]). As *G. frateurii* belongs to the *G. cerinus* group and *G. albidus* to the *G. oxydans* group with *G. oxydans* having a relatively slow growth rate ([Macauley et al., 2001], [Yamada and Yukphan, 2008]), the results obtained from the experiments contradict these group characters.

Related to the slow growth and the low final OD_{590} in fermentations with *K. baliensis*, the differences of final OD which lay between 0.2 and 0.4 seemed to be strongly pronounced (Figure 3.3d). During the first $5\,h$, however, growth was quite similar in all media and increased very slowly, accompanied by almost unchanged yields of sugars and metabolites during the first $6\,h$ which were recognized in long-term fermentations (Figure 3.11). After $5\,h$, growth started and resulted in different OD values, whereby

the strongest increase could be noticed in medium C and D (Sucr + Glc and Sucr + Frc, respectively). This means that growth was promoted more efficiently by glucose or fructose than by maltose or mannitol (medium E and B) in addition to sucrose. However, all of these monosaccharides lead to higher growth rates than sucrose on its own. In medium F (Sucr + SoGlu), OD_{590} increased the slowest compared to the other media and the increase was almost linear throughout the experiment. This can again be due to a regulatory effect of sodium-gluconate on metabolism or to a longer adaption phase to this carbon source, because it has a lower structural similarity to the carbon source used in the preculture medium (glucose) than the other sugars.

In fermentations with *N. chiangmaiensis*, the lowest OD_{590} value after 12 hours lay at 1.5 and the highest at 2.1. First changes in OD_{590} between the fermentations occured already after 3 h and were even more pronounced during the following time. However, this only relates to medium F (Sucr + SoGlu) and D (Sucr + Frc), in which lower growth occured compared to the other media. After 5 h, OD_{590} of medium F started to increase strongly until the highest OD-value was reached in the end of fermentation. One possible explanation for this phenomenon is that sodium-gluconate has a certain regulatory effect retarding growth. Another assumption is that the adaption of *N. chiangmaiensis* metabolism to that carbon source lasts longer than to the other sugars, which have higher structural similarities with xylose used in the preculture medium. In medium D which contained pure D-fructose, the increase in OD_{590} started earlier than in medium F, but was slower, so that it lay at the lowest level in the end of experiment, compared to the other media. It is known that *N. chiangmaiensis* uses D-fructose for acid production and that acid formation out of mannitol and maltose is only weak ([Yukphan et al., 2005]). Acid production leads to a decrease in pH value and can therefore move it from the optimum growth level, which could explain the decreased growth rate in fructose-enriched medium.

4.2.2 *G. frateurii*: Sugar conversions and EPS formation in media with changing carbon sources

In all media, typical metabolic reactions of *G. frateurii* could be observed: Cell counts increased, sugars were degraded and EPS was formed following the degradation of sucrose. At the same time, gluconic acid was produced, followed by increased acetic acid yields accumulating in the medium, whereas gluconic acid was further oxidized. It is known from *G. oxydans*, that glucose can be oxidized via the non-phosphorylative, direct oxidation to gluconic acid and ketogluconic acid, or by initial phosphorylation followed by oxidation via the pentose phosphate pathway. At pH values below 3.5 and also above a threshold value of 5-15 mM glucose, oxidation of glucose and gluconate by the pentose phosphate pathway enzymes is repressed, explaining the rapid accumulation of gluconic acid in the culture medium ([Olijve and Kok, 1979b]).

What should be recognized is that cell counts of *G. frateurii* showed only slight variations after reaching the maximum. Thus the stationary phase of growth lasted until the end of fermentations, when pH values had fallen below pH 3 and acetic acid content was still slightly increasing. On the one hand, this might be a result of high sugar dosages

enabling a prolonged batch life cycle, because sugars have never been used up completely in the end of fermentations. On the other hand, these results show a high resistance of *G. frateurii* towards low pH values.

The maximum EPS yield found in medium F can be explained by the increased pH value (Table 3.1). In experiments carried out to analyse the influence of gluconic and acetic acid on the pH value of the chosen media, it was observed that the same amount of acids led to a lower pH decrease in medium F, indicating a higher buffer capacity caused by sodium gluconate addition. Most known fructosyltransferases have their optimum activity between pH 4.5–7.5 ([Velázquez-Hernández et al., 2009]), with the pH optimum of a fructosyltransferase from *G. diazotrophicus* at 5.0 ([Hernandez et al., 1995], [Trujillo et al., 2001]), from *Lactobacillus panis* at pH 4.0–4.6 ([Waldherr et al., 2008]) and from *Leuconostoc mesenteroides* at pH 6.2 ([Kang et al., 2005]). The data show that a similar or higher pH optimum is most likely for FTF's of *G. frateurii*.

The formation of EPS was accompanied by sucrose utilization, which was cleaved by fructosyltransferase into glucose and fructose, followed by fructan formation. The observed decrease in EPS yields towards the end of fermentations was strongest pronounced in medium A, B and C (Sucr, Sucr + Man and Sucr + Glc). It was not related to remaining sucrose contents, as especially in medium C, there was much sucrose left in the end of fermentation and in medium F, where only low EPS degradation took place, remaining sucrose content was the lowest. The degradation of EPS could be a result of acid hydrolysis, as in medium A, B and C, the most decreased pH values were found (Table 3.1). Another possibility is that the FTF of *G. frateurii* possesses hydrolytic activity. Moreover, another enzyme able to degrade EPS might have been formed, whereby the existence of EPS-degrading enzymes has not been clarified yet for acetic acid bacteria. For *Lactobacillus rhamnosus*, for example, it has been shown that several glycohydrolases are responsible for the decrease in molecular weight and viscosity of EPS ([Pham et al., 2000]). Also for xanthan, which is produced by *Xanthomonas campestris*, there exist specific xanthan lyases, produced by a *Bacillus* and *Corynebacterium* species ([Sutherland, 1987]).

The carbon sources preferred by *G. frateurii* were mannitol and glucose, as they were consumed first in contrast to fructose and maltose. However, their presence did not result in high EPS formation (Figure 3.5), but rather in a strong pH decrease (Table 3.1), which inhibits the formation of high EPS levels as discussed above. Although in both fermentations in medium B, the added mannitol has been consumed completely, mannitol set free by *G. frateurii* as an end product of fructose degradation has not been metabolized but accumulated in the media. Possibly, the bacteria's metabolism was not adapted to this carbon source at that time due to the presence of other sources. This means that either mannitol concentrations were too low to initiate the up-regulation of the corresponding enzymes or that there was not enough time for the bacteria to switch to the mannitol-degrading metabolic pathway.

To discuss these results in terms of EPS optimization, several parameters should be recognized. If EPS are to be isolated and purified from fermentation broth, no further parameters like acetic acid concentration or remaining sugars play a role. Therefore in this case, the medium and time point with the highest EPS level can be chosen, which

were medium A and F with about 81 mM Frc equ/l (average of both fermentations) after 24 h and 30 h, respectively. If the fermentation broth should be further processed in a whole, the parameters acetic acid, gluconic acid and sugar concentration must be considered. However, acetic acid levels were rather low in these experiments (< 10 mM/l). To minimize remaining sugar concentration, broth should be harvested in the end of fermentation, but at this time point, EPS were partly degraded. Only in medium F, where the lowest remaining sucrose content was found (10 mM/l), EPS content was still at maximum after 72 h. This was also the medium with the highest gluconic acid yields, laying at about 82 mM/l (average of both experiments) at t_{48}. In the end of fermentations, about 23 mM/l gluconic acid was left (average of both fermentations). Gluconic acid and its salts are useful materials with a wide range of applications in the pharmaceutical, food, feed, detergent, textile, leather, photographic and other industries ([Das and Kundu, 1987], [Ramachandran et al., 2006]). Most likely, the detected decrease in gluconic acid is due to the further oxidation to ketogluconates: The formation of 2- and 5- ketogluconate and 2,5-diketogluconic acid was found for *G. oxydans*, which is why bacterial gluconic acid production has only limited success at industrial scale ([Weenk et al., 1984], [Ramachandran et al., 2006]). This means that if gluconic acid formation is to be recovered as a by-product in addition to EPS, the optimal time point for harvest has to be considered. On the other hand, ketogluconates also possess industrial importance and therefore oxidation of gluconic acid could be further optimized ([Stubbs et al., 1943], [Kita and Fenton, 1982]).

Another solution to harvest the broth at once and to minimize the concentration of small molecules is the usage of separation processes, e.g. dialysis, ultrafiltration or microfiltration. This way, EPS could be harvested without recognizing yields of other metabolites and no isolation of pure EPS were necessary.

4.2.3 *G. albidus*: Sugar conversions and EPS formation in media with changing carbon sources

When analyzing the two replicates of fermentations with *G. albidus*, the most obvious difference is the presence of high amounts of acetic acid in all media after 48 h in the second trial, which is almost completely degraded until the end of experiment. As *Gluconobacter* are generally unable to further oxidize acetic acid ([De Ley and Swings, 1984]), this is most likely the result of an error occuring either during the fermentation or the analysis of samples. One possibility is the contamination with another strain being able to degrade acetic acid. This must have been a strain with a colony morphology quite similar to *G. albidus*, e.g. *N. chiangmaiensis* or *K. baliensis*, because otherwise the contamination would have been detected on the plates. However, in fermentations with *N. chiangmaiensis* or *K. baliensis*, only very low amounts of acetic acid have been found (Sections 3.3.4 and 3.3.5). Therefore, a measuring mistake during the quantification of acetic acid seems to be most likely.

In all experiments with *G. albidus*, cell counts decreased in the end, with the highest CFU/ml occuring in medium F. Generally, *Gluconobacter* prefer pH values between 5.5 and 6 ([Deppenmeier et al., 2002]). In our results, the drop in pH value was not as

strong in medium F as in the other media (Table 3.1). Data analysis revealed a positive correlation between pH and CFU/ml at t_{72} in both fermentations ($R^2 = 0.8086$ and 0.8375). This shows that *G. albidus* is either more pH-sensitive than *G. frateurii*, wo was still in the stationary phase in all media in the end of fermentations, or has different nutrient requirements.

The conversion rate of consumed sucrose to EPS at maximum EPS levels was independent from the medium and lay at around $40 – 50 \%$. Sucrose consumption tended to be lowest in medium C, and thus lowest EPS yields were measured in this medium. A possible explanation for this is that glucose is metabolized first and sucrose metabolism is suppressed until glucose has been fully consumed. As the consumption of glucose by *Gluconobacter* strains is related to the intense oxidation to gluconic acid, a strong drop of pH value occurs (Table 3.1)([Velizarov and Beschkov, 1994], [Gupta et al., 2001]). In pH-sensitive strains, this can results in a low metabolic activity or even death. Furthermore, these low pH values lead to reduced sucrose degradation and EPS formation, as already shown for *G. frateurii*. This finding is supported by the positive correlation found for EPS and pH at t_{72} in the second fermentation with *G. albidus* ($R^2 = 0.8525$). It can either be caused by an increased production of FTF at elevated pH values or by a higher pH optimum of levansucrases, which has been proven for other species (4.2.2).

In all experiments, no changes in sugar concentrations were detected during t_0 and t_6 and t_{48} and t_{72}. During the first six hours, cells present in the media had to adapt to the medium and to reach a higher cell count for remarkable sugar conversions. In the last 24 h, cell count in all media decreased. The decrease in cell number means that cells died, which was probably either caused by high acidity, the accumulation of toxic metabolites in the medium or a lack of nutrients apart from sugar, as there were still high amounts of sugars left at this time point.

Same as *G. frateurii*, maltose was not metabolized by *G. albidus*, either indicating that *G. albidus* has no genes encoding maltose cleaving enzymes or that under conditions of a sucrose surplus, no up-regulation of such genes takes place. It has been reported that maltose cleavage is possible in *G. oxydans* possessing a point mutation in a glucose dehydrogenase gene, which enables the enzyme to oxidize maltose by replacement of one amino acid ([Cleton-Jansen et al., 1991]).

If *G. albidus* should be used for EPS production, there are again two different approaches: One is to isolate and purify EPS under optimum conditions and the other one is to use the whole fermentation broth, whereby here, also remaining sugars and acid accumulation should be considered. The highest EPS amount was reached after 48 h in medium with sodium gluconate and lay at 70.5 mM Frc equ/l (average of both fermentations), which was about 10 mM/l lower than the maximum found for *G. frateurii*. At this point, an average of 53.9 mM/l sucrose, 34.3 mM/l fructose, 22.0 mM/l glucose and 44.8 mM/l gluconic acid was measured in both replicates. In the first fermentation, the corresponding acetic acid concentration amounted 8.7 mM/l which was the highest level in fermentation one considering all EPS peaks. The sucrose content obtained under these conditions was the lowest of all experiments with *G. albidus*. Therefore in terms of sucrose minimization, the same conditions can be recommended. In terms of acetic acid minimization, medium A and B should be preferred, as no acetic acid was found at

the EPS maximum, which lay at about $50 - 55$ mM Frc equ/l and was reached after 30 and 24 h, respectively. Highest gluconic acid yields were measured in the end of fermentations B and E and amounted $63 - 65$ mM/l. The corresponding EPS contents were 49 and 35 mM Frc equ/l, respectively. A decrease in gluconic acid after reaching the peak was only found in medium C and F, whereas for *G. frateurii*, it was recognized in all fermentations.

4.2.4 *N. chiangmaiensis*: Sugar conversions and EPS formation in media with changing carbon sources

In all fermentations with *N. chiangmaiensis*, almost all initial sucrose was degraded between 6 and 72 h (3.9). The very efficient sucrose metabolism was related to the highest EPS recovery of all previously discussed strains (average of both replicates), whereas EPS peaks were reached quite late after 48 and 72 h. This showed that FTF works independently from cell count which increased until $24 - 48$ h followed by a decrease. As cells secrete the enzyme, which then remains active in the respective environment, actual cell counts do not determine EPS formation. About 50% of consumed sucrose had been converted into EPS at the time of EPS maximum.

