STUDY OF THE POTENTIAL OF (NON-)BAKERY *SACCHAROMYCES CEREVISIAE* STRAINS IN BREAD MAKING AND OF A YEAST STRAIN SPECIFIC FERMENTATION INHIBITING PROTEIN IN WHEAT

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Voorwoord

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Summary

The production of tasty leavened bread is almost inseparably linked to the presence of *Saccharomyces cerevisiae* yeast. The main function of yeast during the fermentation phase is the production of CO_2, resulting in dough leavening. Besides CO_2, the yeast's fermentation metabolism also results in the release of ethanol and other minor constituents, such as glycerol, organic acids and flavor components. These metabolites are known to have a major impact on dough rheology and bread texture, aroma and flavor. The amount and type of metabolites produced by yeast during bread making depend on the ingredients (nutrient availability in flour, salt, etc.), fermentation time and temperature as well as on the pregrowth conditions and genetic background of the yeast strain used.

As a consequence of the growing consumer's interest in a wider variety of tasty and healthy food products, industrial and artisanal bakeries are challenged to improve end products. Strategies to do so include changing dough formulations by, for example, addition of enzymes as bread improvers or wheat bran as a source of dietary fiber. Also yeast technology can be used to change bread properties. The *Saccharomyces cerevisiae* species embraces a lot of different strains, applied in various industrial fermentation processes, offering a large potential for possible application in bread making.

Preliminary experiments in our lab showed a very limited fermentation ability of several *S. cerevisiae* strains in wheat flour dough while studying the impact of strains from diverse industrial categories on dough rheology. As this remarkable observation was left unexplored, the first aim of this doctoral research was to gain insight in this inhibition phenomenon by characterizing it and identifying the inhibition-causing factor(s). The second aim was to evaluate the applicability of (non-bakery) *S. cerevisiae* strains in bread making.

In a first part, the fermentation capacity of a set of 45 *S. cerevisiae* strains originating from 5 yeast-based industrial categories (bread, beer, bio-ethanol, sake & spirits and wine) was screened in different fermentation media. In a liquid fermentation medium, with a water activity adjusted to the level in dough, no industrial category differed significantly from the category of bakery strains, suggesting that they all had good fermentation potential. In wheat flour dough,

variation in fermentation capacity could be noticed amongst the yeast strains. Most remarkable was the very limited fermentation ability of one fifth of the yeast strains. They produced up to 85% less CO_2 than the best bakery strain, clearly demonstrating fermentation inhibition in wheat flour dough. The inhibition-sensitive yeast strains performed significantly better in a gluten-starch model dough. Replacing the aqueous phase of the model dough by a wheat flour extract induced the same degree of inhibition as did wheat flour dough, allowing to conclude that the water extractable fraction of wheat flour contains a yeast-inhibiting component.

In the next part of this PhD work, the yeast inhibition phenomenon was characterized. The representative of the inhibition-sensitive yeast strains showed an inhibited fermentation capacity in the presence of flour from different wheat varieties. Moreover, rye, maize and barley had a similar effect on the yeast's fermentation ability. After further analyses, the inhibition-causing factor turned out to be a water extractable wheat flour protein. Starting from a crude wheat flour extract, the inhibitor was isolated by activity-guided fractionation with cation exchange chromatography and reversed phase-HPLC. Identification of the proteins present in the purest inhibition-active fraction with LC/MS-MS and Edman degradation pointed towards the *thaumatin-like protein* as the inhibition causing factor. This finding was validated by the fact that the recombinantly expressed target protein also showed fermentation inhibition in representative of the sensitive yeast strains. Based on the differences in sensitivity of *S. cerevisiae* strains towards the inhibitor, a mode of action comparable with that of thaumatin-like tobacco osmotin was suggested, mainly affecting the permeability of the yeast cell wall. Although the inability of brewer's yeasts to ferment a wheat flour batter was in the early seventies ascribed to purothionins, this was not conform to our results.

In the last part, the applicability of a selection of (non-bakery) *S. cerevisiae* strains in the bread making process was evaluated. After screening 45 yeast strains for their metabolite and aroma profiles, 10 strains (two from each industrial category), including two inhibition-sensitive strains, were selected for further examination of their impact on dough rheology and their influence on final bread aroma and quality characteristics. As was expected based on the variability in fermentation properties, metabolite analysis of these strains during fermentation also showed different profiles. In spite of these differences and of the fact that individual fermentation metabolites have already been shown to impact rheology in yeastless dough, no clear correlations were found between metabolites produced and rheological changes in yeasted dough. The constant change in density through CO_2 production was assumed to have the

foremost impact on dough properties. Similarly, significantly differing fermentation capacities did not necessarily result in significantly different bread volumes. Furthermore, no clear differences in crumb texture and structure between a control bakery strain and the other inhibition-insensitive yeast strains were observed. The determined variation in fermentation capacity and metabolite production between yeast strains did not seem sufficiently outspoken to result in significant differences in bread volume and texture. All inhibition-insensitive strains in the selection gave rise to breads with similar characteristics to those obtained with the control bakery strain. Aroma profile analysis hinted towards more significant differences between the different strains.

The research in this PhD study gave insight in a yeast specific inhibition phenomenon, caused by the *thaumatin-like protein* during wheat flour dough fermentation. This finding is of clear interest for yeast- and cereal-based fermentation industries, although further work is required on inhibitor quantification and its mode of action. The improvement of existing strains by breeding, the creation of new strains through genetic modification, or genetic modification of wheat to eliminate the harmful component might provide some solutions to enable yeast strains with a beneficial impact on dough and bread quality parameters or with a pleasant volatile compound profile, to be used in the bakery industry.

Samenvatting

Gist en brood zijn onlosmakelijk met elkaar verbonden. De productie van CO_2 om deeg te doen rijzen, wordt vaak gezien als de belangrijkste functie van gist. Tijdens het fermentatieproces zorgt gist echter ook voor de productie van componenten zoals ethanol, glycerol en organische zuren. Van deze fermentatiemetabolieten is geweten dat ze deegreologie, textuur, smaak en aroma van brood kunnen beïnvloeden. De mate waarin deze metabolieten geproduceerd worden tijdens de broodbereiding, is sterk afhankelijk van de ingrediënten (beschikbaarheid van nutriënten in bloem, aanwezigheid van zout,…), de fermentatietijd en –temperatuur, maar ook van de groeicondities en genetische achtergrond van de gebruikte giststammen.

Door het groeiende bewustzijn van de consument voor gezonde en gevarieerde voeding, worden industriële en artisanale bakkers continu uitgedaagd om bestaande eindproducten te verbeteren of broden met vernieuwde smaken of verbeterde nutritionele waarde te ontwikkelen. Hierbij kan de samenstelling van brood gewijzigd worden door, onder andere, toevoeging van enzymen als broodverbeteraars of zemel als bron van dieetvezel. Daarnaast kan ook ingespeeld worden op de gist die aangewend wordt voor broodbereiding. Hiervoor wordt typisch gebruik gemaakt van het species *Saccharomyces cerevisiae*. Dit species omvat echter tal van verschillende stammen die ook in andere industriële fermentatieprocessen toegepast worden, wat de broodindustrie tal van mogelijkheden biedt voor de wijziging van broodeigenschappen.

Voorafgaand aan dit doctoraatsonderzoek werd de impact van *S. cerevisiae* stammen, toegepast in verschillende industrieën, op deegreologie onderzocht. Hierbij kwam aan het licht dat sommige *S. cerevisiae* stammen tarwebloemdeeg slechts in heel beperkte mate konden doen rijzen. Deze observatie werd echter niet verder onderzocht en vormde de eerste doelstelling van deze doctoraatsstudie. Naast het inzicht verwerven in het inhibitiefenomeen door karakterisering en identificatie van de inhiberende tarwebloemfactor(en), werd als tweede doel ook de toepasbaarheid van verschillende (niet-bakkerij) stammen van *S. cerevisiae* voor de broodbereiding geëvalueerd.

In een eerste deel werd de fermentatiecapaciteit van 45 verschillende *S. cerevisiae* stammen, komende uit 5 verschillende industrieën (brood, bier, bio-ethanol, sake & spirits en wijn)

nagegaan in verschillende fermentatiemedia. Na een eerste screening in vloeibaar medium, met dezelfde wateractiviteit als in deeg, bleken de geteste gisten een goed fermentatiepotentieel te vertonen, aangezien geen van de groepen – ondanks de individuele variatie – significant verschilde van de bakkerijstammen. In tarwebloem werd eveneens variatie tussen de individuele stammen waargenomen. Opmerkelijker was dat een vijfde van de giststammen een uiterst beperkte fermentatiecapaciteit vertoonde. Het totaalvolume CO_2 dat geproduceerd werd na twee uur fermentatie lag tot 85% lager in vergelijking met de beste bakkersgist. De inhibitiegevoelige giststammen waren wel in staat om te fermenteren in een modeldeegsysteem bestaande uit gluten en zetmeel. Wanneer de waterige fase van het glutenzetmeeldeeg werd vervangen door een waterig tarwebloemextract, werd opnieuw hetzelfde inhibitiefenomeen als in tarwebloemdeeg waargenomen. Dit toonde aan dat de waterextraheerbare fractie van bloem een gistinhiberende component bevat.

In de volgende fase van het doctoraatsonderzoek werd dit inhibitiefenomeen verder gekarakteriseerd. Naast de fermentatiecapaciteit werd ook de groei van inhibitiegevoelige gisten beïnvloed door de inhibitor. Verschillende tarwevariëteiten en graansoorten werden getest en gelijkaardige inhibitie werd teruggevonden in alle tarwes, rogge, mais en gerst. Verder onderzoek toonde uiteindelijk aan dat de inhibitor een waterextraheerbaar tarwebloemproteïne was.

De inhibitor werd geïsoleerd door fractionering van een ruw tarwebloemextract waarbij de bekomen fracties telkens getest werden op inhibitie-activiteit. Hierbij werd achtereenvolgens gebruik gemaakt van kationuitwisselingschromatografie en reversed phase-HPLC. De proteïnen aanwezig in de zuiverste inhiberende fractie werden geïdentificeerd met behulp van LC-MS/MS en Edmandegradatie. Dit resulteerde in de assumptie dat de inhibitor het *thaumatin-like* proteïne was, wat bevestigd werd na het testen van de inhibitie-activiteit van een recombinant tot expressie gebracht *thaumatin-like* proteïne. De manier waarop het proteïne inwerkt op inhibitie-gevoelige gisten werd niet onderzocht in deze doctoraatsstudie, maar analogie met osmotine, een thaumatine-achtig proteïne uit tabak dat de permeabiliteit van de celwand beïnvloedt, werd gesuggereerd. Hoewel de inhibitie van bepaalde giststammen in een tarwebloembeslag in de jaren zeventig reeds werd toegeschreven aan purothionines, was dit niet in overeenstemming met onze resultaten. Enkele verschilpunten tussen ons onderzoek en de literatuur waren de extraheerbaarheid (petroleumether versus water), de grootte van de inhibitor (5 kDa versus ongeveer 24 kDa) en het verlies van inhibitie-activiteit in aanwezigheid van divalente ionen.

In het laatste deel van dit onderzoek werd de toepasbaarheid van een selectie van (niet-bakkers)gisten in de broodbereiding nagegaan. Uit de 45 stammen werden er 10 geselecteerd, inclusief twee inhibitie-gevoelige. Van deze kleine selectie werd vervolgens de metabolietproductie tijdens de fermentatie, de impact hiervan op deegreologie, alsook de invloed op broodaroma en andere kwaliteitsparameters nagegaan. Ondanks de variatie in de metabolietprofielen en het feit dat de invloed van individuele fermentatiemetabolieten op deegreologie reeds aangetoond werd in niet-gegiste degen, konden deze verschillen niet eenduidig gecorreleerd worden aan reologische veranderingen in gegiste degen. De voortdurende densiteitswijziging door de constante CO_2-productie bleek mogelijke invloeden van metabolieten op de reologie te overstijgen. Analoog kon de variatie in fermentatiecapaciteit van de niet-geïnhibeerde gisten niet rechtstreeks gelinkt worden aan significante verschillen in broodvolume, kruimtextuur en kruimstructuur. Dit deed vermoeden dat de verschillen in fermentatie en metabolietproductie onvoldoende waren om tot uiting te komen in deeg- en broodkwaliteitsparameters. Bijgevolg resulteerde de aanwending van alle geselecteerde inhibitie-ongevoelige gisten in de productie van broden van gelijkaardige kwaliteit als dat van de bakkersgist. De productie van meer gewenste en/of minder ongewenste aromacomponenten zou de finale broodkwaliteit bijgevolg ten goede kunnen komen. Aangezien de aroma-analyses in dit onderzoek echter niet kwantitatief waren en bijgevolg slechts een indicatie gaven van het potentiële aromaprofiel, kon op dit vlak eveneens geen uitspraak gedaan worden over de voorkeur voor bepaalde niet-bakkerijgisten voor de broodbereiding.

Deze doctoraatsstudie gaf inzicht in het gist-specifieke inhibitiefenomeen, veroorzaakt door het *thaumatin-like* proteïne tijdens de fermentatie van tarwebloemdeeg. Deze bevindingen, samen met kennis over de concentratie van de inhibitor in tarwebloem en de manier waarop hij inwerkt op de verschillende gisten, kan de gist- en graangebaseerde fermentatie-industrieën zeker en vast ten goede komen. Modificatie van gisten om ze resistent te maken of van tarwe om de inhibitor te elimineren, kunnen oplossingen bieden om inhibitie-gevoelige gisten met een positieve impact op deeg en brood en die aangename aroma's produceren, toch te kunnen aanwenden in de broodindustrie.