As observed by [Tajima et al., 1998] for *Acetobacter xylinum*, glucose and fructose appeared in addition to levan in the culture broth of *N. chiangmaiensis* when sucrose levels decreased. Furthermore, acetic acid and gluconic acid were formed with the maximum yields preceeding EPS peaks in medium $A - E$. This is a little surprising, as the presence of acids lowers the pH value and this decreases activity of EPS-producing FTF. The high EPS formation of *N. chiangmaiensis* is remarkable also from another point of view, because this strain caused the lowest final pH values measured in all experiments of this series. This means that either the high metabolic activity or a higher FTF formation of *N. chiangmaiensis* plays a more important role for EPS formation than optimal pH levels or that FTF of *N. chiangmaiensis* is not as pH-sensitive as of the two *Gluconobacter* strains. However, the decrease of pH value was slightly slower than in fermentations with *Gluconobacter*. Furthermore, the general positive correlation between EPS contents and pH still holds: In fermentation 1, the lowest pH value in the glucose-containing medium was associated with the lowest EPS yields and medium F (Sucr + SoGlu) in which the highest EPS values were found, was the only medium with maximum acid yields not preceeding the EPS peak. The decrease of pH values in the glucose-containing medium and in all other media where glucose has been set free can again be explained by the conversion of glucose to gluconic acid by membrane bound dehydrogenases: For *G. oxydans* it was shown that without pH control, glucose can be completely converted into gluconic acid ([Olijve and Kok, 1979b], [Weenk et al., 1984]).

In contrast to fermentations with the two *Gluconobacter* strains, EPS was produced throughout the whole fermentation time in most experiments, which is why peaks were reached very late. Degradation of EPS yields after the peak took place only in medium D.

In terms of EPS optimization, the same medium as for *G. frateurii* and *G. albidus* can be recommended, which was medium F. Here, the highest EPS level of about 110 mM

Frc equ/l (average of both fermentations) was detected after 48 h. This means that the EPS optimum was reached later by *N. chiangmaiensis* than by the two *Gluconobacter* strains. Maximum EPS levels exceeded the amounts found for *G. frateurii* in five media and were generally higher than maximum levels found for *G. albidus*. However, after 24 and 48 h where EPS peaks of *G. frateurii* and *G. albidus* lay, EPS yields of *N. chiangmaiensis* were similar. The difference in fermentations with *N. chiangmaiensis* is that FTF maintained its high activity in the following time and further produced EPS. Compared to *Gluconobacter*, only very low amounts of acetic acid were detected, as they never exceeded 5 mM/l. Gluconic acid accumulation was higher than this and amounted up to 53 mM/l. The gluconic acid concentration that corresponded to the maximum EPS yield was 52 mM/l. It was accompanied by about 21 mM/l sucrose, 76 mM/l fructose, 20 mM/l glucose and 19 mM/l mannitol. After 72 h in medium F, sucrose was completely degraded. However, this was related to an increase in fructose (88 mM/l) and mannitol (40 mM/l). In general, fructose and mannitol accumulation tended to be higher in fermentations with *N. chiangmaiensis* compared to *Gluconobacter*. However, due to the high EPS levels compared to both *Gluconobacter* strains tested, *N. chiangmaiensis* seems to be a promising strain for fructan production in laboratory media. An amount of 110 mM Frc equ/l corresponds to about 19.8 g EPS/l.

4.2.5 *K. baliensis*: Sugar conversions and EPS formation in media with changing carbon sources

In the first fermentation with *K. baliensis*, a correlation between pH and CFU/ml was found at t_{48} ($R^2 = 0.897$) and t_{72} ($R^2 = 0.8282$), indicating a strong pH sensitivity of *K. baliensis* growth. Furthermore, a weak positive correlation for EPS and pH ($R^2 = 0.5514$) as well as CFU/ml and EPS ($R^2 = 0.6879$) was found at t_{72} in the first fermentation. Thus it can be assumed that also FTF from *K. baliensis* requires a rather high value for optimum activity. In the second fermentation, the relation between cell viability and pH value could not be observed, because cell counts on the plates decreased strongly at t_{72}. This phenomenon was independent of pH, as pH values were similar at t_{48} and t_{72}, and was most likely caused by an error during diluting or plating or by bad growth on plates. It can be assumed that the drop in pH value has been caused by gluconic acid alone, because acetic acid contents were only minor and lay with < 2 mM/l even below the yields detected for *N. chiangmaiensis*.

The maximum EPS yield reached after 72 h in the first replicate of medium F was about 110 mM Frc equ/l and therefore as high as the maximum found after 48 h for *N. chiangmaiensis*. However, the average of both duplicates in medium F was much lower, because in the second fermentation, sucrose consumption and thus EPS formation was very weak. This experiment can be considered as an outlier, as in all other experiments and also in the first replicate of medium F, more than 90 % sucrose had been degraded throughout the fermentation. Maximum EPS concentrations found in the other media lay around 100 mM Frc equ/ml (average of both replicates) in medium A, D and E and were reached at t_{48}. In medium B and C, they amounted 98 and 93 mM Frc equ/l (average of both replicates) and were reached after 48 and 72 h. This means

that apart from medium F, EPS yields were higher and reached faster or within the same fermentation time compared to *N. chiangmaiensis*. EPS contents also exceeded the maxima found for the tested *Gluconobacter* strains, although fermentation time was rather longer, apart from media E and F. Previous results obtained at our institute show four AAB strains, among them *G. frateurii*, *N. chiangmaiensis* and *K. baliensis*, to produce up to 66.6 mM Frc equ/l in liquid gluconate media containing 80 g/l sucrose ([Jakob et al., 2012b]). The EPS yields found for *K. baliensis* and *N. chiangmaiensis* in this thesis show that these values can be exceeded by suited fermentation conditions. Possibly, even higher EPS yields can be reached by further optimization upon exhaustion.

Sucrose degradation at the point of maximum EPS yield amounted 90 % and was therefore more efficient than the one found for *N. chiangmaiensis*. In fermentations with the two *Gluconobacter* strains, a similarly efficient sucrose consumption was only found in medium F. The fact that pH was always higher in fermentations with *K. baliensis* compared to the other strains can most likely explain this, as discussed in Section 4.2.2.

Maltose and mannitol seem to be no preferred carbon source of *K. baliensis*, as they were degraded only slightly in the respective media. The greatest decrease in mannitol concentration in medium B (Sucr + Man) happend between t_6 and t_{24}, thus it is possible that *K. baliensis* used the sugar alcohol for fast energy gain in the log phase of growth. In the other media, a slight increase in mannitol concentration was observed towards the end of fermentations. There, it was an indicator for the reduction of fructose, leading to mannitol accumulation.

To summarize, one can say that although *K. baliensis* is the strain with the highest EPS formation and the most efficient sucrose degradation, it was the second slowest strain in terms of EPS after *N. chiangmaiensis*. Furthermore, it showed the highest fructose and glucose yields at EPS maximum and also the highest maltose concentration in medium E (Sucr + Mal) at EPS maximum compared to the other tested strains. Acetic acid formation was the lowest of all four strains, whereby mannitol accumulation was lower than *N. chiangmaiensis* in all media apart from medium F, but higher than the other strains in all media apart from medium B. The gluconic acid produced was about as high as found for the other species. This means that in case of a pure EPS optimization and sucrose degradation without the importance of time or the presence of other sugars, *K. baliensis* is the most suited strain out of the four acetic acid bacterial strains tested. Also the low acetic acid accumulation combined with average gluconic acid levels might lead to the conclusion that *K. baliensis* is best suited for further fermentation experiments. However, the longer fermentation time in all media except medium F and the high accumulation of fructose, glucose and mannitol should be included into decision-making. Korakli et al. ([Korakli et al., 2002]) have recovered 14 g/l fructan from fermentations with *L. sanfranciscensis* in sucrose-containing MRS medium. In the same study it was shown that EPS are completely consumed by selected *Bifidobacteria* and increase their growth if pH is controlled.

In sucrose-enriched MRS-based broth, between 5.54 and 7.78 mg/ml EPS have been determined for selected *Weissella cibaria*, *Lactobacillus plantarum* and *Pediococcus pentosaceus* strains ([Di Cagno et al., 2006]). In Homohiochii medium containing 50 g/l

sucrose, EPS-yields of up to 10.1 g/l were produced by selected *Lactobacillus* strains ([Rühmkorf, 2013]).

In another study, 36 g/l glucan were produced by *W. cibaria* in EPS-broth with 100 g/l sucrose ([Lynch et al., 2014]).

4.3 Sourdough fermentations with AAB

In the following section, the dry matter of EPS, its recovery rate from doughs and the adaptability of AAB towards different living conditions in the dough are discussed at first. Then, results of sourdough fermentations in doughs supplemented with sucrose will be analyzed. As metabolite yields like EPS, gluconic acid, acetic acid and remaining sucrose were sometimes quite different between the strains and might be important for the choice of a certain starter culture, discussion is divided into metabolites rather than into different strains.

4.3.1 EPS as easily storeable ingredients and recovery rate from doughs

The constantly high dry matter of EPS from the tested strains indicate that they are, despite their high water solubility, not hygroscopic and can be easily stored at room temperature.

Experiments determining the recovery rate of EPS from doughs demonstrate losses during the isolation and purification procedure (3.13). As no other parameters apart from the bacterial strains were changed, differences in recovery rate must be strain dependent. A strain-dependency concerning the recovery of EPS has already been observed for lactobacilli and explained with differences in the type of polymer released ([Notararigo et al., 2012]). Also in our experiments the observed differences can be a result of the different molecule structure. Furthermore, strain-specific, accompanying substances, which might have modified water solubility of EPS or ethanol precipitation, might be a reason for different recovery rates. Increasing the ethanol:water ratio during precipitation could increase the recovery rate to about 60 %, whereas the strain-dependency remained. Possibly, change of other factors apart from ethanol ratio leads to a further improvement of EPS recovery from doughs. Useful approaches to adjust the method of EPS recovery are given by Smith and Pacett ([Smith and Pacett, 1982]), Ruas-Madiedo and de los Reyes-Gavilán ([Ruas-Madiedo and de los Reyes-Gavilán, 2005]) and Notararigo et al. ([Notararigo et al., 2012]). However, as the isolation of EPS is a time-consuming and costly procedure, the direct usage of fermentation broth, e.g. sourdough, should be preferred. The recovery rate of EPS was not included into the calculation of results to guarantee that the mentioned amounts have been reached for certain. However, it is considered during the discussion for each strain.

4.3.2 Adaptability of AAB towards different sourdough conditions

In growth experiments with various flours and under different oxygen conditions, none of the tested strains was able to become dominant in anaerobic doughs. This was according to our expectations, as AAB are strictly aerobic ([Cleenwerck and De Vos, 2008]). Therefore cultivating AAB in sourdoughs requires constant aeration, e. g. by using a high dough yield and shaking or blowing in oxygen.

Experiments with various flours showed that if inoculated above a certain cell number, all tested strains could grow in most of the doughs even without sugar addition. Added mannitol did not promote the ability of two test strains, *G. frateurii* and *G. albidus*, to become dominant in the dough. Therefore, it has not been further tested for *N. chiangmaiensis* and *K. baliensis*, which became dominant in most doughs also without mannitol addition. This means that the yield of flour originating sugars seems to be sufficient for the bacteria to grow. However, in doughs without sucrose addition, no EPS were determined (Data not shown).

In buckwheat flour, no growth was observed for any of the tested strains. This might have been a result of the high polyphenolic content compared to the other flour varieties (Table 1.2), and shows a contrast between AAB and certain lactobacilli, which grew well in buckwheat dough ([Rühmkorf et al., 2012]).

In oat flour, only *N. chiangmaiensis* could become dominant. It is presumed that this was a result of the high viscosity observed in oat dough in the beginning of fermentations, which was most likely due to the presence of β-glucans ([Doublier and Wood, 1995], [Würsch and Pi-Sunyer, 1997], [Wood et al., 2000]). A higher viscosity decreases the effect of shaking on aeration. As *N. chiangmaiensis* could grow under these conditions, this strain might be more tolerant towards a lower dissolved oxygen (DO) rate or is able to quickly deplete the β-glucans by stronger acid production in the beginning of fermentation.

4.3.3 EPS-optimization in aerobic, gluten-containing sourdoughs

Concerning aerobic, gluten-containing doughs, wheat, whole wheat, spelt and rye flour were tested for *K. baliensis* and *N. chiangmaiensis*. The two *Gluconbacter* strains were cultivated in the same flours apart from whole wheat. Addition of sucrose enabled EPS formation, which is considered as the most important parameter. Therefore the discussion will focus on EPS yields first, followed by the other metabolites. As to our knowledge, no experiments with EPS-producing AAB in sourdoughs have been performed yet, discussion will relate to studies carried out with EPS-forming lactobacilli.

EPS-formation

All of the four strains that produced high EPS amounts in laboratory media were shown to synthesize EPS also in sourdoughs. The general ability of strains producing EPS in laboratory media to produce them in sourdoughs, too, is a similarity towards lactobacilli ([Tieking and Gänzle, 2005]).

The most powerful strain in terms of EPS formation was *K. baliensis*. EPS yields recovered from doughs with this strain amounted up to 49.3 g/kg (SP, t_{48}), followed by 33.0 (WW, t_{24}) and 32.0 – 34.5 g/kg (WT and RY, t_{48}). The second successful strain in this study was *N. chiangmaiensis*, who reached highest EPS yields of 24 – 29 g EPS/kg in spelt and whole wheat doughs. For the two *Gluconobacter* isolates, maximum EPS contents were much lower, but also found in doughs made from spelt flour.