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List of abbreviations

AU	Absorbance units
CEC	Cation exchange chromatography
dm	Dry matter
E. coli	*Escherichia coli*
FODMAP	Fermentable oligo-, di- and monosaccharides and polyols
GC-MS	Gas chromatography – mass spectrometry
GM	Growth medium
GSD(E)	Gluten-starch dough (with flour extract)
HS-SPME	Headspace – solid phase micro extraction
IY	Inactive yeast
LC-MS/MS	Liquid chromatograpy – tandem mass spectrometry
LFM	Liquid fermentation medium
MW	Molecular weight
OD	Optical density
PCA	Principal component analysis
PIR	Proteins with internal repeats
ppm	Parts per million
PR	Pathogenesis- related
PVDF	Polyvinylidene fluoride
r	Correlation coefficient
(r)TLP	(recombinant) *thaumatin-like* protein
(RP-)HPLC	(Reversed phase –) high performance liquid chromatography
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SP SFF	Sulfopropyl sepharose fast flow
TCA cycle	Tricarboxylic acid cycle
TFA	Trifluoroacetic acid
WFD	Wheat flour dough
WFE	Wheat flour extract
YPD	Yeast extract, peptone and dextrose

Context and aims of the PhD

Bread as a staple food represents an important share of our daily western diet. As a consequence of the growing consumer's interest in a wider variety of tasty and healthy food products, industrial and artisanal bakeries are challenged to improve their bread products. Strategies to do so include changing dough formulations by, for example, addition of enzymes as bread improvers or wheat bran as a source of dietary fiber. Also yeast technology could be used to change bread properties. Yeast is an essential bread ingredient and of major importance for the production of tasty leavened bread. The yeast strains typically used for bread making belong to the *Saccharomyces cerevisiae* species and have historically been selected for their fast adaptation to the semi-solid dough environment and the consumption of maltose, and their high CO_2 production capacity. However, the conversion of fermentable sugars by yeast to CO_2 and ethanol during dough fermentation also results in the production of organic acids, glycerol and volatile compounds. These metabolites are known to affect dough rheology, bread quality, flavor and aroma. Likewise, differences in substrate consumption imply changes in final bread composition. In this context, the use of alterative yeast strains, such as *Torulaspora delbrueckii* or *Kluyveromyces marxianus*, has recently been proven useful to alter aroma and flavor or the nutritional value of bread. While most of the research into alternative yeasts focus on the applicability of non-conventional yeast species in bread making, the potential of the wide variety of *S. cerevisiae* strains remains largely unexplored. It forms the background against which this PhD work is situated.

In screening experiments just prior to this doctoral research, *S. cerevisiae* strains from diverse industrial categories were examined in our research group for their impact on dough rheology. A remarkable observation that was left unexplored was the very limited fermentation ability of several *S. cerevisiae* strains in wheat flour dough, whereas their fermentation capacity in liquid fermentation broth was similar to that of other, well performing yeasts. This illustrates that yeast strains not only affect dough and bread characteristics, but - vice versa - also that the dough matrix determines the functionality of yeast. As fermentation potential is key to the use of *S. cerevisiae* in bread making, this fermentation inhibition was further researched as part of this PhD.

The above two elements, the unexplored potential of *S. cerevisiae* strains in bread making and the *S. cerevisiae* fermentation inhibition in wheat dough, are at the basis of the two-fold aim of this study:

(i) Achieve better understanding of the yeast fermentation inhibition occurring during wheat flour dough fermentation and identification of the potential inhibitor(s)

(ii) Gain insight in the impact and evaluate the potential of non-bakery *S. cerevisiae* strains on dough and bread quality

Chapter 1 provides an overview of the scientific literature on yeast in the context of the bread making process. First, literature on yeast and its historical evolution, its potential for bread making and genetic modification or breeding strategies to create new yeast strains is reviewed. Yeast growth phases and metabolic pathways during dough fermentation are discussed, together with the impact of the yeast's fermentation metabolites on dough and bread properties. In a second part, the bread dough matrix as an environment in which yeast needs to operate is discussed. This part covers the nutrient availability in dough and the impact of wheat-inherent components and additional ingredients on yeast.

In **Chapter 2**, the fermentation potential of a set of 45 *S. cerevisiae* strains originating from 5 different yeast-based industrial categories is examined, as a basis for the two research lines in this doctoral work. The first screening involves examination of their fermentation capacity in a liquid fermentation medium to estimate the CO_2 production ability. Fermentation experiments in wheat flour dough and a gluten-starch model dough are performed to assess yeast performance in these more complex systems and tease out the fermentation inhibition that was previously observed.

Characterization of the inhibitory compound is described in **Chapter 3**. It involves examination of the impact of the inhibitor on yeast growth, its sensitivity towards proteolysis and thermal treatment and the concentration dependency. Furthermore, the presence of a similar inhibitory compound in different wheat varieties and other cereal grains is explored.

As results of **Chapter 3** led to believe that the inhibition phenomenon is caused by a wheat flour protein, **Chapter 4** focusses on the identification of the fermentation inhibiting protein. Activity-guided fractionation of proteins in wheat flour extract is performed. A series of chromatographic purification techniques together with LC-MS/MS as an identification tool are used, followed by recombinant expression of the target protein for validation purposes.

In **Chapter 5** the applicability of non-bakery *S. cerevisiae* strains in bread is further explored, after having examined their fermentation capacity in Chapter 2. The metabolite and aroma production of the 45 yeast strains is examined in a model dough system. Based on these results,

10 yeast strains are selected for further analysis in wheat flour dough. The impact of the different strains on dough rheology, bread volume, crumb texture, crumb structure and bread aroma compounds is investigated. An overview of the layout of this doctoral dissertation is given in **Figure 0.1**.

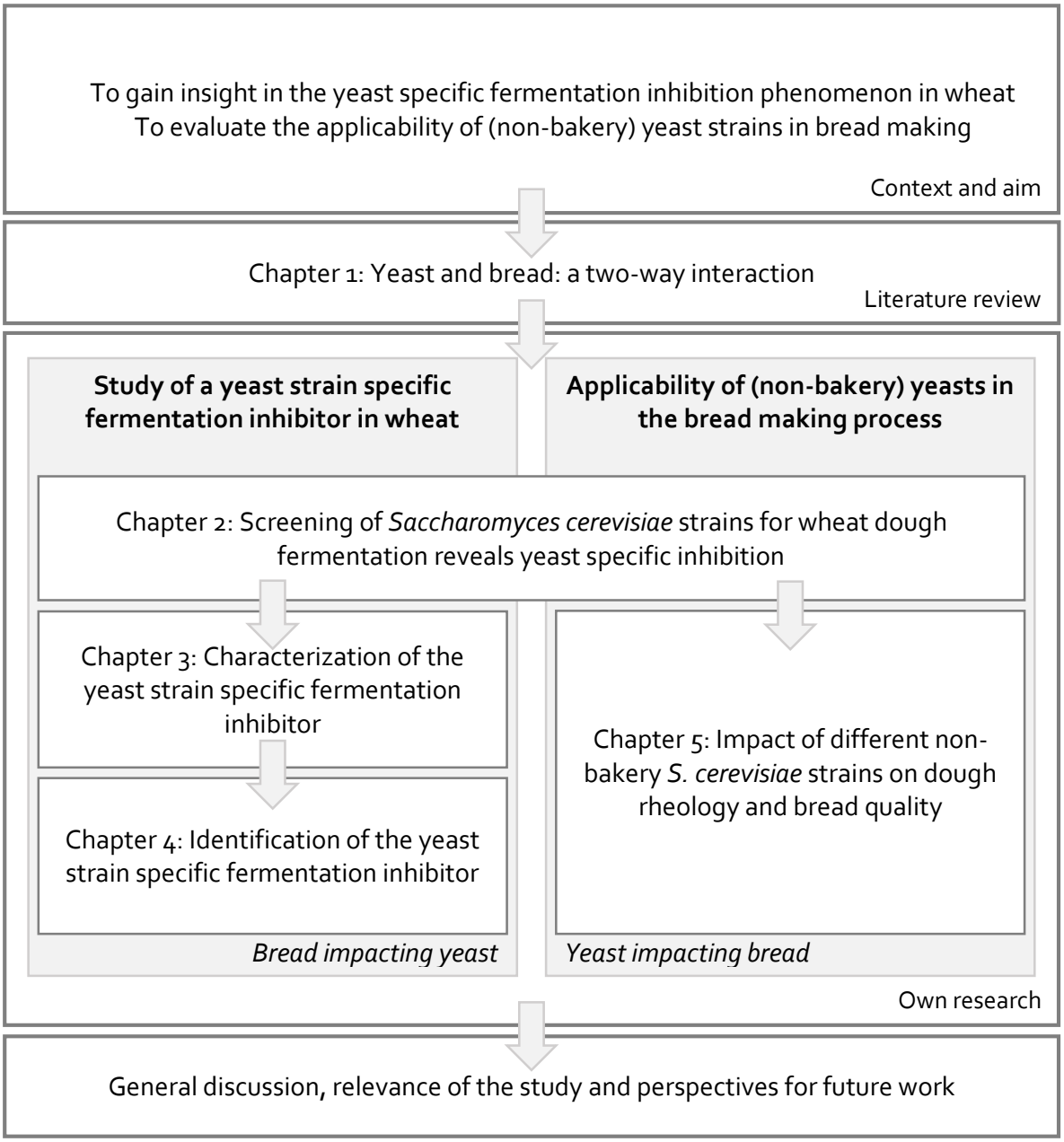

***Figure 0.1**: Schematic overview of the different chapters in this doctoral dissertation.*

PART 1 LITERATURE REVIEW

Chapter 1 Yeast and bread: a two-way interaction

Chapter 1.

Yeast and bread: a two-way interaction

1.1 Introduction

The production of leavened bread is inseparably linked to the presence of yeast. *Saccharomyces cerevisiae* is the most commonly used species for baking purposes. As essential ingredient in the production of bread, yeast has a key role during the fermentation phase (Ali et al., 2012). More specifically, yeast converts the fermentable sugars present in dough into ethanol and carbon dioxide (CO_2), which leads to dough leavening (Mondal and Datta, 2008). However, baker's yeast is more than a leavening agent only. In addition to the CO_2 and ethanol, yeast also produces other metabolites such as organic acids, glycerol and aroma compounds. The production of these fermentation metabolites has important repercussions for dough rheology, texture, flavor and aroma of the final bread (Cho and Peterson, 2010; Jayaram et al., 2013b; Rezaei et al., 2015a). The total amount and type of metabolites produced depend on (i) flour characteristics: the content of sugars, fructans and damaged starch, the presence of enzymes, peptides and amino acids; (ii) other dough ingredients: salt, added sugar, bran; (iii) fermentation time and temperature; and (iv) yeast-inherent factors, such as pregrowth conditions and genetic background of yeast strains (Gabriela and Daniela, 2010; Rezaei et al., 2015a, 2014; Struyf et al., 2017b). Several studies already showed that yeast has a big impact on bread quality parameters due to the metabolites aside from CO_2. Ethanol and succinic acid, for example, have been shown to make dough stiffer and less extensible (Jayaram et al., 2014, 2013b; Meerts et al., 2018a). Analogously, as already mentioned, bread ingredients are determining factors in the fermentation metabolism of yeast. This means that there is a reciprocal interaction between yeast and bread ingredients (**Figure 1.1**).

This is important in the context of product improvement. The increasing consumer's demand for a more diverse assortment of bread and other bakery products, poses a continuous challenge to artisanal and industrial bakeries. On the one hand, this involves quality improvement. Bread improvers such as enzymes can be used as additives to improve bread volume, texture or shelf-life (Caballero et al., 2007; De Gobba et al., 2016; Poutanen, 1997). Amylases and proteases beneficially affect bread texture and flavor by softening the bread crumb and by producing fermentation substrates and aroma precursors (Poutanen, 1997). Moreover, amylases, xylanases and proteases are shown to act as antistaling agents (Caballero et al., 2007), which could be of interest from an industrial point of view. A longer shelf-life, for example, indeed enables industrial bakeries to produce larger quantities of bread (Decock and Cappelle, 2005). Next, non-conventional yeast strains can be added to alter the characteristic flavor and aroma of bread (Aslankoohi et al., 2016). According to Aslankoohi and coworkers (2016), *Torulaspora delbrueckii*

and *Saccharomyces bayanus* not only showed a desirable dough fermentation, their aroma profile in the final bread was also preferred above that of the traditional bakery yeast by a sensory test panel (Aslankoohi et al., 2016).

On the other hand, bread improvement also includes improvement of the health status of the consumer upon consumption of the product by (i) closer adherence to generally accepted dietary guidelines or (ii) by improving specific nutritional aspects of the bread. Wheat (*Triticum aestivum* L.) is one of the most commonly used cereals for bread production. The outer layers of a wheat kernel, called botanical bran (further referred to as bran) provide a source of dietary fiber and form a side-stream when wheat kernels are milled to obtain white flour (Hemdane et al., 2016). Combining all milling fractions that result from milling of wheat yields whole meal. In general, the consumption of bran-enriched products has been proven to be beneficial for consumer's health. However, the daily recommendation of 25 – 35 g of dietary fiber is barely met. Therefore, the consumption of whole meal bread instead of white bread will increase the dietary fiber uptake and help to meet the dietary guidelines (Brouns et al., 2012; Hemdane et al., 2016; Stephen et al., 2017).

Some consumers, for example people suffering from coeliac disease or irritable bowel syndrome (IBS) have specific nutritional requirements when it comes to bread. People with coeliac disease, an autoimmune disorder, are intolerant to gluten proteins, the storage proteins present in wheat flour (Green and Jabri, 2003). In response to their needs, the bread industry offers gluten-free breads made from (mixtures of) other cereals, such as oat, rice or sorghum, or pseudocereals, such as buckwheat and quinoa (Healthgrain consortium, 2013; Masure et al., 2016). Fermentable oligosaccharides as part of FODMAPs (Fermentable oligo-, di- and monosaccharides and polyols), although by some countries seen as dietary fiber (Verspreet et al., 2015), are evidenced to cause abdominal pains through luminal distension by rapid fermentation or by causing water influx in the gut in people suffering from IBS (Whelan et al., 2018). Bread and other wheat derived products naturally contain noticeable amounts of fructans and hence are a major source of FODMAPs in our diet. Recently, Struyf and coworkers (2018a) discovered that a certain *Kluyveromyces marxianus* yeast strain hydrolysed more than 90% of the fructans during bread making, resulting in bread low in FODMAPs. As there is evidence that a FODMAP low diet results in a 70% decrease of the abdominal symptoms in IBS patients, the use of this yeast strain in combination with whole meal bread making offers a lot of potential to help fulfill the nutritional needs for this small population (Struyf et al., 2018).

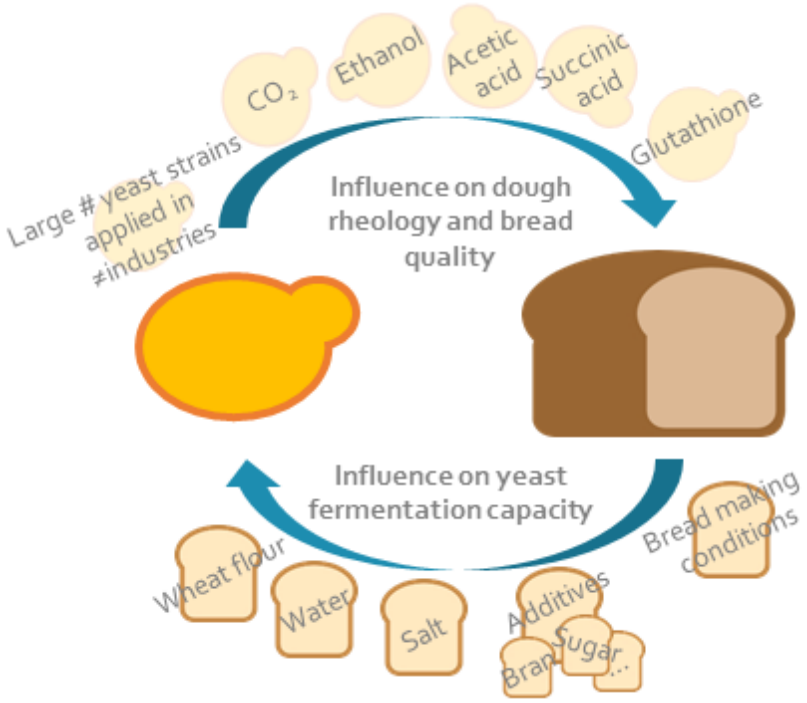

***Figure 1.1**: Reciprocal interaction between yeast and dough/bread ingredients. On the one hand, yeast impacts dough rheology and bread quality at large due to the metabolites produced during bread making. On the other hand, the fermentation capacity of yeast is notably influenced by wheat flour constituents and the other ingredients of bread.*

Because of the considerable impact of yeast on bread quality, using different yeast species and strains will very likely change its characteristics. *S. bayanus* and *S. pastorianus*, together with several *Pichia* species and *Torulaspora delbrueckii* have already been used in a study focusing on the alteration of bread taste by the production of different aroma compounds (Aslankoohi et al., 2016). Because of its high tolerance to osmotic and freeze stress, *T. delbrueckii* has mainly been playing a central role in the search for suitable yeast strains for the production of sweet and frozen dough (De La Cruz Pech-Canul et al., 2019; Hernández-López et al., 2007). The lactose fermenting strain *Kluyveromyces marxianus* has already been shown to possess potential for bread making without negatively influencing the bread flavor (Caballero 1995). Certain strains have even demonstrated the capacity to modify the FODMAP content of bread through the production of inulinase (Struyf et al., 2018).

Although these (non-)*Saccharomyces* species proved their value, the *S. cerevisiae* species itself embraces a broad number of strains that are currently largely neglected in the context of bread making (Gallone et al., 2016; Peter et al., 2018a). Indeed, although they have the same ancestors,

domestication of yeast triggered the development of characteristics which enabled the different strains to control and excel in specific industrial fermentation processes, possibly resulting in the preference for specific fermentation media (Gallone et al., 2016; Sicard and Legras, 2011; Steensels et al., 2014b; Steensels and Verstrepen, 2014). In the past, bakery yeast strains have mostly been selected for their fast fermentation capacity, disregarding the impact they have on other dough and bread characteristics (Giannone et al., 2010). Hence, this opens the door for improvement of the yeast typically used for bread making, especially when it comes to flavor and aroma of the final bread (Randez-Gil et al., 2013, 1999).

The relationship between yeast and bread (dough), as presented in **Figure 1.1**, is the guideline throughout this literature review. A yeast-centered approach is chosen to discuss their interactions. On the one hand, a closer look is taken at yeast in interaction with dough and bread ingredients. Therefore, the diversity of *S. cerevisiae*, the bread making potential of non-*Saccharomyces* and non-conventional *S. cerevisiae* strains and possibilities to genetically alter yeast strains, targeting desirable characteristics are first discussed. Next, yeast growth and the yeast metabolism are covered, followed by yeast fermentation metabolites and the way these affect dough and bread quality parameters. On the other hand, the impact of bread ingredients on the fermentation behavior of yeast is reviewed.

1.2 Yeast diversity

1.2.1 History of use and evolution of *S. cerevisiae*

Consumption of fermented food products dates from thousands of years ago. Evidence shows that our prehistoric ancestors already consumed leavened bread and fermented beverages, such as beer, wine and mead (Legras et al., 2007; Maloney and Foy, 2003; Sicard and Legras, 2011). However, in that time the fermentation processes occurred spontaneously by microorganisms present in the natural environment of the raw materials or the place of production (Steensels and Verstrepen, 2014). Due to the spontaneous character, the fermentation process was rather unpredictable. It could not be controlled, often leading to products of low quality. Over the years, bakers and brewers made use of a process called *backslopping* (Gallone et al., 2016; Steensels and Verstrepen, 2014). A piece of fermented dough or a small volume of fermented beverage from previous preparations was used to inoculate the new, unfermented product, resulting in a faster and more controlled fermentation. Reusing microorganisms for certain fermentation

processes gradually resulted in adaptation towards specific environments created by mankind, finally leading to the isolation of these strains and the development of pure starter cultures.

Saccharomyces species, and *S. cerevisiae* in particular, feature characteristics such as a fast cell growth, an efficient alcoholic fermentation, the production of desired flavor and aroma compounds, etc. (Benítez et al., 1996; Piskur et al., 2006; Rezaei et al., 2015c). As these make them most desirable for industrial applications, these organisms are nowadays dominating bread and fermented beverage industries (Steensels and Verstrepen, 2014). Although yeast strains have been applied to specific industrial processes for centuries, it is thought that their use was based on historical grounds rather than on the optimal performance in the corresponding processes (Steensels et al., 2014b). *S. cerevisiae* can rapidly change from respiration to fermentation in the presence of high glucose concentrations, even when oxygen is present (De Deken, 1966). This phenomenon is called the Crabtree effect and might have developed during evolution, when yeast cells were present in environments with continuously changing availabilities of the needed nutrients. Because of the formation of CO_2, ethanol and organic acids, *S. cerevisiae* created an advantageous environment for itself in the competition with other microorganisms (Gallone et al., 2018; Van Den Brink et al., 2008). The application of specific yeast strains for defined industrial processes involved a certain degree of evolution, resulting in genetic and phenotypic domestication of *S. cerevisiae* strains (Gallone et al., 2018).

1.2.2 Exploring the potential of (non-)conventional yeasts for bread making

Some of the preferred characteristics of yeast for bread making purposes are high biomass yield upon yeast production, fast dough fermentation, ability to ferment maltose and sucrose, tolerance against osmotic stress due to high sugar and salt concentrations, ability to ferment in environments with low water activity and cryotolerance in frozen dough (Benítez et al., 1996; Randez-Gil et al., 1999; Rezaei et al., 2015c; Walker, 1998). Since there is a wide variety of yeast strains within the *S. cerevisiae* species (Gallone et al., 2016; Peter et al., 2018a), this offers a lot of (unexplored) potential. Likewise, dough fermentation was proven not to be restricted to *S. cerevisiae* only but also possible with species of the genera *Pichia*, *Kluyveromyces* and *Torulaspora*. Several comparative studies have already been performed with such *non-Saccharomyces* bakery yeasts, non-bakery *S. cerevisiae* strains as well as with closely related *S. bayanus*. Rezaei and coworkers (2015c) and Heitmann and coworkers (2015) both investigated the impact of non-bakery *S. cerevisiae* strains on dough and bread characteristics, concluding that – although belonging to the same species – the impact is very strain specific. Indeed, the

fermentation ability and metabolite profile in dough of the strains tested by the former research group, showed large differences. In the published score plots yeast strains displayed no clustering by their industrial application, meaning that the metabolite production was not linked to the fermentation process they were originally used for (Rezaei et al., 2015c). Heitmann and coworkers (2015) even showed that certain beer yeasts have a better bread making potential than bakery strains. In an attempt to give more complexity to the aroma profile of bread, Aslankoohi and coworkers (2016) examined the potential of non-conventional yeast strains. They concluded that *T. delbrueckii* and *S. bayanus*, originating from kaoliang wine and champagne, respectively, were adequate candidates for industrial bread making.

T. delbrueckii was also a key organism in the search for strains with a higher freezing and/or osmotic tolerance for frozen and sweet doughs (Hernández-López et al., 2007; Hernandez-Lopez et al., 2003). Some *T. delbrueckii* strains have demonstrated to be both freeze and osmotolerant in combination with high biomass yield, making them very promising for industrial applications. Nevertheless, although this yeast species is commercialized in Japan, it is not regularly used for industrial bread making because of several drawbacks. First, in contrast to the di- or polyploid nature of industrial *S. cerevisiae* strains, *T. delbrueckii* is a haploid yeast resulting in a smaller cell size which causes clogging of the filters during industrial dehydration processes (Hernández-López et al., 2007; Sasaki and Ohshima, 1987). Moreover, *T. delbrueckii* strains show variation in their ability to ferment and assimilate carbon components such as maltose and galactose and good knowledge about their physiology and molecular biology seems to be lacking (Pacheco et al., 2012).

Another non-conventional but researched yeast for traditional or sourdough bread making is *Kluyveromyces marxianus*, phylogenetically related to *S. cerevisiae* (Caballero et al., 1995; Plessas et al., 2008). As this yeast is considered to be safe and food-grade (Generally Regarded As Safe (GRAS) in the United States and Qualified Presumption of Safety (QPS) in the European Union), it has good potential for industrial fermentation applications (Lane and Morrissey, 2010). Several strains of *K. marxianus* display similar dough fermenting capacity as the traditional bakery yeast *S. cerevisiae*. Moreover, although few other aroma compounds were analyzed, they did not alter the sensory perception of bread crumb (Caballero et al., 1995; Struyf et al., 2018). In addition, as already mentioned, the use of certain well-defined *K. marxianus* strains leads to the production of whole meal bread low in FODMAPs (Struyf et al., 2018). The ability of *K. marxianus* strains to

degrade these components, should enable people suffering from IBS to better tolerate yeasted bakery products (Struyf et al., 2018).

1.2.3 Creating diversity by genetic modification of yeast

Next to exploring natural diversity, genetic modification of existing yeast strains can also be a strategy to improve dough and bread quality characteristics, albeit rather for research purposes. The most common modifications focus on (i) altering degradation and consumption of carbohydrates and (ii) increasing osmotolerance and freeze tolerance for application in sweet and/or frozen dough (Dequin, 2001; Sasaki and Ohshima, 1987; Struyf et al., 2017b).

During dough fermentation *S. cerevisiae* prefers to consume glucose and then fructose, which is mainly coming from hydrolysis of fructan present in wheat flour through the action yeast invertase (Carlson, 1987; Struyf et al., 2017a; Verspreet et al., 2013; Verstrepen et al., 2004). After these hexoses are depleted, consumption of maltose becomes the main driving force of dough fermentation. In wheat flour dough this occurs after approximately one hour. The switch of the yeast metabolism from consumption of preferred sugars (glucose, fructose) to consumption of maltose leads to a decrease in CO_2 production rate, called the 'maltose lag' (Hazell and Attfield, 1999; Jenson, 1998). To avoid this maltose lag, bakery yeasts are often modified to enhance their maltose metabolism. Modification of *MAL* genes, their regulators or regulators of the glucose repression pathway (Section 1.3) are the main focus points of such improvement strategies (Lin et al., 2015, 2014; Randez-Gil and Sanz, 1994; Sun et al., 2012; Zhang et al., 2015). Likewise, deletion of the *SUC2* gene, coding for invertase, disables yeast cells to degrade fructans. This benefits the nutritional value of bread, as these carbohydrates are dietary fibers (Roberfroid et al., 2010; Verspreet et al., 2013).