This relation between the strains in terms of maximum EPS formation also remained when EPS recovery rate was included into the calculation, whereby obtained EPS yields generally increased and *G. albidus* reached similar EPS levels as *G. frateurii*. When considering the average EPS recovery rate of each strain, maximum EPS yields of *K. baliensis* increase to 74.7 g/kg (SP, 100 g/l sucrose, t_{48}), 50.4 g/kg (WW, 100 g/l sucrose, t_{24}) and 48.3 – 52.2 g/kg (WT and RY, 50 – 100 g/l sucrose, t_{48}). Highest EPS concentrations of *N. chiangmaiensis* lay at 57.4 g/kg in spelt dough (100 g/l sucrose, t_{24}) and 63.7 g/kg in whole wheat dough (100 g/l sucrose, t_{48}). EPS maxima reached by *G. frateurii* increased to 25.0 g/kg in wheat dough (75 g/l sucrose, t_{48}) and 32.9 g/kg in spelt dough (100 g/l sucrose, t_{48}). For *G. albidus*, inclusion of EPS recovery rate lead to maxima of 22.7 g/kg in rye dough (100 g/l sucrose, t_{24}) and 30.5 g/kg in spelt dough (50 g/l sucrose, t_{48}).

Overall, a rough order of spelt > whole wheat > rye > wheat can be seen concerning EPS yields, whereby in spelt and whole wheat and in rye and wheat doughs they sometimes lay in about the same range. The presence of a preferred flour for EPS formation of all strains is a difference towards EPS-producing lactobacilli, who have been shown to possess strain-specific flours in this matter ([Rühmkorf, 2013]). One explanation for the high EPS yields recovered in this study can be the dough yield (DY = 500) leading to a higher water activity and therefore an increased diffusion of sucrose and enzyme ([Kaditzky and Vogel, 2008]). Furthermore, a reduction of the very high sucrose yields in order to reduce osmotic stress can have played a role for EPS-formation ([Korakli and Vogel, 2006], [Rühmkorf et al., 2012]). In fermentations with lactobacilli, differences in EPS-yield observed between wheat, rye and rye bran dough were further explained with the higher buffer capacity of rye bran flour ensuring a slower decrease of pH and therefore better cell growth ([Kaditzky and Vogel, 2008]). For the flours used in this study, TTA and TTB amounts have been tested, too. Highest TTA and TTB levels were found in spelt dough, which corresponds to maximum EPS formation (3.12). However, TTA of whole wheat dough was lower than of wheat dough, which also relates to TTB and contradicts the theory that EPS yields correspond to buffer capacity. Thus there must be some more parameters influencing EPS formation of AAB. As in experiments with different laboratory media, highest EPS yields were on the one hand recovered from media with higher buffer capacity and on the other hand from media with higher nutrient contents (Section 2.3), the nutrient content might play a role in here. In Table (1.1) it is shown that the order of spelt > whole wheat > wheat relates to the contents of lipids, ash, oleic acid, iron, manganese, zink, magnesium and phosphorus. The most pronounced differences between spelt and wheat flour relate to the yields of phosphorus, iron, zinc, manganese and magnesium and amount 4.0, 4.9, 5.0, 5.8, and even 7.1 times more than in wheat, respectively. Differences between spelt and whole wheat flour are not as strongly pronounced which means that both parameters, nutrient contents

and TTA, must play a role for EPS formation. This finding is in accordance with the results of a study with lactobacilli, where also a combined influence of buffer capacity and flour ingredients on EPS formation has been identified ([Rühmkorf et al., 2012]).

In addition to the dependency of EPS formation on strain and flour, results show that harvest time and sucrose dosage also play a role in this. EPS production increases with sucrose dosage in all gluten-containing flours fermented with *K. baliensis* and *N. chiangmaiensis*, which was similar to *W. cibaria* fermented in sucrose-enriched barley wort ([Zannini et al., 2013]). In fermentations with the two *Gluconobacter* strains, no general relation between EPS and sucrose dosage was discovered, probably due to lower EPS yields and high standard deviations. In opposite to Kaditzky and Vogel ([Kaditzky and Vogel, 2008]), who fermented doughs with lactobacilli and stated that at lower pH (4.0 instead of 4.7), no EPS was detected, we found EPS production at pH values lower than 4.0 for AAB.

The EPS yields recovered in this study are the highest values of bacterial EPS produced in sourdoughs so far: In wheat dough fermentations with sucrose and *L. sanfranciscensis*, about 2 g/kg fructan were detected ([Tieking et al., 2005]). In another study, between 0.5 and 2 g/kg EPS, mostly fructan, were found in sourdoughs fermented with six different *Lactobacillus* strains ([Tieking et al., 2003]). For EPS-forming *Weissella* strains used as starter cultures for sucrose-enriched wheat and sorghum sourdoughs, 1.6–16 g/kg dough EPS were recovered. In the same study, EPS formation observed for *L. sanfranciscensis* amounted about 4.4 g/kg ([Galle et al., 2010]). In other experiments with *W. cibaria* and *L. plantarum*, about 2.5 g/kg EPS were recovered from sucrose-enriched wheat doughs with a dough yield of 160 ([Di Cagno et al., 2006]). The highest EPS values obtained from optimized sourdough fermentations with lactobacilli so far were determined by Rühmkorf ([Rühmkorf, 2013]) and lay at $15.7-17.9$ g/kg. In our study, two to three times more EPS than this have been reached with *N. chiangmaiensis* and *K. baliensis*, and about the same levels by the two *Gluconobacter* strains. In baking trials performed by Jakob et al. ([Jakob et al., 2012b]), EPS isolated from *K. baliensis* and *N. chiangmaiensis* were identified as having the most positive effect on bread volume and staling. Both strains turned out to produce the highest EPS-amount *in situ*, which is why they might be the most promising strains for future work.

It has been shown that the addition of EPS from AAB has desirable technological effects already at a level of 1 %, whereby lower levels have not been tested ([Jakob et al., 2012b]). Other researchers have identified a level of 0.3 % for a remarkable effect of hydrocolloids as dough improvers ([Armero and Collar, 1998]). Furthermore, it was proven that levan produced *in situ* was more effective than externally added levan ([Brandt et al., 2003]). Therefore the yields of more than 30 g/kg (or more than 50 or 60 g/kg, when recovery rate of EPS is considered) obtained in this study mean that effectual levels in the final bread dough can easily be reached by adding about one tenth to one third of sourdough to the bread dough.

Further optimization of EPS formation in sourdoughs by testing different dough yields, sucrose fed-batch and constant pH, e.g. like carried out by Kaditzky and Vogel ([Kaditzky and Vogel, 2008]), seem possible. At this, the dough yield cannot be lowered below a certain amount to ensure a sufficient dissolved oxygen rate in the dough for AAB

to become dominant. The approach of testing sucrose fed-batch seems promising because some strains have consumed nearly the whole sucrose throughout the fermentation, which might be a reason why EPS formation has stopped.

On the other hand, Tamani et al. ([Tamani et al., 2013]) found out that the quantity of EPS production during sourdough fermentation does not correlate with the delay of staling in the final bread. It has been shown that a relation between molecular structure and functionality pf EPS exists ([Jakob et al., 2013]). This means that rather the qualitative nature of EPS may affect bread staling than the total amount. Therefore, the molecular structure of *in situ* produced EPS and also the formation of oligo- and heteropolysaccharides by AAB should be tested in the future. In this matter, *K. baliensis* seems to be the most interesting strain, as the formation of heteropolysaccharides by this strain has already been proven ([Moonmangmee et al., 2008]).

Formation of organic acids

When estimating the required amount of sourdough to reach positive effects on bread, not only EPS content, but also organic acid yields play a role. In common sourdoughs hosting lactobacilli and yeasts, two different organic acids, lactate and acetate, can be detected. To maintain a balanced acid profile and so as not to decrease the positive effects of dough improvers, e. g. hydrocolloids, the whole amount of acids should be considered as well as the ratio between them. The molar ratio of lactate to acetate in bread is usually expressed as the fermentation quotient, and is considered optimum in the range of $2.0 - 2.7$ ([Hammes and Gänzle, 1998]). In fermentations with acetic acid bacteria, gluconic and acetic acid are produced instead of lactic acid. Thus a novel fermentation quotient referring to the ratio of gluconic acid to acetate might be implemented, whose appropriate value must be identified in baking trials.

In our experiments, acetic acid contents showed a general increase throughout the fermentations. They were negatively correlated with sucrose dosage in doughs with *K. baliensis* and *N. chiangmaiensis*, apart from rye dough, where no (*K. baliensis*) or a positive (*N. chiangmaiensis*) relation was found. In spelt dough, where the highest EPS contents were reached, up to 110 mM/kg acetic acid was recovered for *K. baliensis* and only $21 - 38$ mM/kg for *N. chiangmaiensis*. The two *Gluconobacter* strains formed, despite their low EPS production, relatively high acetate yields of up to 130 mM/kg (*G. frateurii*, RY, 100 g/l sucrose, t_{48}) and 127 mM/kg (*G. albidus*, SP, 75 g/l sucrose, t_{48}). Compared to certain studies performed with lactobacilli, where about 32 and 23 mM/kg ([Choi et al., 2012]), 54.2 mM/kg ([Kaditzky and Vogel, 2008]) and 42.4 mM/kg ([Tieking et al., 2005]) acetate were found, the yields recovered in our study are about two to three times higher, apart from *N. chiangmaiensis*. However, we have also determined much higher EPS yields than the ones found in these studies, which allow a lower dosage of sourdough to reach technological effects and therefore results in a lower dosage of acetic acid. Of course, also the time point of EPS-harvest plays a major role for acetic acid dosage, as acetate is the "second" metabolite after gluconate formation and sugar consumption. Therefore maximum acetic acid yields were mostly observed after EPS maxima.

Concerning gluconic acid formation in dough, no comparable studies exist so far. We have recovered up to 165, 119 und 122 mM/kg flour from doughs fermented with *K. baliensis*, *N. chiangmaiensis* and *G. albidus*, respectively. *G. frateurii* produced only up to 15 mM/kg gluconate. In contrast to acetate, yields were rather positively correlated to sucrose dosage. The high gluconate levels in fermentations with three out of four AAB strains show that its influence on bread quality and acid profile should definitely be considered. From our results, suited fermentation conditions for any required acid profile can be selected: If a high gluconate content is to be reached, fermentation should be stopped after 24 h, before further oxidation of gluconic acid takes place. If gluconic acid yield should be rather low, harvest time can be changed to t_{48}, where gluconate contents have been partly degraded in the case of *K. baliensis* and *N. chiangmaiensis*. In fermentations with *G. albidus*, gluconic acid contents increased slower and therefore highest yields were reached after t_{48}. Here, harvest should take place at t_{24} for low gluconate concentrations. Furthermore, *G. frateurii* can be chosen for fermentations with minimum gluconic acid contents.

For lactobacilli, it was observed that higher acid formation took place in flours with higher buffering capacity [Kaditzky and Vogel, 2008]. Relating to the AAB tested in this study, this relation cannot be confirmed, because acid formation was strain- and flour specific, whereas the strain influenced acid formation to a greater extent.

In a study on lactobacilli performed by Galle et al. ([Galle et al., 2010]) it is suggested to apply EPS-forming *Weissella* instead of *Lactobacillus* to synthesize lower amounts of acetate and mannitol. The low acetic acid amounts found for *N. chiangmaiensis* and the high EPS yields determined for *K. baliensis* and *N. chiangmaiensis* enabling the usage of only low sourdough concentrations to reach effectual EPS levels show that these strains might be suited to replace these lactobacilli, too. However, the influence of gluconic acid on dough and bread quality has not been investigated yet. Therefore further research in this field will be needed.

Remaining sugars

In our experiments, sucrose has not fully been depleted throughout the fermentations. Remaining sucrose was always dependent on the start dosage, whereas the speed and amount of consumption was strain-dependent. Final sucrose levels below 50 g/kg were reached in some fermentations with *K. baliensis* and *N. chiangmaiensis*. For the two *Gluconobacter* strains, remaining sucrose levels generally lay above 100 g/kg, apart from fermentations with the lowest and second lowest sucrose dosage using *G. albidus* (SP dough), where 61–85 g/kg sucrose have been determined. In opposite to this, during sourdough fermentations with *Lactobacilli*, no residual sucrose was detected after 48 h ([Kaditzky and Vogel, 2008]). However, if about $20 - 25\%$ sourdough with about 50 g/kg sucrose is added to bread dough, this would lead to a final sucrose content of only $10 - 12.5$ g/kg sucrose. It is known that in typical sourdough co-cultures, the present sucrose is hydrolysed by yeasts and glucose and fructose are liberated which are then rapidly depleted by LAB. Yeasts are able to hydrolyse sucrose about 200 times faster than the released hexoses are fermented, and therefore cause rapid disappearance of sucrose during

sourdough fermentations ([Gobbetti, 1998]). Thus a two-step fermentation for doughs fermented with AAB should be considered: Firstly, an aerobic fermentation with AAB could be performed, which is then followed by an anaerobic fermentation with LAB and yeasts to consume the remaining sugars before baking. Another possible approach could be to perform a stepwise decrease of start sucrose dosage and analyse the influence on EPS, acid formation and bread quality. In doughs fermented with lactobacilli, for example, 1 % sucrose addition to wheat doughs was enough to induce EPS formation to effectual levels ([Brandt et al., 2003]).

In this study, the amounts of other remaining sugars like glucose and fructose have not been detected due to a high background noise during HPLC analysis of sourdough samples. Of course, the possible presence of other sugars than sucrose originating from sucrose depletion or from the flours themselves should not be ignored.

All in all, acceptable sugar levels can only be fixed through baking trials, followed by sensorial analysis.

4.3.4 EPS-optimization in aerobic, gluten-free sourdoughs

The formation of EPS by lactobacilli in gluten-free sourdoughs has been investigated in detail ([Galle et al., 2010], [Galle et al., 2012], [Rühmkorf et al., 2012]), but no study so far investigated the fermentation of gluten-free sourdoughs with AAB. This study presents for the first time data on EPS- and acid formation, as well as sucrose degradation of AAB in gluten-free sourdoughs, combined with the influence of different flours, sucrose dosages and harvesting times. Sourdoughs were produced using fructan-forming *K. baliensis*, *N. chiangmaiensis*, *G. frateurii* and *G. albidus*. For the two *Gluconobacter* strains, millet, corn and rice flour were tested. *K. baliensis* was cultivated only in millet dough and *N. chiangmaiensis* in millet and oat dough, because it was the only strain that could grow in oat dough (Section 4.3.2). Due to the lack of previous studies with AAB in gluten-free sourdoughs, discussion will relate to studies performed with lactobacilli.

EPS-formation

In gluten-free (GF) baking, the addition of hydrocolloids is essential to obtain acceptable product quality in terms of volume, texture and shelf life ([Lazaridou et al., 2007]). Staling is more rapid than in gluten-containing breads, because GF breads are mainly starch based ([Moore et al., 2004]). It has been shown that EPS can increase the textural quality of GF breads that lack wheat gluten or rye pentosans capable of water binding and gas retention and can therefore replace hydrocolloids, e. g. by application of sourdough ([Moore, 2005], [Arendt et al., 2007], [Schwab et al., 2008],[Arendt et al., 2011], [Galle et al., 2011]). For EPS-enriched sourdoughs, decreased resistance of deformation and elasticity has been reported, as well as an increased volume, softer crumb and delayed staling of GF bread ([Schwab et al., 2008], [Galle et al., 2011]). In a study with dextran producing *W. cibaria*, staling rate and crumb hardness of breads were reduced by using buckwheat, teff and wheat sourdoughs, whereas the specific volume and the aroma remained mainly unaffected ([Wolter et al., 2013]). Rühmkorf ([Rühmkorf, 2013])

has found out that also EPS from lactobacilli are able to improve the crumb structure and therefore quality of gluten-free breads.

In our study, doughs were fermented with EPS-forming AAB. EPS amounts of 31.0, 29.5 and 26.8–27.8 g/kg flour were reached by *N. chiangmaiensis* (OA, 100 g/l sucrose, t_{48}), *K. baliensis* (MI, 75 g/l sucrose, t_{48}) and *G. frateurii* (RI and MI, 100 g/l sucrose, t_{48}), respectively. Furthermore, *N. chiangmaiensis* has formed up to 23.4 g/kg EPS in millet dough (75 g/l sucrose, t_{24}) and *G. frateurii* up to 22.2 g/kg EPS in corn dough (100 g/l sucrose, t_{48}). For *G. albidus*, maximum EPS yields amounted only 13.0 g/kg flour and were determined in millet dough with 100 g/l sucrose after 24 h, with even lower yields in corn and rice dough. If the relatively low, strain-dependent recovery rate of EPS from doughs was considered for each strain, maximum EPS yields increased to 44.2–44.7 g/kg (MI, 100–75 g/l sucrose, t_{48}) for *K. baliensis*, to 50.3–52.4 g/kg (MI, 100–75 g/ sucrose, t_{24}) and 58.8–69.5 g/kg (OA, t_{48}) for *N. chiangmaiensis*, to 30.0–39.1 g/kg (MI, 50–100 g/l sucrose, t_{48}) and 40.5 g/kg (RI, 100 g/l sucrose, t_{48}) for *G. frateurii* and to 22.6–23.6 g/kg EPS (MI, 50–100 g/l sucrose, t_{48}) for *G. albidus*.

Similar to experiments with gluten containing sourdoughs (Section 4.3.3), EPS contents of gluten-free flours fermented with AAB are higher than in any other sourdough study so far. For example, in previously studied quinoa, buckwheat and rice sourdoughs fermented with lactobacilli, up to 20.6 g/kg EPS were formed by *L. reuteri* ([Rühmkorf et al., 2012]).

Bread made from oats is known to have high nutritional quality and excellent moisture retention properties that reduce the rate of staling and therefore keep breads fresh for longer ([Hager, 2013]). Most likely, the high nutritional quality has positively influenced EPS-formation by *N. chiangmaiensis* on the one hand and the excellent moisture retention properties have increased viscosity and therefore inhibited growth of the other AAB strains on the other hand. Furthermore, oat had a slightly elevated TTA and TTB value (Figure 3.12) compared to millet and corn flour. Therefore the pH value in oat fermentations did not decrease as fast as in the other flours, enabling a higher activity of EPS-forming fructosyltransferase and therefore higher EPS contents. Due to its high nutritional quality and its positive effects on EPS formation, the other strains could also be tested in oat flour with increased water dosage to decrease start viscosity.

In our experiments, an influence of sucrose dosage on EPS yield could not be seen for *Gluconobacter* strains, which was probably due to high deviations between the replicates. For *K. baliensis* and *N. chiangmaiensis*, higher sucrose concentrations lead to higher EPS contents in millet and oat doughs, respectively, which was similar to previous studies performed with lactobacilli ([Rühmkorf, 2013]). A positive influence of sucrose dosage on EPS formation was also observed by Santos et al. ([Santos et al., 2000]), Ul-Qader et al. ([Ul-Qader et al., 2001]) and Arsköld et al. ([Arsköld et al., 2007]).

In opposite to lactobacilli observed by Rühmkorf et al. ([Rühmkorf et al., 2012]), EPS-expression by AAB in gluten-free flours was not only strain-dependent, but also flour dependent.

As already discussed for gluten containing doughs, further optimization of fermentation conditions could take place for gluten-free doughs, e. g. by applying sucrose fed-batch, different dough yields and pH-control. However, sucrose fed-batch did not

enhance EPS formation of lactobacilli in gluten-free doughs ([Rühmkorf et al., 2012]).

As optimum fermentation time for *in situ* EPS formation with lactobacilli, 24 h was fixed ([Kaditzky and Vogel, 2008], [Rühmkorf et al., 2012]). If AAB are to be applied in sourdough fermentation, the ideal harvest time in terms of EPS formation is reached later, between 30 – 48 h, and depends on the strain/flour/sucrose dosage-combination. Furthermore, the amount of organic acids and residual sugars should be taken into acount when fixing a suited harvest time.

Formation of organic acid

In common sourdoughs, lactic acid and acetic acid are formed during the fermentation. It is known that moderate levels of acetate improve flavour and the shelf-life of bread. Above a certain threshold, however, negative influences on flavour occur ([Kaditzky et al., 2007]). Another effect of organic acids in gluten-free breads is hardening of the bread crumb. However, this can be masked by EPS formation, leading to a softer crumb as demonstrated for fresh and stored sorghum breads ([Galle et al., 2012]). As already discussed for gluten containing sourdoughs, the fermentation quotient being used so far relates to the ideal molar ratio of lactic acid to acetic acid and needs to be adjusted to gluconate and acetate for fermentations with AAB.

In our experiments, acetic acid levels of *K. baliensis* and *N. chiangmaiensis* were quite low and never exceeded about 29 mM/kg flour. During fermentations with the two *Gluconobacter* strains, however, acetate formation was similar to gluten-containing flours. *G. albidus* formed up to 110, 41 and 83 mM/kg acetate in millet, corn and rice dough, respectively, and *G. frateurii* formed up to 95, 18 and 121 mM/kg in the same flours. Sucrose dosage had an influence on acetic acid content only in the case of *G. albidus* and millet flour (positive) and *N. chiangmaiensis* and oat flour (negative). At this, *G. frateurii* was the weakest producer of gluconic acid, reaching about 6 mM/kg at maximum. Due to the low amounts, no influence of sucrose dosage could be found. For the other strains, a positive influence of sucrose dosage on gluconate was recognized, apart from *G. albidus* and millet flour. Gluconic acid yields in the respective flours amounted up to about 25 mM/kg for *K. baliensis* (MI, 75 g/l sucrose, 24 h), up to 64 and 86 mM/kg flour for *N. chiangmaiensis* (OA, 100 g/l sucrose, 48 h and MI, 75 g/l sucrose, 24 h) and up to 58, 31 and 98 mM/kg for *G. albidus* (OA, 100 g/l sucrose, 48 h, CO, 75 g/l sucrose, 48 h, and RI, 100 g/l sucrose, 48 h). In GF sourdoughs with EPS-forming *L. reuteri*, 148 – 150 mM/kg acetate, 208 – 212 mM/kg lactate and up to 16.0 g EPS per kg flour were formed ([Galle et al., 2012]). In the same study, a positive effect of EPS-enriched sourdoughs compensating the negative effect of acids was already observed upon an addition of 10 % EPS positive sourdough. In another study, lactate levels between 142 – 257 mM/kg flour and acetate levels of about 104 mM/kg flour have been detected for *L. reuteri* and *L. curvatus* ([Rühmkorf et al., 2012]). This data suggests that in addition to the determination of a novel fermentation quotient, also the optimum ratio between EPS and organic acids for the most positive effect on breads should be defined. At this, our fermentations show that among the strains studied, an AAB strain can be found for each possible EPS/acid combination: *K. baliensis* is a high EPS, low acetic, average

gluconic acid producer in millet dough. *N. chiangmaiensis* can serve as a high EPS, low acetic, increased gluconic acid producer in oat dough. Concerning the two *Gluconobacter* strains, *G. frateurii* has produced higher EPS yields, while forming very low gluconic and high acetic acid yields. The lowest ratio between EPS and organic acids has been reached by *G. albidus* due to its low EPS and relatively high acid formation.

Remaining sugars

Similar to gluten containing doughs fermented with AAB, remaining sugar amounts in GF doughs were again correlated with sucrose dosage and in fermentations with *K. baliensis* and *N. chiangmaiensis*, again the lowest remaining sugar levels were detected. They lay at around 57, 58 and 63 g/kg flour in MI (*K. baliensis*, 50 g/l sucrose and *N. chiangmaiensis*, 50 g/l sucrose) and oat flour (*N. chiangmaiensis*, 50 g/l sucrose), respectively. The two *Gluconobacter* strains reached final sucrose levels below 100 g/kg only in one rice (*G. frateurii*, 50 g/l sucrose) and one millet dough (*G. albidus*, 50 g/l sucrose) each. In our fermentations, residual sucrose lay at about the same level as in a study performed by Rühmkorf ([Rühmkorf, 2013]). However, the higher EPS yields produced by the AAB in our study enable a lower sourdough dosage and therefore a decrease in final sucrose. Of course, also gluten-free sourdoughs fermented with AAB could be exposed to further optimization steps as already discussed for gluten containing flours in Section 4.3.3. However, this will not substitute future baking experiments to evidence the presumed positive effects of AAB-fermented sourdoughs on final gluten containing and gluten-free breads.

4.3.5 Competitiveness of *G. frateurii* and *K. baliensis* upon backslopping in aerobic spelt dough

Without the addition of a starter culture and by performing daily back-slopping in wheat and spelt sourdoughs, a stable microbiota is established within a week. During this, no significant differences have been observed between the two flour varieties ([Van der Meulen et al., 2007]). However, this relates to traditional sourdoughs with an anaerobic microflora. In our experiments, aerobic strains were used which is why doughs had a higher dough yield and were aerated by shaking. Under these conditions, we tested the strains *K. baliensis* and *G. frateurii*. It was shown that they were able to become dominant in type I sourdoughs with daily backslopping using 10 % of dough over a period of eight days (3.21). However, cell counts decreased during the experiment, which means that a further optimization of fermentation conditions will be necessary. Possibly longer periods of time between two refreshments or a higher percentage of backslopped dough can help to maintain cell counts and to stabilize the sourdough culture for longer. All other studies which have been performed on backslopping of sourdoughs so far were derived under anaerobic conditions. Therefore the development of backslopped, aerobic sourdoughs using acetic acid bacteria opens up a new field for further research. So far, the most important group of sourdough fermenting bacteria still consists of lactobacilli ([Corsetti and Settanni, 2007]). However, this might get additions by the use of EPS

producing AAB in the future.

4.4 WKP as a novel hydrocolloid

In this section, the overall and functional properties of WKP are discussed at first. They play an important role in assessing WKP as novel hydrocolloid for general applications. Then, the suitability of WKP as baking agent is considered. In the part discussing fresh breads, the focus lays on the potential of WKP to improve flavour and to reduce sodium. When discussing 48 h old breads, the influence of WKP on staling is paramount.

4.4.1 Overall and functional properties of WKP

With a dry matter of $93.66 \pm 0.07\%$, WKP contains less water than most cereals, whose dry matter lays between 86.3 and 88.4% ([Belitz et al., 2009]). Therefore it can easily be stored at room temperature for at least one year. If milled under the conditions mentioned, it has good pouring properties, is very easy to dose and not dusty. In opposite to other hydrocolloids which have a neutral taste and aroma, WKP possesses a slightly sour, starchy and fermented flavour.

During soaking, WKP particles take up water and increase their size (Figure 3.22 and 3.23), which causes a thickening effect in liquid suspensions. The water binding capacity of WKP lays at around 919% and is therefore higher than of starch (82%), but lower than of xanthan (1580%), whereas no temperature-dependency exists in the range between 20 and $75\,^{\circ}\mathrm{C}$.

As WKP exists as angular particles which – similarly to original water kefir granules – do not form bonds between each other, no gel-forming properties have been observed. However, particles can get caught during shearing due to their angular shape, which increases viscosity in mixing and stirring processing steps.

In freeze-thaw experiments, the WKP dosage required for 95% water binding lay at 8%. With 10% and 12% WKP, even 96.7% and 98.8% water binding could be reached, respectively, which relates to only 3.2% and 1.2% syneresis. This shows that at a lower WKP addition, there is too much surplus water which is not bound strong enough to avoid separation by freezing. At higher WKP dosages, almost all water is bound and does not freeze out during one step of freeze-thawing.

As the product is made by drying pure water kefir grains being grown in fermentation broth, it contains a certain amount of organic acids. In watery suspensions with $1 - 2\%$ WKP, the pH value decreased to below 4.0. It is known that below pH $= 4.0$, living cells of almost all food spoiling bacteria are killed and no toxins are produced, whereas bacterial spores can survive these conditions ([Heiss and Eichner, 2002]). By this condition, also most of the yeasts and even a few fungi are affected, whereas aflatoxin production can still take place ([Heiss and Eichner, 2002], [Ternes, 2011]). Therefore, in addition to its thickening effects, WKP application can be a useful means for food preservation due to its natural richness in organic acids.