Most of the strategies to improve osmo- and freeze tolerance coincide and are based on the accumulation of stress protectants such as glycerol, proline and trehalose (Shima and Takagi, 2009; Struyf et al., 2017b). Glycerol is the main factor for osmoregulation in *S. cerevisiae*. Together with proline and trehalose, it makes the yeast cell tolerant to osmotic stress caused by high sugar/salt concentrations and to freeze-thaw stress (Nevoigt and Stahl, 1997; Shima and Takagi, 2009; Takagi et al., 1997). Freeze-thaw tolerance can also be enhanced by antifreeze peptides and overexpression of functional aquaporins, both playing a role in intracellular ice crystal formation (Panadero et al., 2005; Shima and Takagi, 2009; Struyf et al., 2017b).

1.2.4 Breeding to create new yeast strains

Next to genetically modifying yeast strains to obtain the preferred characteristics, breeding of yeast strains can also result in hybrid strains showing the desired phenotype. Parental strains are carefully chosen, each possessing an interesting phenotype. Mating these two parents can result in segregants with strong qualities of both parental cells. Examples of such breeding processes for the bakery industry are the rare mating of two *S. cerevisiae* strains resulting in an intraspecific hybrid with higher leavening capacity and the intergeneric hybridization of *S. cerevisiae (var. diastaticus)* and *Z. rouxii* resulting in osmotolerant strains (Steensels et al., 2014b).

1.3 Pregrowth conditions and yeast cell growth[1]

Besides the phylogenetic diversity, the pregrowth conditions are also a determining factor for the performance of yeast during dough fermentation. Many different growth media are available and suitable for yeast growth. Essentially, they contain glucose or sucrose, ammonium salts as a nitrogen source, vitamins and minerals (Bergman, 2001; Dymond, 2013; Gélinas, 2012; Zonneveld, 1986). In laboratory experiments, a rich medium composed of yeast extract, bacterial peptone and glucose/dextrose (YPD) is often used (Bergman, 2001). On industrial scale, beet and cane molasses are mostly used for yeast production (Gélinas, 2012; Randez-Gil et al., 2013, 1999). Because molasses not always provide the desirable amount of nitrogen for yeast growth, free amino acids, small peptides, ammonia or ammonium salts are added (Albers et al., 1996; Reed, 1991).

When glucose is sufficiently available in the growth medium – albeit *as such* or after hydrolysis of sucrose into glucose and fructose by yeast invertase –, yeast switches automatically to the fermentative metabolism even in the presence of oxygen, an effect known as the Crabtree effect (De Deken, 1966; Otterstedt et al., 2004; Randez-Gil et al., 2013). A undesirable consequence of the Crabtree effect during growth, is a reduced biomass production (Otterstedt et al., 2004; Van Hoek et al., 1998; Verstrepen et al., 2004). This can be avoided by growing yeast in an aerated fed-batch reactor, in which glucose is supplied well controlled and in low concentrations (Fiaux et al., 2003; Otterstedt et al., 2004; Randez-Gil et al., 2013; Verstrepen et al., 2004). The glucose repression mechanism leads to a lag phase in CO_2 production at the moment that yeasts have to

[1] This paragraph is based on the section *Modification of yeast pregrowth conditions* from the following reference: Struyf, N., Van der Maelen, E., Hemdane, S., Verspreet, J., Verstrepen, K. J., & Courtin, C. M. (2017). Bread Dough and Baker's Yeast: An Uplifting Synergy. *Comprehensive Reviews in Food Science and Food Safety*, *16*(5), 850–867.

switch their metabolism to the consumption of fermentable sugars such as maltose, resulting in longer fermentation times (Higgins et al., 1999; Randez-Gil et al., 2013; Verstrepen et al., 2004).

In liquid medium, the growth curve of yeast can be divided into five phases (**Figure 1.2**). First, yeast cells have to adapt to the environment during a lag phase. After adaptation to the new conditions, they start to grow exponentially on their preferred carbon sources, mainly glucose and fructose. As already mentioned, due to high glucose or fructose concentrations present in the growth medium, yeast cells prefer the fermentative metabolism for biomass production above the energetically more efficient respiration (De Deken, 1966; Fiaux et al., 2003; Smets et al., 2010). During this exponential growth phase, yeast cells are less stress resistant (Smets et al., 2010; Verstrepen et al., 2004; Werner-washburne et al., 1993). Although the accumulation of ethanol during the fermentative metabolism could be detrimental for microbial growth and hence lead to a competitive advantage (Smets et al., 2010; You et al., 2003), it serves as a carbon source when glucose is depleted. As a consequence, *S. cerevisiae* switches from the fermentation of glucose to the aerobic respiration of ethanol. This is called the *diauxic shift*, the third growth phase of yeast (Alvarez-Vasquez et al., 2007; Galdieri et al., 2010; Smets et al., 2010). This shift includes major genetic changes, after which growth continues at a slower pace during the post-diauxic growth phase (Geistlinger et al., 2013; Werner-washburne et al., 1993). Finally, when also ethanol is depleted and no carbon sources are available anymore, yeast cells enter the stationary phase, a resting state in which there is no growth anymore but cells are still viable. Starvation for other nutrients, such as nitrogen, phosphorus or sulfur, could also bring them into the stationary phase (Galdieri et al., 2010; Herman, 2002; Werner-washburne et al., 1993). In this state, they are able to survive without nutrients. Adding the nutrients they were starved for (carbohydrates, nitrogen, phosphorus or sulfur), enables stationary-phased yeast cells to grow again (Werner-washburne et al., 1993).

The physiological, biochemical and morphological characteristics of yeast cells differ between the different growth stages (Galdieri et al., 2010; Herman, 2002; Werner-washburne et al., 1993). Rezaei *et al*. (2014) showed that the physiological state of yeast at the time of harvesting is important for its performance during dough fermentation. The highest dough volume was reached when cells were harvested during the diauxic shift or the post-diauxic growth phase. Indeed, yeast cells harvested at these points in the growth phase were shown to have a higher fermentation rate and production of ethanol and organic acids, probably affecting dough extensibility (Rezaei et al., 2014).

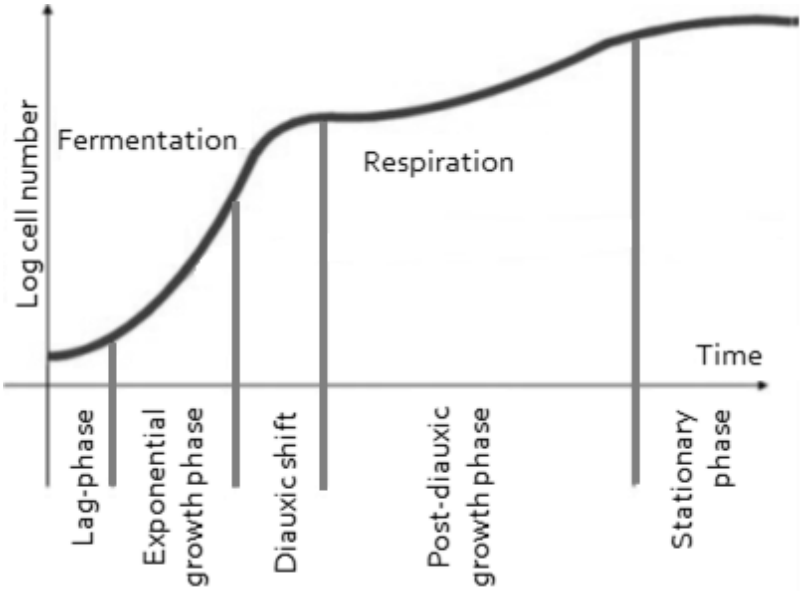

Figure 1.2: *Growth curve of Saccharomyces cerevisiae. The graph represents the log cell number in function of time during the growth process of yeast. Five important growth phases can be distinguished: (i) lag-phase, (ii) exponential growth, (iii) diauxic shift, (iv) post-diauxic growth and (v) the stationary phase (adapted from Smets et al., 2010).*

1.4 Metabolic pathways of yeast during dough fermentation

The conversion of sugars to CO_2 and ethanol is the main biochemical event during dough fermentation. However, besides these two metabolites, other fermentation metabolites are produced such as glycerol and several organic acids. All metabolites are intermediates or products of the glycolysis, alcoholic fermentation, the tricarboxylic acid (TCA) cycle or the glyoxylate shunt (**Figure 1.3**) (Jayaram et al., 2013a; Rezaei et al., 2015a). The main pathway is the glycolysis, catabolizing glucose into pyruvate with the release of NADH and two molecules ATP. A crucial intermediate of the glycolysis is dihydroxyacetone phosphate because it can subsequently be reduced and dephosphorylated to form glycerol, a relevant molecule during dough fermentation (Aslankoohi et al., 2015; Dickinson and Kruckeberg, 2006). Glycerol functions as an osmolyte, protecting the yeast cell against osmotic stress in a dough environment, and is known to have a beneficial influence on dough fermentation and the gas retention capacity of dough (Aslankoohi et al., 2015).

Pyruvate fulfills a central role in the metabolism of yeast. On the one hand, pyruvate enters the alcoholic fermentation. It is catabolized into CO_2 and acetaldehyde, which is reduced to ethanol by alcohol dehydrogenase. Acetaldehyde can also be oxidized to acetic acid, one of the by-products of fermentation and also a precursor for cytosolic acetyl-coenzyme A, used for lipid

and amino acid biosynthesis or transported to mitochondria (Dickinson and Kruckeberg, 2006). A part of the produced acetic acid is excreted by the yeast cells and can be detected in the fermentation medium (Jayaram et al., 2013a; Rezaei et al., 2015c). On the other hand, pyruvate can directly be transported from the cytosol to the mitochondria, in which case it is converted to acetyl-coenzyme A, entering the TCA cycle (Dickinson and Kruckeberg, 2006; Kruckeberg and Dickinson, 2004; Pronk et al., 1994).

Although yeast prefers fermentation in the presence of high glucose levels – regardless of the presence of oxygen –, respiration via the TCA cycle is not completely shut down during dough fermentation. This is important to note because succinic acid, a product of the this cycle, has an important impact on dough rheology (more information, Section 1.5.3.2). Yeast has three pathways to produce succinic acid: (i) the oxidative (clockwise) and (ii) reductive (counterclockwise) branch of the TCA cycle within mitochondria and (iii) the glyoxylate shunt in the cytosol (Camarasa et al., 2003; Rezaei et al., 2015a). With the help of the enzyme isocitrate lyase, the latter pathway converts isocitrate – an intermediate from the TCA cycle that migrates to the cytosol – into succinate and glyoxylate, which is further metabolized to malate together with acetyl-coenzyme A by malate synthase (Kruckeberg and Dickinson, 2004; Kunze et al., 2006). The former two pathways lead to the production of succinic acid by resp. oxidizing α-ketoglutarate and reducing fumarate (Rezaei et al., 2015a). The functionality of these three pathways during fermentation was shown by Rezaei *et al.* (2015a) by using knockout mutants of *S. cerevisiae*. The absence in these mutants of enzymes crucial for the production of succinic acid resulted in significantly lower levels of succinic acid compared to the wild-type lab strain.

Although some yeast strains in certain industrial fermentation processes, e.g. sake production, are known to use the three ways to produce succinic acid (Arikawa et al., 1999), Rezaei and coworkers (2015a) showed that during bread making, succinic acid is only produced in the oxidative pathway of the TCA cycle and the glyoxylate shunt.

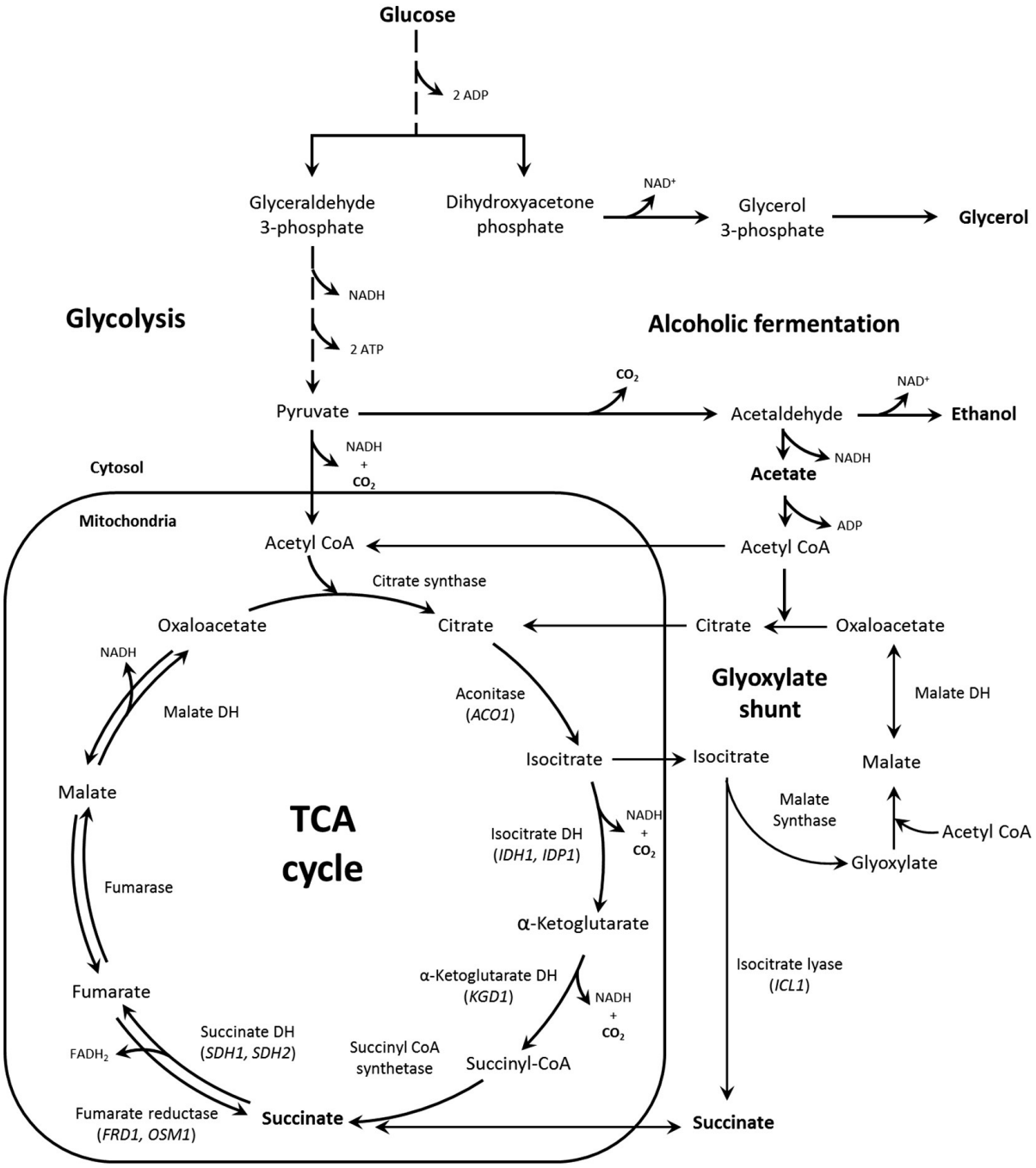

***Figure 1.3**: Schematic overview of the metabolic pathways of yeast leading to the most important metabolites of fermentation. Glycolysis breaks down glucose into building blocks for alcoholic fermentation, with production of CO_2 and ethanol on the one hand, and for the TCA cycle, producing organic acids on the other hand. Intermediates of the alcoholic fermentation at their turn are important for the glyoxylate shunt* (Rezaei et al., 2015a).

1.5 Impact of yeast metabolites on dough and bread properties

As illustrated in the section above, the fermentation metabolism of yeast during bread making results in more metabolites than only CO_2 and ethanol. Before describing the impact of the most

important fermentation metabolites on dough and bread characteristics, the different phases of the bread making process are shortly described.

1.5.1 The bread making process

Different bread making procedures exist, such as (i) the sponge-and-dough process, (ii) the straight-dough process and (iii) the Chorleywood process. In this work only the straight-dough process is discussed and used, as this is the most commonly used bread making process in Western Europe. In general, bread is made up of four essential ingredients: flour, water, yeast and salt. In the straight-dough bread making process, the ingredients are all mixed in one step. During this mixing phase, flour particles are hydrated and a visco-elastic dough network is formed, in which gas cells are incorporated (Delcour and Hoseney, 2010). The wheat flour components responsible for the formation of this visco-elastic network are gluten proteins. Gluten proteins consist of glutenins (large polymers built up of glutenin subunits, with each subunit having a molecular weight of 80,000 and higher) and gliadins (monomers with a molecular weight between 30,000 and 80,000) (Goesaert et al., 2005). Upon hydration of flour particles and through energy input during mixing, glutenins form inter- and intramolecular disulfide bonds resulting in a continuous elastic network (Delcour and Hoseney, 2010). The gliadins are discontinuously spread within this network, imparting viscosity to the gluten network. In dough, starch functions only as an inert filler within the gluten network, while it becomes more importance during the baking phase (Goesaert et al., 2005).

When all ingredients are homogeneously mixed and optimal hydration has occurred, the fermentation phase can start. Yeast cells consume fermentable sugars present in dough and convert them into ethanol, CO_2 and other metabolites. At first, CO_2 is dissolved in the aqueous phase until saturation. After saturation, the CO_2 that is additionally produced migrates to the air cells that were incorporated in the dough during mixing, leading to expansion of these cells. Due to the dough matrix composition and the visco-elastic properties of the gluten network, dough is able to hold the expanding gas cells. Punching during the fermentation phase is necessary to redistribute the ingredients and to subdivide the existing gas cells into smaller and more homogeneously spread cells (Delcour and Hoseney, 2010; Dobraszczyk and Morgenstern, 2003).

After molding and additional fermentation, the leavened dough is ready to be baked. Once in the oven, dough undergoes a rapid expansion during the first 10 minutes (Delcour and Hoseney, 2010). This so-called oven spring is caused by (i) elevated yeast activity due to the increasing dough temperature, until approximately 55°C is reached and yeast cells are inactivated; (ii)

evaporation of water and ethanol; (iii) decreased solubility of CO_2 in the aqueous phase and consequent migration to the gas cells and (iv) expansion of gasses at higher temperatures (Delcour and Hoseney, 2010). Starch embedded in the protein network gelatinizes at temperatures around 65°C, depending on the water content of the medium. During baking and as a result of starch gelatinization, starch granules swell due to the uptake of water. As the water availability in dough is limited, water is withdrawn from the gluten proteins, stiffening the gluten network and disabling further expansion (Delcour and Hoseney, 2010). As a result, the dough structure is set and the formerly discontinuously spread gas cells open up and form a continuous air phase within the dough (**Figure 1.4**) (Delcour and Hoseney, 2010; Gan et al., 1995). As water is present in the crumb at all times, the dough temperature here does not exceed 100°C. Only the dough surface reaches higher temperatures with crust formation as a consequence. Maillard reactions between amino acids and reducing sugars are responsible for the crust color (Delcour and Hoseney, 2010).

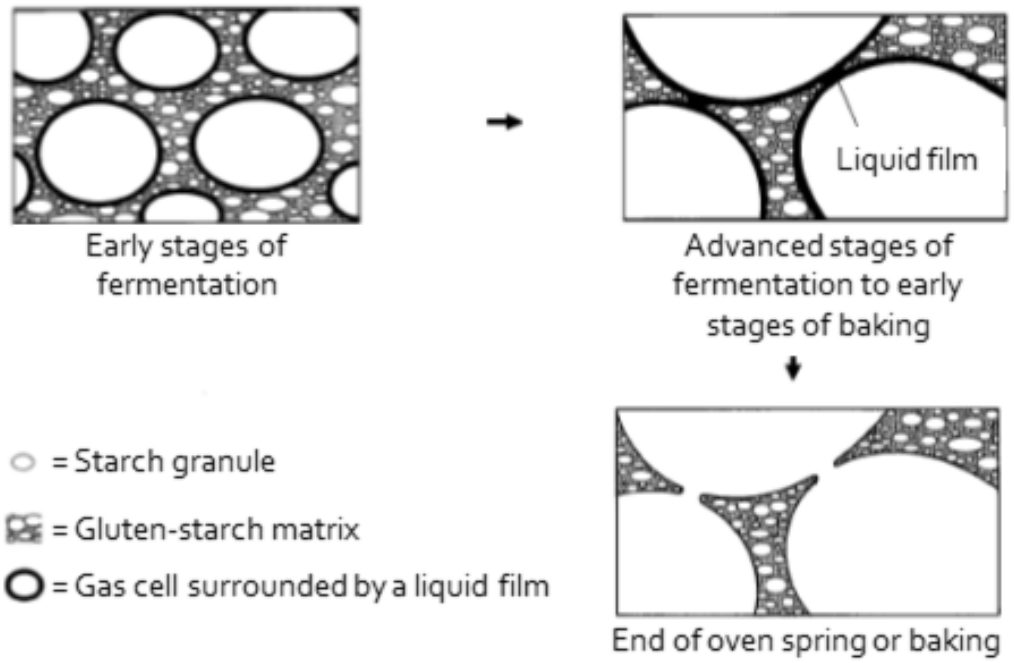

***Figure 1.4**: Evolution of gas cells throughout the bread making process. Gas cells first form a discontinuous phase within the continuous gluten matrix during fermentation. After expansion of the cells and during baking, the liquid lamellae disappear and a continuous gas phase is formed (adapted from Gan et al., 1995).*

1.5.2 Primary fermentation metabolites

1.5.2.1 CO_2

The most important function of CO_2 in bread production is without doubt the leavening of dough. As mentioned in Section 1.5.1, prior to migration to the air cells, CO_2 is dissolved in the aqueous phase until saturation. Because yeast is incapable of creating new gas cells, incorporation of sufficient gas cell nuclei into dough during mixing is crucial (Elmehdi et al., 2007; Gan et al., 1995; Verheyen et al., 2014b). After saturation of the aqueous phase, additionally produced CO_2 diffuses to the incorporated air cells (Delcour and Hoseney, 2010). As a consequence, dough density throughout the fermentation process decreases compared to the dough immediately after mixing (Dobraszczyk and Morgenstern, 2003; Elmehdi et al., 2007).

To avoid gas loss from the dough, a first important parameter to consider is the size distribution of the incorporated gas cells. The size distribution cannot be too large. Typically, the diameter varies between 20 to 200 µm. As a consequence of this rather broad range, diffusion of CO_2 from small to large cells – known as Ostwald ripening – can occur (Dobraszczyk and Morgenstern, 2003; Dobraszczyk and Roberts, 1994; Van Vliet et al., 1992). When large gas cells grow at the expense of small ones, a coarse final bread crumb is obtained.

Next to the size distribution, stability of the gas cells is crucial to prevent premature failure of the cells along the bread making process and produce bread with a light and even crumb (Dobraszczyk et al., 2003; Gan et al., 1995). Right after mixing, gas cells are discontinuously embedded in and stabilized by the gluten-starch matrix (**Figure 1.4**) (Gan et al., 1995; Sroan et al., 2009). As a consequence of the continuous CO_2 production by yeast during fermentation and gas evaporation during the baking phase, gas cells expand. After considerable expansion, at the end of the proofing period or at the beginning of baking, the gluten-starch matrix fails to continuously surround the gas cells. Consequently, at certain points, gas cells are only separated from each other by a liquid film. The rheological characteristics of this surrounding film determine the stability of the gas cells and the degree of gas cell expansion in the late fermentation and early baking stage (Dobraszczyk and Morgenstern, 2003; Gan et al., 1995; Ktenioudaki et al., 2009). Gas cell expansion continues during baking until the liquid films break, resulting in the transition of a foam-like dough to a sponge-like bread (Gan et al., 1995).

According to Verheyen and coworkers (2014b) the retention of CO_2 in the dough matrix is not affected by the gas production rate. Rezaei and coworkers (2014), however, observed that the onset of CO_2 loss during dough fermentation is correlated with yeast's fermentation rate, but not

with the total amount of CO_2. They suggest that the production of other metabolites (ethanol and organic acids) might play a role in the gas holding capacity of dough (see further).

1.5.2.2 Ethanol

The total amount of ethanol produced by yeast during dough fermentation depends on several parameters such as yeast dosage, sugar content of the dough and fermentation time. Under the conditions they used, Jayaram and coworkers (Jayaram et al., 2013a) found that yeast produced up to 60 mmol ethanol per 100 g of wheat flour in straight-dough formulated bread. Although ethanol is a primary metabolite during dough fermentation, it is only recently that studies have been performed on the impact of ethanol in these concentrations on dough rheology (Jayaram et al., 2014; Meerts et al., 2018b; Rezaei et al., 2016). Supplementing unyeasted dough with a concentration of 60 mmol ethanol per 100 g of wheat flour made it less extensible. This behavior was accompanied by a higher force required to extend the dough but a lower energy required for breakage, and more stiffness (Jayaram et al., 2014; Meerts et al., 2018b).

Jayaram and coworkers (2014) showed that at the low ethanol concentrations encountered in dough aqueous ethanol serves as a good solvent for some gliadins. ω-gliadins, which are more hydrophilic compared to the α- and γ-gliadins (Jayaram et al., 2014), were selectively dissolved. Elimination of these gluten proteins from the gluten network was suggested to result in a less viscous and less well aggregated network (Delcour and Hoseney, 2010; Meerts et al., 2018b; Robertson et al., 1999). These findings were in line with the suggested molecular mechanisms behind the impact of much higher ethanol concentrations (more than 30% versus less than 3% in dough) on gluten proteins, as extensively examined by Robertson and coworkers (1998, 1999, 2002).

Ethanol plays not only a role during fermentation, it is also of importance during baking. As a result of its evaporation due to elevated temperatures during baking, ethanol contributes to the oven spring and consequently to the final bread texture (Delcour and Hoseney, 2010).

A small but important side note: although ethanol is one of the most important fermentation metabolites of yeast, high concentrations do not only have an impact on dough rheology but can also be detrimental to the yeast itself (Ding et al., 2009). Ethanol is for example known to increase plasma membrane fluidity in yeast cells to destructive levels (Ding et al., 2009; Ma and Liu, 2010). The presence of the unsaturated fatty acids palmitoleic acid and oleic acid and of ergosterol in the membrane and of some amino acids, e.g. proline, in the medium or

biosynthesized within the yeast cell, are factors determining the tolerance of the yeast cell to ethanol (Ding et al., 2009; Ma and Liu, 2010; Shima and Takagi, 2009; You et al., 2003).

1.5.3 Secondary fermentation metabolites

As was already clear from Section 1.4 and **Figure 1.3**, CO_2 and ethanol are not the only metabolites produced by yeast during fermentation. The following sections provide an overview on the impact of glycerol, organic acids, aroma compounds and other metabolites, such as glutathione, on dough and bread characteristics and on final bread quality.