As WKP is a highly hydrophilic material, it did not solve in organic solvents. However, also in pure water the solubility amounted only 3.2%. This can be explained

with the original purpose of the special grain structure: Water kefir grains represent biofilms which host the microbial consortium during fermentation. Whereby granules are designed to take up a certain amount of water and enable water supply and material transport sufficient for the inherent microbes, only their attribute of not being solvable allows this protective function. Under slightly acid and alkaline conditions, solubility increased to about 10.9 % (0.1 M NaOH) and 7.1 % (0.1 M HCl). However, this is rather a result of hydrolysis reactions than of solubility, as many EPS can be hydrolysed by bases or acids ([Kang et al., 1982], [Morin, 1998],[Sutherland, 1990]).

WKP has been found to possess a certain fat binding capacity (129 %), which lies between soy protein, lecithin and gum arabic. As this had been determined by employing a centrifuge method and as WKP consists of angular particles, this result can be a consequence of the filling of cavities between WKP particles with oil, which was not removed by centrifugation. This means that no real molecular interchanges are necessary to detect a fat-binding effect determined by this method, as also the pure structure of WKP with its cavities and hollows between particles can have lead to this result. This supposition is supported by the fact that no emulsifying and foam-forming properties and no solubility in organic solvents have been found, which means that there are no detectable hydrophobic interactions with other molecules or surfaces. However, as in many biofilms, the molecular structure of water kefir EPS is difficult to analyse and has not been fully defined yet.

4.4.2 Influence of WKP on texture, oven loss, loaf height and volume of fresh and 48 h old breads

The addition of 1 %, 2 % and 5 % WKP lead to significantly decreased crumb firmness of fresh and 48 h old breads, whereas no clear effects on crust crispness and loaf weight have been found (Table 3.2). In fresh breads, crumb firmness was decreased by 15.1 % (1 % WKP), 20.6 % (2 % WKP) and 16.0 % (5 % WKP), compared to standard. Crumb firmness of 48 h old breads was influenced to a higher extent and lay at 20.9, 32.4 and 31.4 % below the corresponding standard for 1, 2 and 5 % WKP addition.

The height of breads with 5 % WKP was decreased and correspondingly, their volume was decreased, compared to standard breads.

Breads with 1 and 2 % WKP-addition did not show a difference in loaf maximum height, but a tendency of increased volume could be observed. In addition, loaf weight tended to be higher than in breads without WKP, meaning that fewer oven losses have occurred, which is most likely a result of the high water binding capacity of WKP.

Another advantage of WKP-addition to wheat breads is that the increased water content leads to a reduced flour:water ratio and therefore to reduced flour consumption and calories. With 1, 2 and 5 % WKP, dough yield increased from 160 (standard recipe) to 164 (1 % WKP), 167 (2 % WKP) and 177 (5 % WKP).

As the most positive effect of WKP on crumb firmness and therefore bread staling have been noticed for 2 % WKP addition and as no significant negative effects on loaf height, weight, volume or crust crispness have been noticed for this dosage, the addition of 2 % WKP to wheat bread doughs can be generally recommended.

4.4.3 Suitability of WKP to improve flavour and to reduce sodium in wheat breads

The level of 2 % WKP, which has been found most suited to increase bread texture and overall properties, has been used for sensorial analyses of fresh and 48 h old breads. Tastings of fresh breads focused on overall characters and sodium reduction potential of WKP and tastings of 48 h old breads focused on bread staling. A potential for sodium reduction was assumed due to the slightly sour, starchy and fermented taste of WKP, which is why it could serve as a saltiness enhancer and also increase the overall taste of breads.

In our experiments, a sensorial evaluation with an untrained panel did not show many effects of 2 % WKP on taste of fresh and 48 h old wheat breads and also no effect on sodium perception. Fresh WKP-enriched breads even tended to taste less sour, less aromatic and more bland than breads without WKP (Figure 3.24). These effects were more likely a result of sodium reduction, meaning that WKP was neither able to balance the loss in saltiness perception nor in aromatic and sour notes. Therefore, WKP could not be confirmed as a saltiness or taste enhancer for fresh breads.

However, after 48 h storage, WKP-enriched breads were rated higher than standard breads with full sodium content in terms of aromatic and sour smell. Here, a slight quality improvement for sodium reduced breads could be reached by adding WKP, because the decision to buy was answered with "Yes" by six out of 14 panellists, compared to only two panellists who would buy sodium-reduced breads without WKP-addition.

As tastings were performed by an untrained panel, the validity of this data is limited. These results mean that on the one hand, that a certain threshold value laying above 2 % is needed for a positive (or negative) sensorial impact of WKP on wheat breads. On the other hand, results show that WKP has virtually no effect on bread taste and that the 2 % WKP addition which are necessary to decrease bread staling can be added to wheat breads without being noticed.

Furthermore, positive effects of water kefir liquid on gluten-free baking have been described ([Kane, 2013]), which is a hint that also WKP might have a positive effect on gluten-free products.

5 Summary

Bread counts as one of the most popular staple foods and is consumed in many countries all over the world. Over the past 150 years, changes in the breadmaking process from small artisan bakeries to a high technological bakery industry have led to a standardization, often accompanied by a decrease, of bread quality. Among the most important quality parameters of bread are aroma, texture and shelf-life, relating to microbial stability and staling. Difficulties concerning the bread quality summit in gluten-free breads. These are mainly starch-based and serve as nutrition for coeliacs, who possess a permanent intolerance for dietary gluten. In gluten-free breads, it is even more difficult to reach a high product quality. For traditional bread production, sourdough is used to leaven and acidify wheat and rye breads due to its beneficial effects on flavour, texture, shelf life and nutritional properties. Besides acidification, the production of exopolysaccharides (EPS) by sourdough originating lactobacilli and yeasts during fermentation is a basic reason for its positive effects on bread and other baked goods. EPS belong to hydrocolloids, which influence bread texture and retard staling due to their high water binding capacity. Due to their wide range of chemical structures resulting in new-fangled applications, the demand for EPS has increased recently.

Therefore, the objective of this work was to investigate new EPS-producing bacterial species as well as a novel, EPS-based hydrocolloid, for baking applications. For the exploitation of new EPS-producing species, six EPS-forming acetic acid bacterial strains isolated from water kefir and other food sources were chosen. At first, they were tested for their robustness of EPS-formation using different laboratory media. The strains *K. baliensis* DSM 14400, *N. chiangmaiensis* NBRC 101099, *G. frateurii* TMW 2.767, *G. albidus* TMW 2.1191 produced more than 20 g/l EPS in one or more of the tested media. In fermentations with *G. cerinus* DSM 9533T and *G. oxidans* TMW 2.339, however, not more than 5 g/l EPS was found in any of the tested media, which is why they were excluded from further experiments.

With the other four strains, experiments in laboratory media with varied carbon sources in addition to sucrose were carried out to test the influence of carbon source and also interactions of other parameters on EPS formation. The media A (sucrose only), B (sucr + mannitol), C (sucr + glucose), D (sucr + fructose), E (sucr + maltose) und F (sucr + sodium gluconate) were chosen. It was shown that EPS-formation depended on the strain, on time and also on the carbon source. Fot this, the varying acid formation from carbon sources relating to the strains as well as the buffer capacity of the media were mainly held responsible. They resulted in different courses of pH value and therefore in a different activity of EPS-building fructosyltransferases. In fermentations with *K. baliensis*, the pH value was always significantly higher than in fermentations with *G. albidus*, *G. frateurii* and *N. chiangmaiensis*, which were basically not different from

each other. Furthermore, pH was significantly lower in the medium with glucose than with mannitol and lower in both of them compared to the other media. In the medium with sodium gluconate, pH-course was significantly higher than in all other media. As influences of medium and strain overlapped each other, *K. baliensis* was the strongest EPS producer and formed maximum EPS yields of at least 18 g/l in the medium with sucrose, fructose, maltose and sodium gluconate. The second strongest EPS producer was *N. chiangmaiensis*. It reached more than 18 g/l in medium F and $16.0 - 16.7$ g/l in medium A, B, D and E (average from two replicates). *G. albidus* did not produce more than 12.8 g/l EPS in any of the tested media. The strain *G. frateurii* produced between $14.8 - 14.9$ g/l EPS only in medium A and F and up to 9.9 g/l in the other media. For *K. baliensis* and *N. chiangmaiensis*, time points of maximum EPS formation lay at $48 - 72$ h, for the two *Gluconobacter* species at $24 - 48$ h. For all strains, a partial degradation of EPS after reaching the maximum was detected. *K. baliensis* showed a very efficient sucrose degradation of more than 90 % in all media. For *N. chiangmaiensis*, sucrose degradation lay at $85 - 89$ % in medium $A - E$ and at 100 % in medium F. The two *Gluconobacter* isolates degraded $34 - 79$ % of sucrose in medium $A - E$. Only in medium F, sucrose degradation was more efficient and amounted $96 - 97$ %. *K. baliensis* was the weakest acetic acid producer, because for this strain, not more than 2 mM/l acetic acid were detected in any fermentation. *N. chiangmaiensis* and *G. frateurii* produced up to 5 and 10 mM/l acetic acid, respectively. *G. albidus* was the strongest acetic acid producer and produced up to 23 mM/l. Concerning the formation of gluconic acid, strains behaved differently in the different media. In medium F, where gluconate was already present at the beginning, *K. baliensis* did not produce any additional gluconic acid, in contrast to the other strains. *N. chiangmaiensis* produced 52 mM/l, *G. albidus* 55 mM/l and *G. frateurii* 82 mM/l. In the other media, maxima of $28 - 47$ mM/l were detected for *K. baliensis*, $9 - 53$ mM/l for *N. chiangmaiensis*, $25 - 65$ mM/l for *G. albidus* and $11 - 63$ mM/l for *G. frateurii*. For all tested strains, a partial degradation of gluconic acid after the maximum could be recognized. The consumption of mannitol and maltose was low for all isolates, whereby small amounts of mannitol accumulated towards the end of fermentations in sodium gluconate-free media.

In the following, sourdough fermentations with gluten containing and gluten-free flours were carried out with *K. baliensis*, *N. chiangmaiensis*, *G. albidus* and *G. frateurii*. At first, the influence of oxygen supply, flour and mannitol addition on growth was investigated. Wheat-, rye-, whole wheat-, spelt-, oat-, golden millet-, wild browntop millet-, corn-, rice- and buckwheat flour were selected for the experiments, whereby not all strain-flour-combinations were tested. Afterwards, suited doughs were used for the variation of sucrose concentration and harvest time while observing the parameters growth, pH-value, EPS- and acid-formation, as well as sucrose degradation.

If inoculated with a sufficient start cell count, all strains reliably became dominant in aerobic doughs and produced high amounts of EPS under sucrose supplementation. The only exceptions were buckwheat- and oat flour: In buckwheat dough, none of the tested strains could remain and in oat dough, only *N. chiangmaiensis* could grow. As mannitol addition did not result in improved growth for the two *Gluconobacter* strains, this approach was not followed any further.

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Statistical data analysis of gluten containing doughs showed numerous influences of the variables flour, start sucrosedosage and time on the investigated parameters. Relating to EPS- and acid formation of the different strains, the same tendencies like for the experiments with different carbon sources could be recognized. Therefore *K. baliensis* was again the strongest EPS-producer. Due to its high buffer capacity as well as its high nutrient content, spelt flour was the most suited for EPS formation. In spelt flour, *K. baliensis* reached up to 49 g/kg EPS, followed by whole wheat (33 g/kg), wheat- and rye dough (32 − 36 g/kg). *N. chiangmaiensis* produced 24 − 29 g/kg EPS in spelt and whole wheat doughs. Maximum EPS yields formed by the two *Gluconobacter* strains were lower than this, but were also detected in spelt doughs. Furthermore, the production of EPS was positively correlated with start sucrose dosage for *K. baliensis* and *N. chiangmaiensis*. In contrast to this, a rather negative relation between acetic acid formation and start sucrose was found for both strains, and a positive relation with fermentation time. In spelt doughs, *K. baliensis* and *N. chiangmaiensis* produced up to 100 and 21 − 38 mM/kg acetic acid, respectively. Acetic acid yields reached in spelt dough by the two *Gluconobacter* strains lay at 119 − 127 mM/kg. The formation of gluconic acid was – in contrast to acetic acid formation – positively correlated with start sucrose dosage. *G. frateurii* was with up to 15 mM/kg the weakest gluconic acid producer. *K. baliensis*, *N. chiangmaiensis* and *G. albidus* produced at maximum 165, 119 and 122 mM/kg flour. The remaining sucrose dosage was always positively correlated with start sucrose dosage, whereby the speed of sucrose degradation and the consumed amount depended on strain and flour. *K. baliensis* and *N. chiangmaiensis* reached remaining sucrose amounts below 50 g/kg, whereby for the two *Gluconobacter* strains, final sucrose levels were generally higher than this.

Concerning gluten-free doughs, fermentations with sucrose were carried out for *K. baliensis* only in millet, for *N. chiangmaiensis* in oat and millet and for the two *Gluconobacter* isolates in millet, corn- and rice doughs. Maximum EPS yields reached here lay at 31, 30 and 28 g/kg flour and were determined for *N. chiangmaiensis* (oat), *K. baliensis* (millet) and *G. frateurii* (rice and millet). Also in gluten-free doughs, a positive correlation between start sucrose dosage and EPS-yield could be recognized. In opposite to gluten containing doughs, acetic acid amounts produced by *K. baliensis* and *N. chiangmaiensis* were lower in gluten-free doughs and never exceeded 29 mM/kg flour. Relating to the two *Gluconobacter* strains, no essential differences were found. Concerning gluconic acid formation, *G. frateurii* was again the weakest producer while reaching up to 6 mM/kg, whereby also the other strains produced rather lower amounts than in gluten containing flours. However, a positive influence of start sucrose dosage on gluconic acid could again be found for *K. baliensis*, *N. chiangmaiensis* and *G. albidus*. Also relating to the connection between start and remaining sucrose contents, the same observations like in gluten containing flours were made.

A subsequent experiment with *K. baliensis* and *G. frateurii* in backslopped doughs over eight days showed the general suitability of acetic acid bacteria for industrial sourdough fermentations.