1.5.3.1 Glycerol

As shown in **Figure 1.3**, glycerol is produced out of an intermediate of the glycolysis. When the yeast cell experiences osmotic stress as a result of the hyperosmotic dough environment, the high osmolarity glycerol (HOG) pathway is activated and glycerol is produced (Aslankoohi et al., 2015; Hohmann, 2002; Rodríguez-Peña et al., 2010). The osmotic shock that yeast cells encounter when brought into dough hence results in the production of glycerol at the expense of CO_2 and ethanol (**Figure 1.3**). Glycerol functions as an osmoprotectant, controlling the intracellular water activity as a response to the high salt and/or sugar – e.g. sweet dough – concentrations in the dough (Nevoigt and Stahl, 1997; Shima and Takagi, 2009). Similarly, yeast cells in frozen dough systems are also characterized by elevated intracellular glycerol concentrations. Freezing enhances the formation of ice crystals, which are harmful for yeast cells. Subsequent thawing could cause high turgor pressure inside the intact cells or cell leakage in ice crystal damaged cells. Owing to the presence of glycerol, the cells are guarded against dehydration and other osmotic stress related damage (Izawa et al., 2004b, 2004a; Nevoigt and Stahl, 1997; Shima and Takagi, 2009).

Glycerol, together with ethanol and non-dissociated forms of organic acids, is able to diffuse freely through the yeast's cell membrane and has a transmembrane efflux through the channel protein Fps1 (Hohmann, 2002; Kruckeberg and Dickinson, 2004; Oliveira et al., 2003). This enables glycerol to be released into the dough where it can influence dough and bread characteristics. To fully understand the impact of glycerol on dough properties, Aslankoohi and coworkers (2015) added different concentration of glycerol – ranging from 1.6 to 31.2 mmol/100 g of flour – to yeastless dough and performed uniaxial extensibility tests. An increased extensibility and a decreased maximum force for the extension, resulting in a softer dough, were pointed out as the effects of glycerol on dough rheology (Aslankoohi et al., 2015). This was confirmed by Meerts and coworkers (Meerts et al., 2018b). Glycerol was also found to lower the firmness of

bread, and hence giving it a longer shelf life. This was ascribed to plasticization of the macromolecular matrix (Barrett et al., 2000).

1.5.3.2 Organic acids

The most important organic acids produced by yeast during dough fermentation are acetic acid and succinic acid (Jayaram et al., 2013a; Rezaei et al., 2014). As stated in Section 1.4, respiration through the TCA cycle is not completely shut down during fermentation, with residual production of succinic acid as a result. This is of great importance in the production of fermented beverages like sake and wine. Indeed, succinic acid functions as a taste component in sake and is known to be an important determinant in the organoleptic properties and acidity of wine (Arikawa et al., 1999; Camarasa et al., 2003). As acetic acid is one of the main detected acids amongst the aroma compounds of bread crumb (Aslankoohi et al., 2016; Pico et al., 2015), it might also be of relevance for bread aroma and flavor.

Organic acids impact dough rheology mainly by decreasing dough pH. This causes proteins to be more positively charged, resulting in electrostatic repulsion between gluten proteins and thus reducing dough extensibility and gluten network strength (Jayaram et al., 2013b; Meerts et al., 2018b). Important to note is the combined effect of organic acids and salt on dough properties. As salt ions have the ability to shield charges, it is presumed that the presence of salt may minimize the detrimental impact of an acidic pH on dough rheology (Galal et al., 1978; Meerts et al., 2018b).

1.5.4 Other metabolites

Next to the previously described fermentation metabolites, yeast cells also contain components with (intra)cellular functions which can be released into the dough environment after cell lysis. Main examples shortly discussed below are glutathione, hydrogen peroxide (H_2O_2) and fatty acids of the cell membrane.

1.5.4.1 Glutathione

Glutathione or γ-L-glutamyl-cysteinyl-glycine is a tripeptide of amino acids glutamate, cysteine and glycine and is especially of importance inside the yeast cell. It is essential for yeast growth, maintains the cellular redox balance and functions as antioxidant (Bachhawat et al., 2009). As glutathione is not able to pass intact cell membranes, it remains intracellular until lysis occurs as a result of cell death. During the production process of commercial yeast, the cells are subjected to several stresses (thermal and oxidative), potentially resulting in cell death for part of the yeast

population (Verheyen et al., 2014a). Verheyen and coworkers (2014a) determined the glutathione content of commercial fresh and dry yeasts and reported concentrations up to 81 mg/g dry sample. Consequently, when using commercial yeast for bread making, certain amounts of glutathione are added and can affect dough rheology immediately from the beginning. This is in contrast to the other yeast metabolites, that are continuously produced during fermentation (Jayaram et al., 2013a; Verheyen et al., 2014a). Glutathione is a reducing agent that weakens the gluten network by interchanging thiol-disulfide groups with polymeric gluten, thereby increasing the extensibility of dough (Delcour and Hoseney, 2010; Dong and Hoseney, 1995; Meerts et al., 2018b).

1.5.4.2 Hydrogen peroxide

Reactive oxygen species (ROS) such as H_2O_2 are commonly present as by-products of the oxidative metabolism in aerobically growing organisms (Jamieson, 1998; Liao et al., 1998). H_2O_2 has long been thought to have the largest share in the impact of yeast on dough rheology (Jamieson, 1998; Takasaki et al., 2005). Even at low concentrations, it was supposed to strengthen dough and increase its elasticity (Liao et al., 1998). However, recent research of Rezaei and coworkers, (2015b) using a much more selective method for H_2O_2 quantification, showed that no H_2O_2 could be detected in a yeast containing fermentation broth or yeasted dough. Moreover, *S. cerevisiae* cells are known to possess enzymes, such as catalases and cytochrome c peroxidase, catalyzing degradation of H_2O_2 and other ROS (Izawa et al., 1996; Lushchak and Gospodaryov, 2005). Together with the recently obtained knowledge about the strong impact of primary and secondary yeast metabolites on dough rheology, this suggests that rheological changes of dough during fermentation cannot be attributed to the presence of H_2O_2 (Rezaei et al., 2015b).

1.5.5 Combined impact of all fermentation metabolites

In order to study the individual effects of the produced metabolites on dough rheology, researchers made use of unyeasted dough, to which metabolites were added in relevant concentrations. However, what matters in the end is the final rheological behavior of dough in the presence of all metabolites that are produced throughout the fermentation process. Rezaei and coworkers (Rezaei et al., 2016) and Meerts and coworkers (Meerts et al., 2018b) also investigated the impact of yeast metabolites on the rheology of fermented dough. In general, they both concluded that fermented dough was less extensible, had a lower spread and tore more easily when stretched (Meerts et al., 2018b; Rezaei et al., 2016). When comparing this

behavior with the effects induced by individual metabolites, most of these characteristics could be ascribed to the contribution of ethanol. It was concluded, though, that the final rheological behavior of fermented dough cannot simply be predicted based on the sum of the individual effects of the single metabolites (Jayaram et al., 2013b; Meerts et al., 2018b).

1.5.6 Aroma compounds

Crucial metabolites for the consumer's perception of bread are the aromas produced during yeast fermentation. They complement the aroma compounds in the bread crumb that are the products of lipid oxidations and Maillard reactions. Indeed, next to the above mentioned fermentation metabolites, yeast also produces volatile compounds such as higher alcohols, aldehydes, esters, organic acids and some ketones which can all contribute to the aroma of the final bread (Birch et al., 2014; Dickinson et al., 2003). Each volatile component is characterized by a specific threshold concentration and odor activity value, defined as the ratio of total concentration of the aroma compound in bread to the threshold concentration of that compound (Birch et al., 2014; Pico et al., 2015). An overview of the most important bread crumb aroma compounds, produced by yeast, and their corresponding odor is presented in **Table 1.1**.

Yeast cells produce aldehydes mainly via the Ehrlich pathway, starting from wheat flour amino acids (Derrick and Large, 1993; Hazelwood et al., 2008). Reduction of the aldehydes gives rise to the corresponding alcohols. The most important aroma-active aldehydes and alcohols formed by yeast activity are 3-methylbutanal and 3-methyl-1-butanol – derived from leucine – and phenylacetaldehyde and 2-phenylethanol – derived from phenylalanine (Birch et al., 2014; Pico et al., 2015). Another group of components perceptible in bread aroma are organic acids. Acetic acid and butanoic acid are products of the fatty acid synthase pathway in yeast. 3-methylbutanoic acid is produced via an alternative route, being the oxidation of aldehydes from the Ehrlich pathway (Berry and Watson, 1987; Birch et al., 2014; Pico et al., 2015). Another group of volatile components are the esters, interesting because of their agreeable odors (Birch et al., 2014). To form esters, acetyl-CoA derivatives of short to medium chain fatty acids enzymatically react with free alcohols. Equal to the formation of previous compounds, these reactions take place inside the yeast cell. In bread crumb, ethyl acetate, ethyl hexanoate, ethyl octanoate and isoamyl acetate are the most important esters (Aslankoohi et al., 2016; Birch et al., 2014; Pico et al., 2015). A last group of aroma compounds are the ketones. Of importance here are 2,3-butanedione (diacetyl) and 3-hydroxy-2-butanone (acetoin) (Aslankoohi et al., 2016; Pico et al., 2015). Different from aldehydes, alcohols, acids and esters, these two ketones are formed

outside the yeast cell. The building block is α-acetolactate, an intermediate for the production of amino acids valine and leucine that is leaked out of the yeast cell where it undergoes a non-enzymatic reaction to form diacetyl and acetoin (Berry and Watson, 1987; Birch et al., 2014).

As the yeast's fermentation metabolism is partly responsible for the production of aroma compounds, the choice of yeast strain for bread making also determines the final bread aroma (Birch et al., 2014). As already mentioned, Aslankoohi and coworkers (2016) analyzed the aroma production of several non-conventional yeast strains during bread making, pointing out the potential of *T. delbrueckii* and *S. bayanus* as worthy alternatives for *S. cerevisiae*. Steensels and coworkers (2014a) evaluated the aroma-active compounds of hundreds of different *S. cerevisiae*, *S. paradoxus* and *S. pastorianus* yeast strains.

Table 1.1*: Important aroma compounds produced by yeast during dough fermentation, with their corresponding odor. The information is based on following references: Aslankoohi et al.* (2016)*, Birch et al.* (2014, 2013b)*, Pico et al.* (2015) *and Burdock et al.* (2005)*.*

Chemical group	Aroma compound	Odor	Odor threshold in water (µg/kg)
Aldehydes	3-methylbutanal	Malty, roasty, fermented	0.2
	Phenylacetaldehyde	Honey-like, sweet	4
Alcohols	3-methyl-1-butanol	Alcoholic, malty, balsamic	250
	2-phenylethanol	Flowery, honey-like	1,100
Acids	Acetic acid	Sour, pungent, acid	32,300
	Butanoic acid	Rancid, sweaty	240
	3-methylbutanoic acid	Sweaty, cheesy, rancid	120
Esters	Ethyl acetate	Sweet, fruity, pineapple	6,200
	Ethyl hexanoate	Apple peel, fruity	1
	Ethyl octanoate	Sweet, soap, fresh, fruity	92
	Isoamyl acetate	Banana	43
Ketones	2,3-butanedione (diacetyl)	Buttery, caramel	6.5
	3-hydroxy-2-butanone (acetoin)	Buttery, creamy	10,000

The production of, for example, isoamyl acetate and ethyl acetate – two important aroma compounds – showed extreme differences between the different yeast strains. The high natural variation offered the opportunity to create new strains by breeding, resulting in yeasts which could produce a new set of aroma compounds (Steensels et al., 2014a). While the purpose of Steensels and co-workers was to create new strains for more flavorful beers, this knowledge is also of importance for other yeast-based fermentation products.

1.6 Influence of dough ingredients and fermentation conditions on yeast performance

In order to produce its primary and secondary metabolites, yeast needs to have access to the nutrients that are crucial for its fermentation metabolism. The complex dough matrix is normally adequate to meet yeast's nutritional needs. However, not all dough ingredients necessarily impact the fermentation capacity of yeast in a positive way. Furthermore, not only the presence or absence of nutrients are determinant for a proper functionality of yeast during bread making. Also fermentation conditions and the genetic background of yeast determine the fermentation capacity of yeast in dough. In the following sections, the impact of bread ingredients and fermentation conditions on yeast's functionality is reviewed.

1.6.1 Nutrients and nutrient availability

By definition, bread is minimally composed of four essential ingredients: flour, water, yeast and salt (Delcour and Hoseney, 2010). Each of these ingredients has its own specific function in dough, thereby affecting final dough and bread properties.

The interaction of flour and water is crucial for visco-elastic network formation (Section 1.5.1). The aqueous phase within the dough matrix allows yeast mobility and enzyme activity. Both are important for optimal dough leavening. Thanks to its mobility, yeast has access to the nutrients present in the dough aqueous phase. Normally, no supplementation of nutrients is needed, as wheat flour intrinsically contains sufficient nutrients to enable yeast fermentation in dough. However, they are not all instantly available. Flour- and yeast-associated enzymes play an important role in nutrient availability, especially when it comes to enzymatic degradation of carbohydrates to fermentable sugars (Struyf et al., 2016). The following sections provide an overview of the different components needed for fermentation.

1.6.1.1 Carbohydrates

The presence of fermentable sugars is of utmost importance for fermentation in dough. Yeast cells preferably consume glucose, in second instance fructose and in third instance maltose. In the presence of glucose, the uptake of other carbohydrates is repressed (Carlson, 1999). As the native amounts of mono- and disaccharides in wheat flour – ranging from 0.05% for glucose, fructose and maltose to 0.2% for sucrose – are too low to enable sufficient fermentation, fermentable sugars need to be generated by enzymatic degradation of larger carbohydrates endogenously present in flour (Sahlström et al., 2004; Struyf et al., 2016). In lean dough, when

no sugars are added, damaged starch and fructans from wheat flour are the preferred substrates (Struyf et al., 2017a).

Starch, present in amounts ranging from 70 to 75%, is the most abundant constituent of wheat flour. As native starch is resistant to digestion by amylases, it does not serve as a source of fermentable sugars (Goesaert et al., 2005; Tester et al., 2006). During milling of wheat kernels, however, 5 to 8% of the starch granules are damaged, making the starch more accessible for hydrolysis by amylases (Delcour and Hoseney, 2010; Goesaert et al., 2005; Van Der Borght et al., 2005). Eventually, subsequent actions of α- and β-amylases on damaged starch lead to the generation of maltose and small amounts of glucose. Struyf and coworkers (2016) showed that the maltose concentration in unyeasted dough increased from almost 0.05% dry matter (dm) in flour to 1.0% dm after mixing and further to 2.0% dm after three hours of resting or fermentation, illustrating its availability during lean dough fermentation. Maltose is taken up by the yeast cell by maltose transporters and intracellularly degraded to glucose by yeast maltase before entering the fermentation metabolism (Struyf et al., 2017b). Next to damaged starch, fructan should not be neglected as carbohydrate source for yeast fermentation, especially in whole meal bread (Struyf et al., 2017a; Verspreet et al., 2012). Wheat grain fructan levels range from 0.7% to 2.9% dm, of which the majority is located in the wheat bran fraction (Huynh et al., 2008; Verspreet et al., 2012). *S. cerevisiae* invertase, which is present in the yeast periplasm, extracellularly degrades fructans and sucrose to glucose and fructose (Nilsson et al., 1987). The importance of fructan and damaged starch and their degradation for yeast-mediated dough fermentation was shown by Struyf and coworkers (Struyf et al., 2017a). They showed that 44% of the sugars consumed in wheat flour dough during two hours of fermentation were the product of invertase activity. This amount even increased up to 70% in whole meal bread dough. The rest of the consumed sugars were released by the action of amylases on damaged starch (Struyf et al., 2017a).

1.6.1.2 Nitrogen sources, minerals, vitamins

As yeast cells do not have the ability to fix molecular nitrogen, the presence of nitrogen rich nutrients is required for proper yeast growth and functionality (Walker, 1998). Organic as well as inorganic nitrogen sources are therefore often supplied to growth and fermentation media. The same is true for minerals and vitamins. In the following paragraphs, the general impact of nitrogen sources, minerals and vitamins on yeast's metabolism during fermentation is described.

The presence of nitrogen in fermentation media is important for two reasons: (i) it affects yeast's biomass production during fermentation and (ii) it has an impact on the fermentation rate. In

alcoholic beverage fermentations, the lack of nitrogen results in stuck or sluggish fermentations (Mas et al., 2014). *S. cerevisiae* prefers nitrogen rich sources such as glutamine, asparagine and ammonia. Presence of these components represses the uptake of other *poor* nitrogen sources, a phenomenon known as nitrogen catabolite repression (Hofman-Bang, 1999) and similar to what is observed with glucose. For the incorporation of nitrogen into the metabolism of yeast, ammonium salts can serve as an inorganic source, often supplied to the growth media of yeast as essential ingredient (Section 1.3) (Walker, 1998). Yeast leavened bread formulations have also been supplied with improvers, including ammonium salts, resulting in increased yeast activity. This has a positive effect on bread volume (Stigler, 1973). Like with ammonia, uptake of glutamine and asparagine supports maximal growth rate (Dickinson, 2004; Mas et al., 2014). The presence of free amino acids is highly dependent on the activity of the peptidases endogenous to wheat flour during dough mixing and fermentation (Thiele et al., 2002). Collar and coworkers (1991) noticed a decrease in the content of free amino acids in the first phase of dough fermentation, due to low peptidase activity together with uptake of amino acids by yeast. Next to their importance for yeast's cell growth and fermentation, amino acids also function as precursors for aroma components and are therefore of relevance for the final bread flavor (Section 1.5.6).

There are some mineral requirements for yeast growth, such as potassium and magnesium. Their importance for the fermentation performance of yeast cannot be neglected (Walker and Stewart, 2016). In alcoholic beverages, the metal ions Mg^{2+}, K^{+}, Ca^{2+} and Zn^{2+} have been shown to influence yeast during the fermentation process (Walker et al., 2006). Concerning vitamins, yeast is supposed to be self-sufficient, synthesizing them starting from simple precursors (Maloney and Foy, 2003). Biotin is considered as essential for yeast as the organism is not able to synthesize it, although it is involved in several enzymatic reactions and the nitrogen metabolism of yeast (Bohlscheid et al., 2006; Knowles, 1989; Lindegren, 1945; Oura and Suomalainen, 1978; Walker, 1998). Biotin might be limiting in molasses and is therefore often supplied when growing bakery yeast (Maloney and Foy, 2003; Walker and Stewart, 2016). The biotin content of wheat flour should be sufficient to meet the yeast's needs for multiplication and dough fermentation (Walker, 1998). Thiamine (vitamin B1) can be synthesized by *S. cerevisiae* itself, but it is seen that addition to fermentation medium increases the fermentation rate and yeast resistance against several stresses (Walker, 1998; Wolak et al., 2014).

1.6.2 Other wheat peptides and proteins

Wheat flour contains in general 10-12% proteins, of which 80% are gluten proteins (gliadins and glutenins). The remaining 20% are the non-gluten proteins (albumins and globulins). The former group functions as storage proteins in cereals and is of main importance for the formation of the visco-elastic network during mixing. The latter group of proteins has physiological functions such as enzyme activity (Delcour and Hoseney, 2010; Goesaert et al., 2005). The previous section elucidated the importance of amino acids for yeast metabolism. As already stated, the amount of amino acids available for yeast metabolism depends on the activity of wheat endogenous proteinases (Thiele et al., 2002). These enzymes prefer acidic environments and thus show a rather low activity at the beginning of dough fermentation when pH is still around 6 (Collar et al., 1991; Jayaram et al., 2013a; Thiele et al., 2002). The decrease of pH along the fermentation process consequently involves an increased release of amino acids (Thiele et al., 2002). Proteinases are not the only important wheat flour enzymes affecting the metabolism of yeast. Another example of enzymes crucial for a proper functioning of yeast cells are the earlier discussed amylases as they have a clear influence on the release of glucose and maltose, two of the most important nutrients of yeast (Section 1.6.1.1).

Next to proteins that have beneficial effects on yeast and yeast functionality, cereals are also known to contain pathogenesis-related (PR) proteins, first defined by Antoniw and coworkers (Antoniw et al., 1980b) as proteins "coded for by the host plant but induced only in pathological or related situations" (Antoniw et al., 1980b; Ebrahim et al., 2011; Muthukrishnan et al., 2001). These proteins are part of the defense system of plants and are induced as a response to pathogen attacks (Ebrahim et al., 2011). They are now classified into 17 families of PR proteins, according to their amino acid sequence, serological properties and enzymatic or biological activity (Muthukrishnan et al., 2001; Sudisha et al., 2012; van Loon et al., 1994). An overview of PR protein families is given in **Table 1.2**.

Antoniw et al (1980a) were the first to observe PRs in tobacco (*Nicotiana tabacum*) plants after infection with tobacco mosaic virus. First, the classification was based on the decreasing electrophoretic mobility of the proteins, resulting in groups PR-1 to PR-5 containing proteins

***Table 1.2**: Classification of PR proteins*

Family	Type member	Properties	Reference
PR-1	Tobacco PR-1a	Antifungal protein	Antoniw et al., 1980b
PR-2	Tobacco PR-2	β-(1,3)-glucanase	Antoniw et al., 1980b
PR-3	Tobacco P, Q	Chitinase type I, II, IV to VII	Van Loon, 1982
PR-4	Tobacco 'r'	Chitinase type I, II	Van Loon, 1982
PR-5	Tobacco S	Thaumatin-like protein	Van Loon, 1982
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	Green and Ryan, 1972
PR-7	Tomato P69	Endoproteinase	Vera and Conejero, 1988
PR-8	Cucumber chitinase	Chitinase type III	Métraux et al., 1988
PR-9	Tobacco peroxidase	Peroxidase	Lagrimini et al., 1987
PR-10	Parsley 'PR1'	'ribonuclease-like'	Somssich et al., 1986
PR-11	Tobacco 'class V' chitinase	Chitinase type I	Melchers et al., 1994
PR-12	Radish Rs-AFP3	Defensin	Terras et al., 1992
PR-13	Arabidopsis THI2.1	Thionin	Epple et al., 1995
PR-14	Barley LTP4	Lipid-transfer protein	García-Olmedo et al., 1995
PR-15	Barley OxOa (germin)	Oxalate oxidase	Zhang et al., 1995
PR-16	Berlay OxOLP (germin-like)	'Oxalate oxidase-like'	Wei et al., 1998
PR-17	Tobacco PRp27	Unknown	Okushima et al., 2000

detected in tobacco plants (Van Loon, 1999; van Loon et al., 1994). Later on, PR proteins were also identified in many other plant species (Van Loon, 1999) finally resulting in 17 classes of PRs. As it was impossible to classify all identified proteins only according to their mobility in SDS-PAGE, the characteristics on which the grouping was based, were extended to serological properties and biochemical activity of the proteins (van Loon et al., 1994).

These proteins can potentially be induced during growth and maturation of wheat kernels on the field and could consequently be present in wheat flour used for bread making. Although some of the PR proteins are known to be induced after viral infection of the plant (tobacco mosaic virus), not all classes possess antiviral activity. Classes currently known to defend plants against viral attacks are PR-9, PR-10 and PR-12 to 14 (Musidlak et al., 2017; Tam et al., 2015).

The table above shows that almost one third of the PR families (PR-2 to PR-4, PR-8 and PR-11) have chitinase or β-1,3-glucanase activity. These two enzymes are of major importance during pathogen attack, as chitin and β-1,3-glucan are important structural components of the fungal

cell wall (Ebrahim et al., 2011). Although not always clearly defined, the mode of action of PR families 5, 12, 13 and 14 might be involved in permeabilization or disruption of the pathogen's cell membrane (Florack and Stiekema, 1994; Selitrennikoff, 2001; Sinha et al., 2014). Other mechanisms of action have an indirect antifungal impact by cross-linking the cell wall of the host, which is the case in PR-9, PR-15 and PR-16 (Lagrimini et al., 1987; Vidhyasekaran, 2008; Wei et al., 1998; Zhang et al., 1995) or inhibiting digestive proteinases of the attacking insects (PR-6) (Green and Ryan, 1972). Although the general function of the proteins belonging to families PR-7, PR-10, PR-14 and PR-17 is known, their mechanism of action remains unclear (García-Olmedo et al., 1995; Selitrennikoff, 2001; Vidhyasekaran, 2008).

Several studies have shown that PR proteins of the families 1 to 6, 9, 12 to 14, 16 and 17 are present in wheat (Muthukrishnan et al., 2001; Okushima et al., 2000; Stotz et al., 2009; Thomma et al., 2002; Vidhyasekaran, 2008). Plant PRs belonging to classes PR-1 to PR-5, PR-8, PR-10 and PR-14 have been demonstrated to be food allergens, which can be of human interest when consuming cereals containing proteins of these families (Sinha et al., 2014).

1.6.3 Salt

Salt is an essential ingredient of bread and is added in a concentration of 1-2%. It adds to taste, and strengthens the gluten network by shielding the charged gluten molecules (Delcour and Hoseney, 2010; Mondal and Datta, 2008). While salt decreases yeast viability and fermentation capacity and hence results in longer fermentation times (Randez-Gil et al., 2013), it enables control of the fermentation rate by avoiding excessive CO_2 production (Belz et al., 2012).The presence of salt in dough is also undeniably linked with the osmotic stress on yeast cells during bread making with a consequent increase of intracellular glycerol (Section 1.5.3.1) (Aslankoohi et al., 2013). It further adds to shelf life of the baked product, slowing down microbial infection.

1.6.4 Bran

Addition of wheat bran to bread formulations does not only have a positive impact on human health as a source of dietary fiber (Hemdane et al., 2016), it also improves yeast's fermentation capacity (Struyf et al., 2017a). In dough systems where sugars were not limiting (6% sucrose added), the addition of bran resulted in a higher fermentation rate (Struyf et al., 2017b). Wheat bran is known to contain higher fermentable carbohydrate levels, mainly sucrose and fructans, positively influencing the fermentation metabolism of yeast during bread making when there is a limited availability of sugars (Struyf et al., 2017b; Verstrepen et al., 2004). Moreover, as the majority of wheat enzymes is located in the bran layers of the wheat kernel, α-amylases present

in bran can also cause an increased release of maltose during fermentation (Hemdane et al., 2016; Struyf et al., 2017b). According to De Brier and coworkers (2015), the proportion of most of the minerals in wheat bran is significantly higher compared to their concentration in wheat flour. One exception is Ca^{2+}, which is equally present in both (De Brier et al., 2015). As certain minerals are known to beneficially impact yeast fermentation (Section 1.6.1.2), it might be thought that adding bran to the ingredient bill improves dough fermentation. However, minerals are known to be thermostable. As heat treatment of bran was shown to abolish its positive effect, it is rather unlikely that they cause the higher fermentation rate of yeast in the presence of wheat bran.