A further optimization of doughs by carrying out a second anaerobic fermentation with traditional sourdough starters, meaning successive fermentation with several bacterial

strains, is a promising approach to affect acid contents and remaining sugars, as well as other dough properties. An example for a fermentation with several bacterial strains is water kefir. Water kefir is formed by a microbial consortium of different bacteria and yeasts, which are embedded into an EPS-matrix and assault as waste as the consortium grows. As WKP contains EPS-forming acetic acid bacteria and as most of the strains used in this study were isolated from water kefir, the exploitation of water kefir granules as a novel hydrocolloid and for baking applications was another objective of this work. Therefore granules were processed to a white to cream-coloured powder termed WKP (water kefir powder) by sterilizing, lyophilizing and milling. The powder was tested for its functional properties and its influence on the quality of wheat breads in baking experiments.

WKP has a dry matter of 93.66 % and is not hygroscopic, which is why it can be stored at room temperature for several months. In aqueous suspensions, a pH decrease to 3.89 and 3.79 was observed for 1 and 2 % WKP addition, respectively. WKP has a water binding capacity of 919 % and therefore a thickening effect on liquids. At this, no gel-forming, foam-forming or emulsifying activity was detected. The addition of WKP to wheat breads lead to significantly decreased crumb firmness of fresh and 48 h old breads, whereas no clear effects on crust crispness and loaf weight have been found. As ideal dosage, 1 % WKP and a dough yield of 164 was identified. A therefore reduced flour:water ratio further decreased flour consumption and bread calories.

This is the first study on sourdough fermentations with EPS-producing acetic acid bacteria, as all previous studies in this field have been conducted with lactobacilli. Furthermore, this is the first study on hydrocolloid properties of processed water kefir granules. Therefore, further research activities will be needed to fully explore the potential and the wide range of applications of EPS from these bacteria, and also of WKP. For example, baking experiments using sourdoughs which were fermented with AAB, the definition of a new fermentation quotient, which relates to the ideal ratio between gluconic acid and actic acid, as well as experiments about the ideal EPS/gluconic acid/acetic acid ratio in bread could follow. At this, the results of this work enable the selection of various EPS/gluconic acid/acetic acid-ratios in different sourdoughs and further show numerous connections between the observed variables. EPS yields found here are two- to threefold higher than any other EPS yields recovered from sourdoughs so far, whereby the ideal time point for further usage of doughs is reached after $30-48$ h. As EPS-contents between $0.3-1$ % are recommended for positive effects on doughs and as *in situ* produced EPS are more effective than added ones, technologically effective levels can be reached through the addition of low sourdough amounts, especially when considering the low recovery rate for EPS from sourdoughs. Due to the small required dosage, remaining sucrose contents should not impact baking abilities of final breads. In case that no remaining sucrose is accepted in the final product, e. g. in applications for diabetics, WKP can be applied instead of using sourdoughs with acetic acid bacteria. Here, further research could focus on the influence of different particle sizes on bread staling and also on the potential of WKP for other industrial applications.

6 Zusammenfassung

Brot zählt als eines der wichtigsten Grundnahrungsmittel und wird in zahlreichen Ländern der Erde verzehrt. Während der letzten 150 Jahre führten Veränderungen im Prozess der Brotherstellung von kleinen, handwerklichen Bäckereien hin zu einer hochtechnisierten Backindustrie zu einer Standardisierung, oft einhergehend mit einer Verringerung der Brotqualität. Zu den wichtigsten Qualitätsparametern zählen dabei das Aroma, die Textur und die Haltbarkeit, welche sich auf die mikrobielle Stabilität und das Altbackenwerden bezieht. Schwierigkeiten in der Brotqualität existieren auch bei glutenfreien Broten. Diese sind hauptsächlich stärkebasiert und dienen als Nahrung für Zöliakiekranke, welche eine permanente Intoleranz für aufgenommenes Gluten besitzen. In glutenfreien Broten ist es noch schwieriger, eine hohe Produktqualität zu erreichen. Bei der traditionellen Brotproduktion wird zur Lockerung und Säuerung von Weizen- und Roggenbroten Sauerteig verwendet, da er positive Effekte auf Geschmack, Textur, Haltbarkeit und Nähreigenschaften hat. Neben der Säuerung ist hierbei die Produktion von Exopolysacchariden (EPS) durch sauerteigeigene Laktobazillen und Hefen während der Fermentation ein wichtiger Grund für seine positiven Effekte auf Brot und andere Backprodukte. EPS gehören zu den Hydrokolloiden, welche aufgrund ihrer hohen Wasserbindekapazität die Brottextur beeinflussen und das Altbackenwerden verzögern. Wegen der Vielzahl ihrer chemischen Strukturen, welche in neuartigen Anwendungsmöglichkeiten resultieren, ist die Nachfrage nach EPS kürzlich stark gestiegen.

Daher wurden in dieser Arbeit sowohl neue, EPS-produzierende Bakterienstämme, als auch ein neuartiges, EPS-basiertes Hydrokolloid, für Anwendungen in Backwaren untersucht. Für die Erschließung neuer EPS-produzierender Spezies für Back- und andere Anwendungen wurden sechs aus Wasserkefir und anderen Lebensmittelquellen isolierte, EPS-produzierende Essigsäurebakterienstämme ausgewählt. Zu Beginn wurden sie in einem erweiterten Screening in verschiedenen Labormedien auf die Robustheit ihre EPS-Produktion getested. Die Stämme *K. baliensis* DSM 14400, *N. chiangmaiensis* NBRC 101099, *G. frateurii* TMW 2.767 und *G. albidus* TMW 2.1191 produzierten mehr als 20 g/l EPS in einem oder mehr Medien. Hingegen wurde in Fermentationen mit *G. cerinus* DSM 9533T und *G. oxidans* TMW 2.339 nicht mehr als 5 g/l EPS gefunden, weshalb sie von weiteren Experimenten ausgeschlossen wurden.

Mit den anderen vier Stämmen wurden anschließend Experimente in Labormedien mit veränderten C-Quellen zusätzlich zu Saccharose durchgeführt, um den Einfluss der C-Quelle auf die EPS-Produktion zu testen. Ausgewählt wurden die Medien A (nur Saccharose), B (Sacch + Mannit), C (Sacch + Glukose), D (Sacch + Fruktose), E (Sacch + Maltose) und F (Sacch + Natriumgluconat). Es zeigte sich, dass die EPS-Produktion sowohl Stamm-, als auch C-Quellen-spezifisch war. Hierfür wurden vor allem die unterschiedliche Säurebildung der Stämme aus den verschiedenen C-Quellen sowie

die unterschiedliche Pufferkapazität der Medien verantwortlich gemacht. Diese resultierten in verschiedenen Verläufen des pH-Wertes, und somit in unterschiedlicher Aktivität der EPS-bildenden Fructosyltransferasen. In Fermentationen mit *K. baliensis* lag der pH-Wert stets signifikant höher als bei *G. albidus*, *G. frateurii* und *N. chiangmaiensis*, welche sich grundsätzlich nicht voneinander unterschieden. Zudem war der pH im Medium mit Glucose niedriger als in dem mit Mannitol und in beiden niedriger als in allen anderen Medien. Im Medium mit Natriumgluconat war der pH-Verlauf signifikant höher als in allen anderen. Da sich die Einflüsse der Medien und Stämme überlagerten, war *K. baliensis* der stärkste EPS-Bildner und produzierte höchste EPS-Mengen von mindestens 18 g/l in den Medien mit Saccharose, Fructose, Maltose und Natriumgluconat. Der zweitstärkste EPS-Bildner war *N. chiangmaiensis*. Er erreichte in Medium F über 18 g/l EPS und in A, B, D und E 16.0 – 16.7 g/l (Durchschnitt aus 2 Replikaten). *G. albidus* bildete in keinem der Medien mehr als 12.8 g/l EPS. Der Stamm *G. frateurii* produzierte nur in in Medium A und F 14.8 – 14.9 g/l EPS und in den anderen Medien bis zu 9.9 g/l EPS. Für *K. baliensis* und *N. chiangmaiensis* lagen die Zeitpunkte für maximale EPS-produktion zwischen 48 – 72 h, für die zwei *Gluconobacter* Stämme zwischen 24 – 48 h. Bei allen Stämmen wurde ein teilweiser Abbau der EPS nach dem Maximum festgestellt. *K. baliensis* zeigte in allen Medien einen sehr effizienten Saccharoseabbau von über 90 %. Bei *N. chiangmaiensis* lag dieser bei 85 – 89 % in den Medien A – E und bei 100 % in Medium F. Die zwei Isolate aus *Gluconobacter* bauten in den Medien A – E 34 – 79 % der Saccharose ab. Nur in Medium F war der Saccharoseabbau für diese Stämme effizienter und betrug 96 – 97 %. Der schwächste Acetatbildner war *K. baliensis*, denn bei diesem Stamm wurde in keiner Fermentation mehr als 2 mM/l Essigsäure nachgewiesen. *N. chiangmaiensis* und *G. frateurii* bildeten maximal 5 und 10 mM/l Essigsäure. *G. albidus* war der stärkste Acetatbildner und erreichte bis zu 23 mM/l. Bezüglich der Gluconsäurebildung verhielten sich die einzelnen Stämme je nach Medium unterschiedlich. In Medium F, in dem bereits Gluconat vorhanden war, bildete *K. baliensis* im Gegensatz zu den anderen Stämmen keine zusätzliche Gluconsäure. *N. chiangmaiensis* erreichte hier 52 mM/l, *G. albidus* 55 mM/l und *G. frateurii* 82 mM/l. In den anderen Medien wurden für *K. baliensis* maximal 28 – 47 mM/l, für *N. chiangmaiensis* 9 – 53 mM/l, für *G. albidus* 25 – 65 mM/l und für *G. frateurii* 11 – 63 mM/l Gluconsäure detektiert. Für alle untersuchten Stämme konnte nach dem Maximum eine teilweise Oxidation der Gluconsäure festgestellt werden. Die Mannit- und Maltose-Verwertung aller Stämme war gering, wobei in Natriumgluconat-freien Medien gegen Ende der Fermentation geringe Mengen Mannit akkumulierten.

Im Anschluss wurden mit allen dieser vier Stämme Sauerteigfermentationen mit glutenhaltigen und glutenfreien Mehlen durchgeführt. Zunächst wurde der Einfluss der Sauerstoffverfügbarkeit, des Mehles und von Mannitzugabe auf das Wachstum untersucht. Für die Experimente wurden Weizen-, Roggen-, Vollkornweizen-, Dinkel-, Hafer-, Goldhirse-, wilde Braunhirse-, Mais-, Reis- und Buchweizenmehl ausgewählt, wobei nicht alle Stämme in allen Mehlen getestet wurden. Anschließend wurden geeignete Teige zur Variation der Saccharosekonzentration sowie des Erntezeitpunktes verwendet und die Parameter Wachstum, pH-Wert, EPS- und Säurebildung, sowie Saccharoseabbau untersucht.

Bei ausreichender Anfangskeimzahl setzten sich alle vier Stämme zuverlässig in aeroben Teigen durch und produzierten bei Sacharose-Zugabe hohe Mengen an EPS. Davon ausgenommen waren Buchweizen- und Hafermehl: In Buchweizen konnte sich keiner der untersuchten Stämme und in Hafermehl nur *N. chiangmaiensis* durchsetzen. Da die Zugabe von Mannit bei den zwei *Gluconobacter* Spezies keine Verbesserung des Wachstums erreichte, wurde dieser Ansatz nicht weiter verfolgt.

Die statistische Datenanalyse glutenhaltiger Teige ergab zahlreiche Einflüsse der Variablen Mehl, Start-Saccharosemenge und Zeit auf die untersuchten Parameter. Bezüglich der EPS- und Säureproduktion der verschiedenen Stämme konnten dieselben Tendenzen wie für die Experimente mit variierter C-Quelle festgestellt werden. Daher erwies sich *K. baliensis* abermals als der stärkste EPS-Bildner. Aufgrund seiner hohen Pufferkapazität sowie seines hohen Nährstoffgehalts war Dinkelmehl am geeignetsten zur EPS-Produktion. In Dinkelteig erreichte *K. baliensis* bis zu 49 g/kg EPS, gefolgt von Vollkornweizen- (33 g/kg), Weizen- und Roggenteig (32 – 36 g/kg). *N. chiangmaiensis* bildete 24 – 29 g/kg EPS in Dinkel- und Vollkornweizenteigen. Die maximalen EPS-Gehalte der zwei *Gluconobacter* Spezies waren geringer, aber wurden auch in Dinkelteigen detektiert. Die Produktion von EPS war außerdem für *K. baliensis* und *N. chiangmaiensis* positiv mit dem Start-Saccharosegehalt korreliert. Im Gegensatz dazu wurde für beide Stämme ein eher negativer Zusammenhang zwischen Essigsäurebildung und der Start-Saccharosedosierung gefunden, sowie ein positiver Zusammenhang mit der Fermentationszeit. In Dinkelteig produzierte *K. baliensis* und *N. chiangmaiensis* bis zu 110 und 21 – 38 mM/kg Essigsäure. Die von den zwei *Gluconobacter*-Stämmen produzierten Mengen lagen in Dinkelteig bei 119 – 127 mM/kg. Die Gluconsäureproduktion war hingegen wieder positiv mit der Start-Saccharosemenge korreliert. *G. frateurii* war mit bis zu 15 mM/kg Mehl der schwächste Gluconsäureproduzent. *K. baliensis*, *N. chiangmaiensis* und *G. albidus* produzierten maximal 165, 119 und 122 mM/kg Mehl. Der Rest-Saccharosegehalt war stets positiv mit der Start-Saccharosedosis korreliert, wobei die Geschwindigkeit des Saccharoseabbaus und die konsumierte Menge stamm- und mehlspezifisch waren. *K. baliensis* und *N. chiangmaiensis* erreichten Rest-Saccharosemengen unter 50 g/kg, wobei die Rest-Saccharosemengen für die zwei *Gluconobacter* Spezies generell darüber lagen.

Bezüglich glutenfreier Teige wurden Fermentationen mit Saccharose für *K. baliensis* nur in Hirse-, für *N. chiangmaiensis* in Hafer- und Hirse-, und für die zwei *Gluconobacter* Spezies in Hirse-, Mais- und Reisteigen durchgeführt. Die hier erreichten, maximalen EPS-Gehalte von 31, 30 und 28 g/kg Mehl wurden in Teigen mit *N. chiangmaiensis* (Hafer), *K. baliensis* (Hirse) und *G. frateurii* (Reis und Hirse) nachgewiesen. Auch in glutenfreien Teige konnte eine positiver Zusammenhang zwischen Saccharosedosierung und EPS-Menge gefunden werden. Im Gegensatz zu glutenhaltigen Teigen waren die in glutenfreien Teigen von *K. baliensis* und *N. chiangmaiensis* produzierten Essigsäuremengen niedriger und betrugen nie mehr als 29 mM/kg Mehl. Für die zwei *Gluconobacter* Stämme wurden diesbezüglich keine wesentlichen Unterschiede gefunden. Der Einfluss der Saccharosemenge war hierbei weniger eindeutig als in glutenhaltigen Teigen. Bezüglich Gluconsäure erwies sich mit Mengen bis zu 6 mM/kg abermals *G. frateurii* als der schwächste Produzent, wobei auch die anderen Stämme eher etwas geringere Men-

gen produzierten. Ein positiver Einfluss von Start-Saccharosegehalt auf Gluconsäure konnte jedoch auch hier für *K. baliensis*, *N. chiangmaiensis* und *G. albidus* gefunden werden. Auch bezüglich der Beziehung zwischen Start- und Rest-Saccharosegehalt wurden gleiche Beobachtungen wie in glutenhaltigen Mehlen gemacht.

Ein anschließendes Backslopping-Experiment mit *K. baliensis* und *G. frateurii* über acht Tage hinweg zeigte darüber hinaus die generelle Eignung von Essigsäurebakterien für industrielle Sauerteigfermentationen.

Eine weitere Optimierung der Teige durch eine zweite, anaerobe Fermentation mit traditionellen Sauerteigstartern, also aufeinander folgende Fermentationen mit verschiedenen Bakterienstämmen, ist ein vielversprechender Ansatz, um Säure- und Restzuckergehalte, sowie andere Teigeigenschaften, zu beeinflussen. Ein Beispiel für eine Fermentation mit verschiedenen Bakterienstämmen ist Wasserkefir. Wasserkefirgranulen beherbergen ein mikrobielles Konsortium verschiedener Bakterien und Hefen. Diese sind in eine EPS-Matrix eingebettet und fallen als Müll an, da das Konsortium wächst. Da WKP EPS-produzierende Essigsäurebakterien enthält und da die meisten der in dieser Arbeit verwendeten Stämme aus Wasserkefir isoliert wurden, war die Erschließung von Wasserkefirgranulen als ein neuartiges Hydrokolloid und für Backanwendungen ein weiteres Ziel dieser Arbeit. Hierfür wurden die Granulen durch Sterilisieren, Gefriertrocknen und Vermahlen zu einem weiß bis cremefarbenen Pulver verarbeitet, welches WKP (Wasserkefirpulver) genannt wurde. Das Pulver wurde bezüglich seiner funktionellen Eigenschaften sowie auf seinen Einfluss auf die Qualität von Weizenbroten getestet.

WKP besitzt eine Trockenmasse von 93.66% und ist nicht hygroskopisch, weshalb es für mehrere Monate bei Raumtemperatur lagerfähig ist. In wässrigen Suspensionen wurde für 1 und 2% WKP eine Absenkung des pH-Wertes zu 3.89 und 3.79 beobachtet. Zudem hat WKP eine Wasserbindekapazität von 919% und somit einen verdickenden Effekt auf Flüssigkeiten. Dabei konnten keine gelbildenden, schaumbildenden oder emulgierenden Eigenschaften detektiert werden. Die Zugabe von WKP zu Weizenbroten führte zu signifikant erniedrigerter Krumenhärte von frischen und 48 h alten Broten, wohingegen keine klaren Effekte auf die Krustenknusprigkeit und das Laibgewicht gefunden wurden. Als ideale Dosis wurden 1% WKP und eine Teigausbeute von 164 ermittelt. Ein dadurch reduziertes Mehl:Wasser Verhältnis verringerte den Mehlverbrauch sowie den Kaloriengehalt der Brote.

Dies ist die erste Studie über Sauerteigfermentationen mit EPS-produzierenden Essigsäurebakterien, da alle vorherigen Studien in diesem Bereich mit Laktobazillen durchgeführt wurden. Darüber hinaus ist dies die erste Studie über die Hydrokolloid-Eigenschaften verarbeiteter Wasserkefirgranulen. Daher sind weitere Forschungsaktivitäten notwendig, um das Potential und das weite Anwendungsfeld für die EPS dieser Bakterien und auch für WKP komplett zu erschließen. Beispielsweise können die Definierung eines neuen Fermentationsquotienten, welcher sich auf das ideale Verhältnis zwischen Gluconsäure und Essigsäure bezieht, sowie Versuche zum idealen EPS/Gluconsäure/Acetat-Verhältnis im Brot als Ansatz für zukünftige Forschungsaktivitäten dienen. Dabei ermöglichen die Ergebnisse dieser Arbeit die Auswahl verschiedenster EPS/Gluconsäure/Acetat-Verhältnisse in verschiedenen Sauerteigen und zeigen zudem zahlreiche Zusammenhänge zwischen den betrachteten Einflussgrößen auf.

Die hier gefundenen EPS-Gehalte sind zwei- bis dreimal höher als die bisher jemals aus Sauerteigen isolierten Mengen, wobei der ideale Zeitpunkt für die Weiterverarbeitung der Teige nach $30 - 48\,$h erreicht ist. Da im Allgemeinen EPS-Gehalte zwischen $0.3 - 1\,\%$ für positive Effekte auf Teige empfohlen werden und zudem *in situ* produzierte EPS effektiver sind als zugesetzte, können – auch unter Berücksichtigung der niedrigen Wiederfindungsrate für EPS aus Teigen – technologisch wirksame EPS-Mengen bereits durch Zugabe geringer Mengen Sauerteig erreicht werden. Die Rest-Saccharosegehalte dürften sich aufgrund der niedrigen erforderlichen Teigdosis nur gering auf die Backeigenschaften der Brote auswirken. Für den Fall, dass keinerlei Restsaccharose im Endprodukt akzeptabel ist, z. B. in Anwendungen für Diabtiker, kann WKP anstelle von Sauerteigen mit Essigsäurebakterien angewendet werden. Hierbei könnten sich zukünftige Forschungsaktivitäten auf den Einfluss verschiedener Partikelgrößen auf das Altbackenwerden von Brot oder auf das Potential von WKP für andere industrielle Anwendungen konzentrieren.

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7 Appendix

Figure 7.1 – Questionnaire for sensorial evaluation of fresh breads.

Sensorische Prüfung von Brot

Prüfanleitung:

Es werden insgesamt 3 Proben gereicht. Jede Probe ist gesondert zu bewerten. Bitte bewerten Sie die Kriterien in Abstufungen von 1 bis 6, wobei 1 für wenig ausgeprägt und 6 für stark ausgeprägt steht. Bewerten sie <u>NICHT</u> mit Null. Die Proben sind mit den Buchstaben A, B, C gekennzeichnet, welche auf den Probentellern zu finden sind. Tragen Sie bitte die jeweilige Zahl in das jeweils vorgesehene Kästchen in der Tabelle ein.
Wiederholungen bezüglich der Merkmale sind zulässig.

1. Geruch

	A	B	C
Aromatisch			
Säuerlich			
Muffig			
Bemerkungen			

2. Geschmack

	A	B	C
Aromatisch			
Salzig			
Säuerlich			
Fade, pappig			
Bemerkungen			

3. Kaugefühl/ Kruste

	A	B	C
Trocken/fest			
Frisch/elastisch			
Bemerkungen			

4. Angenommen, Sie sind ein Weißbrot-Esser, würden Sie die Brote kaufen? Ja/ Nein?

	A	B	C
Bewertung			

Vielen Dank !

Figure 7.2 – Questionnaire for sensorial evaluation of 48 h old breads.

flour: WT						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log			
Intercept	β_0	0.8402789	0.685007		457.9508	6.589e+00
		0.2820973	0.278561		142.8840	2.012e-01
t	β_1	0.1377176		1.9215	-19.2861	-1.287e-01
		0.0298519		0.3341	6.8066	2.157e-02
s	β_2				10.1404	
					1.7012	
t^2	β_3	-0.0017193			0.2431	1.860e-03
		0.0005853			0.1335	4.175e-04
s^2	β_4					
t\cdot s	β_5					-2.747e-04
						8.703e-05
	Fermentation					
	Note					

flour: SP						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log		log	log	
Intercept	β_0	0.8299156	2.14528	1.7181927	5.813152	6.705e+00
		0.3137517	1.07466	0.4535097	0.223103	1.677e-01
t	β_1	0.2214452	0.88745	0.1603320	-0.013886	-1.648e-01
		0.0249985	0.11372	0.0311622	0.002897	1.375e-02
s	β_2				0.015244	-1.661e-02
					0.002723	6.784e-03
t^2	β_3	-0.0030874	-0.01606	-0.0021178		2.312e-03
		0.0004373	0.00223	0.0004865		2.607e-04
s^2	β_4					1.398e-04
						6.905e-05
t\cdot s	β_5					-2.742e-04
						8.777e-05
	Fermentation					
	Note					

flour: RY						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model			log		log	
Intercept	β_0				6.513e+00	6.936e+00
					5.347e-02	1.153e-01
t	β_1	1.162239	0.2757525	4.60449	-2.232e-02	-1.964e-01
		0.493660	0.0812477	1.10197	2.912e-03	9.447e-03
s	β_2				8.820e-03	-1.172e-02
					6.364e-04	4.662e-03
t^2	β_3	-0.017529	-0.0033886	-0.04196	3.020e-04	2.666e-03
		0.009679	0.0013631	0.02161	5.765e-05	1.792e-04
s^2	β_4					9.630e-05
						4.746e-05
t\cdot s	β_5		-0.0014274			-1.683e-04
			0.0005607			6.033e-05
	Fermentation					
	Note					

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Figure 7.3 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with gluten containing flours and *G. frateurii*.

flour: MI						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log		log	
Intercept	\beta_0		9.927e-01		6.487e+00	6.415e+00
			7.666e-02		7.459e-02	2.291e-01
t	\beta_1	0.1504743		4.82563	-2.995e-02	-1.400e-01
		0.0432776		0.59426	3.288e-03	2.456e-02
s	\beta_2				1.021e-02	
					9.494e-04	
t^2	\beta_3	-0.0021119	-4.531e-04	-0.05961	1.462e-04	1.920e-03
		0.0008167	1.085e-04	0.01165	4.612e-05	4.753e-04
s^2	\beta_4					
t\cdot s	\beta_5		1.953e-04		1.225e-04	-2.269e-04
			6.756e-05		3.064e-05	9.908e-05
	Fermentation					
	Note					

flour: RI						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log		log	
Intercept	\beta_0	2.38538	1.1449371		6.5384087	6.985e+00
		0.23717	0.2171753		0.2364942	1.996e-01
t	\beta_1	0.04387	0.0455120	2.1467	-0.0380962	-1.594e-01
		0.00751	0.0229818	0.2342	0.0081458	1.419e-02
s	\beta_2				0.0107791	-2.292e-02
					0.0030374	6.552e-03
t^2	\beta_3		-0.0011673			2.803e-03
			0.0004506			2.620e-04
s^2	\beta_4					2.039e-04
						6.272e-05
t\cdot s	\beta_5				0.0003392	-4.871e-04
					0.0001028	9.213e-05
	Fermentation					
	Note					

flour: CO						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log	log	log	
Intercept	\beta_0	1.5586158	1.242421	3.978928	6.313781	7.269e+00
		0.1411736	0.175427	0.249201	0.161665	3.115e-01
t	\beta_1	0.1431521	-0.023929	-0.066969	-0.005127	-1.351e-01
		0.0149392	0.005661	0.006567	0.002357	2.215e-02
s	\beta_2				0.011878	-2.857e-02
					0.001992	1.023e-02
t^2	\beta_3	-0.0017161				2.145e-03
		0.0002929				4.088e-04
s^2	\beta_4					2.597e-04
						9.788e-05
t\cdot s	\beta_5					-5.336e-04
						1.438e-04
	Fermentation			A, B		
	Note					

Figure 7.4 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with gluten-free flours and *G. frateurii*.

flour: WT							
	response	eps		gluconicacid	aceticacid	sucrose	pH
Model		log		log	log	log	
Intercept	\beta_0	-1.5030054			5.065e+00	5.359659	6.709e+00
		0.4486623			5.067e-01	0.289290	1.492e-01
t	\beta_1	0.2599814		0.1410147		-0.010060	-1.736e-01
		0.0218124		0.0243968		0.003756	1.223e-02
s	\beta_2	0.0126635			-3.030e-02	0.010327	-1.492e-02
		0.0055047			1.437e-02	0.003531	6.035e-03
t^2	\beta_3	-0.0037189		-0.0022125			2.324e-03
		0.0004229		0.0004093			2.319e-04
s^2	\beta_4				1.850e-04		1.287e-04
					9.511e-05		6.143e-05
t\cdot s	\beta_5			0.0005737	1.016e-04		-2.723e-04
				0.0001684	3.004e-05		7.809e-05
	Fermentation						
	Note						
flour: SP							
	response	eps		gluconicacid	aceticacid	sucrose	pH
Model				log	log	log	
Intercept	\beta_0			1.225190	4.368038	5.6791492	6.451e+00
				0.292094	0.046896	0.0904664	9.650e-02
t	\beta_1	4.20259		0.119661	0.005607	-0.0314083	-1.662e-01
		0.82016		0.030910	0.001236	0.0095733	1.035e-02
s	\beta_2						
t^2	\beta_3	-0.03527		-0.001341		0.0004635	2.350e-03
		0.01376		0.000606		0.0001877	2.002e-04
s^2	\beta_4						
t\cdot s	\beta_5	-0.01005					-3.085e-04
		0.00566					4.174e-05
	Fermentation				A, B	A, B	
	Note						
flour: RY							
	response	eps		gluconicacid	aceticacid	sucrose	pH
Model		log		log	log	log	
Intercept	\beta_0	0.7089329		0.6271979	4.368e+00	5.262564	6.771e+00
		0.2455401		0.1411601	1.962e-01	0.188040	8.411e-02
t	\beta_1	0.1752338		0.1241455		-0.009153	-2.149e-01
		0.0259834		0.0174579		0.002442	9.017e-03
s	\beta_2			-0.0015685		0.012819	
				0.0002929		0.002295	
t^2	\beta_3	-0.0023724		0.0003215			3.004e-03
		0.0005095		0.0001205			1.745e-04
s^2	\beta_4						
t\cdot s	\beta_5				4.244e-05		-2.057e-04
					6.723e-05		3.638e-05
	Fermentation						
	Note			lin also reasonable			

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Figure 7.5 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with gluten containing flours and *G. albidus*.

flour: MI							
	response	eps		gluconicacid	aceticacid	sucrose	pH
Model		log		log	log	log	
Intercept	\beta_0			1.760986	4.054008	4.555e+00	6.355e+00
				0.216212	0.180989	3.180e-01	1.187e-01
t	\beta_1	0.2516402		0.040147	0.002328	-3.558e-02	-1.792e-01
		0.0476336		0.006977		4.651e-03	1.272e-02
s	\beta_2				0.006034	4.695e-02	
					0.002328	8.745e-03	
t^2	\beta_3	-0.0034490				2.378e-04	2.483e-03
		0.0009242				6.523e-05	2.462e-04
s^2	\beta_4					-2.280e-04	
						5.761e-05	
t\cdot s	\beta_5					1.353e-04	-2.353e-04
						4.333e-05	5.133e-05
	Fermentation						
	Note						

flour: RI							
	response	eps		gluconicacid	aceticacid	sucrose	pH
Model		log		log	log	log	
Intercept	\beta_0			1.3770206	4.78958	4.0206438	6.858e+00
				0.1485441	0.56931	0.5412563	1.344e-01
t	\beta_1	0.1588536		0.0685488	-0.02624	-0.0205232	-2.008e-01
		0.0454332		0.0183711	0.01543	0.0058453	9.555e-03
s	\beta_2					0.0602448	-1.051e-02
						0.0152524	4.411e-03
t^2	\beta_3	-0.0021647		-0.0011320		0.0002213	2.810e-03
		0.0008908		0.0003082		0.0001146	1.764e-04
s^2	\beta_4						9.393e-05
							4.222e-05
t\cdot s	\beta_5			0.0004814		-0.0003114	-1.847e-04
				0.0001268		0.0001012	6.203e-05
	Fermentation				A, B		
	Note						

flour: CO							
	response	eps		gluconicacid	aceticacid	sucrose	pH
Model				log		log	
Intercept	\beta_0	0.9499488		1.2292116		5.4532814	6.884e+00
		0.3390195		0.4378441		0.0745915	2.828e-01
t	\beta_1		2.605223	-0.0645741	2.42502	-0.0032535	-1.079e-01
			0.167022	0.0351135	0.72920	0.0009685	2.011e-02
s	\beta_2					0.0152017	-1.931e-02
						0.0009105	9.284e-03
t^2	\beta_3		-0.027206		-0.03504		1.663e-03
			0.003275		0.01430		3.712e-04
s^2	\beta_4						2.123e-04
							8.886e-05
t\cdot s	\beta_5	0.0007364		0.0008376			-5.390e-04
		0.0001407		0.0004286			1.305e-04
	Fermentation	A, B	F				
	Note						

Figure 7.6 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with gluten-free flours and *G. albidus*.

flour: WT						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log	log		
Intercept	\beta_0	-1.4034296		2.8142932	494.673344	7.643e+00
		0.4443631		0.6870243	32.626255	3.608e-01
t	\beta_1	0.1975977	1.880e-01		-22.017135	-1.791e-01
		0.0267507	3.753e-02		2.438477	2.172e-02
s	\beta_2	0.0751915		-0.0172184		-3.859e-02
		0.0125641		0.0095738		1.020e-02
t^2	\beta_3	-0.0029466	-3.489e-03	0.0007004	0.265721	3.112e-03
		0.0004737	7.359e-04	0.0003237	0.034323	3.846e-04
s^2	\beta_4	-0.0006016	1.241e-04		0.057827	3.238e-04
		0.0001107	5.276e-05		0.004655	8.989e-05
t\cdot s	\beta_5	0.0003375			-0.039825	-7.061e-04
		0.0001872			0.022634	1.520e-04
	Fermentation					
	Note					
flour: WW						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model			log	log	log	
Intercept	\beta_0		-1.1993083	4.970292	4.794e+00	7.972e+00
			0.5594003	0.521452	1.174e-01	2.254e-01
t	\beta_1	5.50490	0.2365474	0.033875	-6.950e-02	-2.286e-01
		2.49597	0.0353891	0.012600	6.367e-03	1.357e-02
s	\beta_2		0.0162272	-0.028741	4.012e-02	-2.566e-02
			0.0076732	0.006758	4.056e-03	6.373e-03
t^2	\beta_3	-0.11187	-0.0052332		6.363e-04	3.528e-03
		0.04661	0.0006267		1.249e-04	2.403e-04
s^2	\beta_4	-2.562e-04			-1.633e-04	2.014e-04
		2.842e-05			3.268e-05	5.616e-05
t\cdot s	\beta_5		0.0007305			-3.930e-04
			2.338e-04			9.494e-05
	Fermentation					
	Note					
flour: SP						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log			log	
Intercept	\beta_0	-1.0331872			4.125e+00	7.3897390
		0.4928869			1.021e-01	0.2736872
t	\beta_1	0.2319815	1.979494	1.106081	-5.338e-02	-0.1470577
		0.0296718	0.879901		5.537e-03	0.0164760
s	\beta_2	0.0444003	-0.773140	1.231727	5.512e-02	-0.0425078
		0.0139361	0.413267	0.446163	3.527e-03	0.0077383
t^2	\beta_3	-0.0041297	-0.052234		3.766e-04	0.0026807
		0.0005254	0.015581		1.086e-04	0.0002918
s^2	\beta_4	-0.0004335	0.009213	-0.011894	-2.562e-04	0.0003635
		0.0001228	0.003642	0.004418	2.842e-05	0.0000682
t\cdot s	\beta_5	0.0010926	0.028126			-0.0006982
		0.0002076	0.006156			0.0001153
	Fermentation					
	Note					

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Figure 7.7 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with gluten containing flours and *K. baliensis*.

flour: RY						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log		log	
Intercept	β_0				4.555e+00	7.098e+00
					1.197e-01	1.701e-01
t	β_1	1.347e-01	0.1361859		-2.353e-02	-2.139e-01
		2.223e-02	0.0263046		7.342e-03	1.024e-02
s	β_2	4.058e-02	0.0456400		3.906e-02	-2.009e-02
		1.044e-02	0.0123546		3.511e-03	4.810e-03
t^2	β_3	-2.531e-03	-0.0031641	0.029138	3.023e-04	3.581e-03
		3.937e-04	0.0004658	0.008536	1.297e-04	1.814e-04
s^2	β_4	-3.322e-04	-0.0003887		-1.366e-04	1.995e-04
		9.202e-05	0.0001089		3.092e-05	4.239e-05
t\cdot s	β_5	7.339e-04	0.0011841		-1.920e-04	-4.720e-04
		1.556e-04	0.0001840		5.476e-05	7.166e-05
	Fermentation					
	Note					
flour: MI						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log		log	
Intercept	β_0	-1.0019032	1.8999386		5.8080514	7.301e+00
		0.4002335	0.2780608		0.2463743	3.748e-01
t	β_1	0.3557431	-0.0949430	0.3522475	-0.0581808	-1.543e-01
		0.0423533	0.0133872	0.0458203	0.0108606	2.256e-02
s	β_2				0.0116398	-4.242e-02
					0.0031358	1.060e-02
t^2	β_3	-0.0051760		-0.0026029	0.0003514	2.943e-03
		0.0008304		0.0008984	0.0001523	3.995e-04
s^2	β_4					3.930e-04
						9.338e-05
t\cdot s	β_5		0.0013172		0.0002857	-8.799e-04
			0.0001619		0.0001012	1.578e-04
	Fermentation					
	Note					

Figure 7.8 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with rye and millet flour and *K. baliensis.*

flour: WT

	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log		log	
Intercept	β_0				5.627487	7.584e+00
					0.416249	3.162e-01
t	β_1	0.1828206	0.1583049	0.713600	-0.037558	-1.588e-01
		0.0519464	0.0349741	0.187056	0.006700	1.903e-02
s	β_2	0.0607358			0.013680	-4.789e-02
		0.0251772			0.005309	8.939e-03
t^2	β_3	-0.0032266	-0.0040148			3.695e-03
		0.0010185	0.0006533			3.370e-04
s^2	β_4	-0.0004288				4.409e-04
		0.0002381				7.878e-05
t\cdot s	β_5		0.0008724	-0.003843		-1.240e-03
			0.0001585	0.002262		1.332e-04
	Fermentation					
	Note					

flour: WW

	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log	log	log	
Intercept	β_0		-2.370124		5.8515272	6.9828038
			0.370451		0.4051619	0.2183562
t	β_1	0.0951948	0.166083	1.186e-01	-0.0579932	-0.1726443
		0.0371615	0.028204	1.817e-02	0.0162046	0.0240871
s	β_2	0.0625236	0.024334		0.0128707	
		0.0176245	0.004193		0.0054463	
t^2	β_3	-0.0019624		-8.755e-04		0.0033942
		0.0006073	0.000553	2.677e-04		0.0004531
s^2	β_4	-0.0005717		9.584e-05		
		0.0001449		3.806e-05		
t\cdot s	β_5	0.0010747		-4.350e-04	0.0003963	-0.0007542
		0.0002614		1.229e-04	0.0002122	0.0001108
	Fermentation					
	Note					

flour: SP

	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log	log	log	
Intercept	β_0	-1.3202938	1.5375059	3.863602	5.240e+00	7.288e+00
		0.4660048	0.3068786	1.755922	2.486e-01	3.161e-01
t	β_1	0.1703827	0.1138826		-9.383e-02	-1.708e-01
		0.0280535	0.0338521		9.733e-03	1.903e-02
s	β_2	0.0798559			3.372e-02	-3.539e-02
		0.0131760			7.814e-03	8.938e-03
t^2	β_3	-0.0030373	-0.0034516	0.010724	1.134e-03	3.345e-03
		0.0004968	0.0006367	0.001781	1.945e-04	3.370e-04
s^2	β_4	-0.0006303			-1.278e-04	3.204e-04
		0.0001161			5.922e-05	7.877e-05
t\cdot s	β_5	0.0007098	0.0008172	-0.002954		-8.446e-04
		0.0001963	0.0001558	0.001098		1.332e-04
	Fermentation					
	Note					

Figure 7.9 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with gluten containing flours and *N. chiangmaiensis*.

flour: RY						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log		log	log	
Intercept	\beta_0			1.633316	5.711915	6.949e+00
				0.300153	0.278626	1.664e-01
t	\beta_1	0.0869484	3.535047	0.020339	-0.023767	-1.956e-01
		0.0303789	1.753917	0.005712	0.004485	1.002e-02
s	\beta_2	0.0296454		0.005693	0.015013	-2.089e-02
		0.0141205		0.002970	0.003554	4.704e-03
t^2	\beta_3	-0.0014444	-0.067930			3.422e-03
		0.0005424	0.034389			1.774e-04
s^2	\beta_4	-0.0002279	0.005038			2.163e-04
		0.0001255	0.002465			4.145e-05
t\cdot s	\beta_5	0.0005104				-6.167e-04
		0.0002072				7.007e-05
	Fermentation					
	Note					

flour: MI						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model			log	log	log	
Intercept	\beta_0		1.5020415	1.0969694	6.0990608	7.3665160
			0.3534526	0.4041888	0.3642190	0.4141266
t	\beta_1	4.87807		0.0926515	-0.0872115	-0.1699486
		2.46725		0.0360300	0.0167794	0.0249305
s	\beta_2				0.0116088	-0.0401141
					0.0046352	0.0117092
t^2	\beta_3	-0.09951	-0.0019177	-0.0015492	0.0008295	0.0031123
		0.04641	0.0003584	0.0006635	0.0002477	0.0004415
s^2	\beta_4					0.0003648
						0.0001032
t\cdot s	\beta_5	0.02497	0.0011353		0.0002612	-0.0008495
		0.01135	0.0002211		0.0001496	0.0001744
	Fermentation					
	Note					

flour: OA						
	response	eps	gluconicacid	aceticacid	sucrose	pH
Model		log	log		log	
Intercept	\beta_0	-3.1753931	-1.8684907		5.639e+00	7.497e+00
		0.8006270	0.8584194		2.150e-01	2.561e-01
t	\beta_1	0.2011426	0.1295266	0.543773		-1.317e-01
		0.0403614	0.0517436	0.111637		1.542e-02
s	\beta_2	0.0858246	0.0470949		1.583e-02	-3.162e-02
		0.0205936	0.0242565		2.639e-03	7.242e-03
t^2	\beta_3	-0.0030661	-0.0023966		-4.181e-04	1.892e-03
		0.0006908	0.0009235		7.228e-05	2.731e-04
s^2	\beta_4	-0.0006419	-0.0004196			2.864e-04
		0.0001641	0.0002182			6.382e-05
t\cdot s	\beta_5	0.0007790	0.0010343	-0.004801		-5.543e-04
		0.0002956	0.0003742	0.001350		1.079e-04
	Fermentation					
	Note					

Figure 7.10 – Chosen models and β-values for t, s, t^2, s^2 and $t \cdot s$ for selected parameters during sourdough fermentations with rye, millet and oat flour and *N. chiangmaiensis.*