1.7 Conclusion

Next to the primary fermentation metabolites CO_2 and ethanol, yeast fermentation also results in the production of glycerol, organic acids, glutathione and aroma compounds, all having their own impact on dough rheology and bread characteristics. Using different yeast strains could have considerable consequences on dough and bread characteristics. Conversely, slight changes in the ingredient bill of bread can completely alter yeast's performance. The composition of wheat flour, the amount of fermentable sugars and salt and the addition of bran can all impact the fermentation capacity for the worse or for the better.

In the search for alternative strains, *Torulaspora delbrueckii* and *Kluyveromyces marxianus*, for example, have recently been proven useful to alter aroma and flavor or the nutritional value of bread. However, the potential of the diversity of *S. cerevisiae* remains largely unexplored. Because of the advantages *S. cerevisiae* strains have over the alternative yeasts, both during dough fermentation as well as during industrial production, knowledge about the impact of *S. cerevisiae* strains with different industrial backgrounds on dough and bread, can be expected to open up new possibilities regarding yeast-based bread quality improvement. This forms the starting point of this dissertation.

PART 2 EXPERIMENTAL WORK

Chapter 2 Screening of *Saccharomyces cerevisiae* strains for their wheat dough fermentation potential reveals yeast strain specific inhibition

Chapter 3 Characterization of the yeast strain specific fermentation inhibitor in wheat

Chapter 4 Activity-guided fractionation of a wheat flour extract and identification of a novel yeast strain specific fermentation inhibitor in wheat

Chapter 5 Impact of non-bakery *Saccharomyces cerevisiae* strains on dough rheology and bread quality

Chapter 2.

Screening of *Saccharomyces cerevisiae* strains for their wheat dough fermentation potential reveals yeast strain specific inhibition

This chapter is based on the following reference: Van der Maelen, E., Rezaei, M.N., Struyf, N., Proost, P., Verstrepen, K.J., Courtin, C.M. (2019). Identification of a wheat thaumatin-like protein that inhibits Saccharomyces cerevisiae. Journal of Agricultural and Food Chemistry, 67 (37): 10423-10431.

2.1 Introduction

S. cerevisiae bakery strains have a great impact on bread quality parameters due to the metabolites they produce during the crucial leavening phase of bread making. Next to CO_2 and ethanol also organic acids, glycerol and aroma components are produced as secondary metabolites. Their presence has an important influence on dough rheology, texture, flavor and aroma of the final bread (Ali et al., 2012; Cho and Peterson, 2010; Jayaram et al., 2013b; Rezaei et al., 2015a). Bread-inherent factors, like the presence or absence of ingredients and conditions during dough fermentation, as well as yeast-inherent factors, like pregrowth conditions and genetic background of yeast strains determine the extent of metabolite production (Gabriela and Daniela, 2010; Rezaei et al., 2015a, 2014; Struyf et al., 2017b).

Industrial and artisanal bakeries are continuously challenged to improve existing or create new end products. To achieve this, next to changing the composition of the ingredient bill, also the yeast strain used for dough leavening could be altered (Aslankoohi et al., 2016; Ausar et al., 2003; Brouns et al., 2012; Caballero et al., 2007; De Gobba et al., 2016; Hernández-López et al., 2007; Struyf et al., 2018). While the *S. cerevisiae* species itself embraces a broad number of strains (Gallone et al., 2016; Peter et al., 2018b), only few of these are used in dough fermentation. Other strains are used in beer brewing, bio-ethanol production, cocoa fermentations, wine and spirits production (Gallone et al., 2016; Legras et al., 2007; Lin and Tanaka, 2006; Steensels and Verstrepen, 2014).

In this study, we examined the CO_2 production capacity of 45 different *S. cerevisiae* strains. These strains were randomly selected from the extensive collection present at the VIB – KU Leuven Laboratory for Systems Biology & CMPG Laboratory for Genetics and Genomics. All yeast strains in the collection were assigned to categories according to the industrial fermentation process they were used for. The selected strains were applied in the production of beer, bio-ethanol, bread, cocoa, sake & spirits or wine.

In the course of this research, some yeast strains were identified as being interspecies hybrids between *S. cerevisiae* x *S. kudriavzevii*. However, this was of minor importance as some of these hybrids are also applied in the bread making industry. Our aim was to gain insight in the applicability of *S. cerevisiae* strains from different industrial categories in the dough fermentation process. Before analyzing the strains' capacity in wheat flour dough, we performed a first screening in a liquid model system.

2.2 Materials and methods

2.2.1 Materials

Wheat grain (mixture of varieties Joker and Profilius) was provided by EVD (Jodoigne, Belgium). The grains were milled into flour with a Bühler MLU-202 laboratory mill with a yield of 69.4%. Commercial wheat gluten and wheat starch were provided by Tereos Syral (Aalst, Belgium). Forty-five industrial *S. cerevisiae* strains from various applications were obtained from the collection of the VIB Laboratory for Systems Biology (KU Leuven, Belgium). Bacteriological agar, yeast extract and balanced peptone were provided by Lab M (Brussels, Belgium), commercial sugar and salt by Tiense Suiker (Tienen, Belgium) and Everyday (Gellingen, Belgium), respectively. All other chemicals, reagents and solvents were purchased from Sigma-Aldrich and were of analytical grade.

2.2.2 Flour characterization

The protein content of the wheat flour and the gluten was determined using an automated Dumas protein analysis system (VarioMax Cube N, Elementar, Hanau, Germany) according to an adapted version of AOAC method 990.03 (AOAC, 1995). The flour had a protein content (N x 5.7) of 11.6% on dry matter base. The protein content (N x 5.7) of the gluten was 78.6% on dry matter base. The moisture content was determined following the AACC International Approved Method 44-19.01 (AACC International, 2000) and was 13.7%, 4.8% and 11.1% for wheat flour, gluten and starch respectively. A model dough system (gluten-starch dough) was composed of 13.4% gluten and 86.6% starch. The optimal water absorption and mixing time of wheat flour dough and gluten-starch dough were determined with the Farinograph (Brabender, Duisburg, Germany) and Mixograph (National Manufacturing, Lincoln, NE) according to AACC International Approved Methods 54-21.02 and 54-40.02, respectively (AACC International, 2000), and were 59.0% and 2 min 45 s, respectively for wheat flour dough and 57.0% and 3 min 00 s, respectively, for gluten-starch dough. All measurements were carried out in triplicate.

2.2.3 Preparation of yeast cells for dough fermentation

Yeast cells from a glycerol stock were grown on yeast peptone dextrose (YPD) agar plates, composed of 1.0% w/v yeast extract, 2.0% w/v balanced peptone, 2.0% w/v dextrose and 2.0% w/v agar in deionized water. Next, yeast precultures were made by transferring a single colony from the plate to 5 mL YPD medium. After overnight shaking at 250 rpm and 30°C, 1.0 mL preculture was suspended in a baffled Erlenmeyer flask containing 100 mL YPD medium. The

yeast cultures were again incubated overnight under the same circumstances. After 16h, the optical density (OD) at 595 nm of each yeast suspension was measured with a microtiter plate reader (Bio-Rad Laboratories, Nazareth, Belgium). The cells were harvested after the diauxic shift, at an OD of 1.0 – 1.2. This was done by centrifuging the suspensions using a benchtop centrifuge (model EBA 21, Hettich Lab Technology, Massachusetts, USA) for 3 min at 870 g and washing the obtained pellet with deionized water. The time of occurrence of the diauxic shift of a yeast strain was derived from its growth profile, determined using a Bioscreen C device (Thermo Fisher Scientific, Aalst, Belgium). The OD was in this case measured at 600 nm, every 15 min for 96h at 30°C with continuous shaking. All measurements were performed in triplicate.

2.2.4 Preparation of wheat flour extract

A wheat flour extract was made with deionized water as extraction solvent. To 10.0 g of flour, 15.0 mL of deionized water was added. The suspension was stirred for five minutes and centrifuged at room temperature (21000 g, 15 min). The supernatant (approximately 10 mL) was collected as the wheat flour extract.

2.2.5 Composition of liquid fermentation medium

A liquid fermentation medium for the analysis of the fermentation capacity of different yeast strains was composed. It contained 6.0% sucrose, 0.15% sodium chloride, 1.0% peptone and 20% sorbitol in deionized water. Sorbitol was added to adjust the water activity (a_w) of the solution to the level of dough, being approximately 0.97. Peptone was necessary to provide yeast with a nitrogen source.

2.2.6 Dough making

Two different types of dough were used in this study: (i) wheat flour dough and (ii) gluten-starch dough as a model system. In both cases, dough was prepared according to AACC International Approved Method 10-10.03 with following ingredients: 10.0 g flour or gluten-starch blend (14% moisture base), 6.0% (w/w) sucrose, 1.5% (w/w) sodium chloride and 5.3% (w/w) fresh yeast. Additionally, 1.0% (w/w) balanced peptone was added to gluten-starch dough to provide yeast with sufficient nitrogen. When harvested, the fresh yeast pellet has a moisture content of approximately 80-85%. The amount of water used for making wheat flour dough and gluten-starch dough was respectively 59.0% (v/w) and 57.0% (v/w). The ingredients were mixed with a 10 g pin bowl mixer (National Manufacturing) with respective mixing times of 2 min 45 s and 3 min 00 s.

2.2.7 Gas production analysis

The volume of gas produced by different yeast strains during fermentation in dough and fermentation medium was measured with the Risograph (National Manufacturing). Doughs and fermentation media were prepared in triplicate as described earlier, put in sealed buckets and analyzed in the Risograph at 30°C for 3 or 16h, depending on the experiment. The CO_2 production is measured every minute, as the difference in pressure in the headspace of the sample bucket and a reference bucket. When closing the buckets at the beginning of the experiment, air is included. This causes a steep dropdown in the first 10 minutes of the fermentation profiles. Consequently, as this dropdown is an artefact caused by the way the fermentation rate is measured, it should be ignored.

2.2.8 Statistical analysis

All fermentation experiments were performed in triplicate. For this purpose, three biological replicates of each yeast strain were analyzed. Significant differences in total volume of CO_2 produced after 3h of fermentation were indicated by a one-way ANOVA using JMP Pro software 14 (SAS Institute, Cary, NC, USA). Mean values were compared using a post-hoc Tukey HSD test with a significance level of 0.05.

2.3 Results and discussion

2.3.1 Fermentation capacity of *S. cerevisiae* strains in liquid fermentation medium

Liquid model systems are often used to screen different yeasts for their capacity to ferment dough (Attfield et al., 2000; Bell et al., 2001; Pérez-Torrado et al., 2010). Here, we measured the fermentation capacity of 45 *S. cerevisiae* strains from 6 different industrial categories in a liquid model system. Using sorbitol, the a_w of the liquid medium was brought to 0.97, the approximate value in dough. **Figure 2.1A** shows the total volume of CO_2 produced at the end of the fermentation period for all the strains. The different strains are classified into industrial categories depending on their applications, being bread (orange; referred to as Bro1 to Bro4), beer (yellow; Be01 to Be16), bio-ethanol (blue, BioE01 to BioE05), cocoa (brown, C01), sake & spirits (green; S01 to S08) and wine (red; W01 to W11). Overall, the total volume of CO_2 produced after three hours of fermentation ranged from 62 to 124 mL CO_2 with one exception (yeast S01, 28 mL). Despite the variation within each industrial category, the categories themselves did not differ significantly from each other. The bakery strains showed a fermentation capacity between 70 and 112 mL CO_2 after three hours of fermentation. This range covered almost 90% of all strains

tested in this experiment. It could be concluded that, regardless of their industrial background, all yeast strains performed rather similarly to the bakery strains in liquid fermentation medium.

2.3.2 Fermentation capacity of *S. cerevisiae* strains in wheat flour dough

To make a comparison between the fermentation capacity of all yeast strains in liquid fermentation medium and in wheat flour dough and to investigate whether predictions based on yeast behavior in the former medium could be extrapolated to their behavior in the latter, the 45 yeast strains were evaluated in wheat flour dough (**Figure 2.1B**). The tested yeast strains belonging to the bakery, bio-ethanol and wine categories and the cocoa strain were all able to ferment dough and reached higher volumes of CO_2 produced compared to the liquid model system, after addition of the same amount of yeast pellet. Amongst the bakery strains, differences in fermentation capacity could be noticed with average CO_2 volumes varying between 109 mL and 147 mL. Somewhat remarkable, the overall highest volume of CO_2 in wheat flour dough was not produced by a bakery strain. The W07 wine strain showed the strongest performance, with a production of 166 mL CO_2 in 3h. With exception of the best beer strain (Be16, 159 mL), W06 differed significantly from the best performers of the other categories, being strains Bro2 (147 mL), BioE05 (150 mL), C01 (116 mL) and S07 (151 mL).

It was remarkable to note that not all yeast strains were able to leaven dough at least to the extent that was expected from the liquid model system screening. Eight out of 45 yeast strains in this experiment produced significantly less CO_2 after three hours than the average bakery yeast, and also less than all the other strains tested in general. Within this less performing group of strains, a further distinction could be made between strains that performed really weakly (85% less CO_2 produced than the overall average) and strains with intermediate performance (50% less CO_2). The six weakly performing strains are used in the industrial beer production and the two intermediate dough fermenting strains in the industrial sake & spirits production. Although strains Be13 and Be14 belonged to the most promising beer strains in the liquid model system with a CO_2 volume comparable with that of the best bakery strain, this behavior could not be extrapolated to their behavior in wheat flour dough, in which they produced less than 12% of the CO_2 volume produced by the best bakery strain. Together with the weak correlation between CO_2 production in the liquid model system and in wheat flour dough (R^2 = 0.19), this suggests that liquid fermentation medium does not always provide a good estimate of a strain's dough fermentation capability.

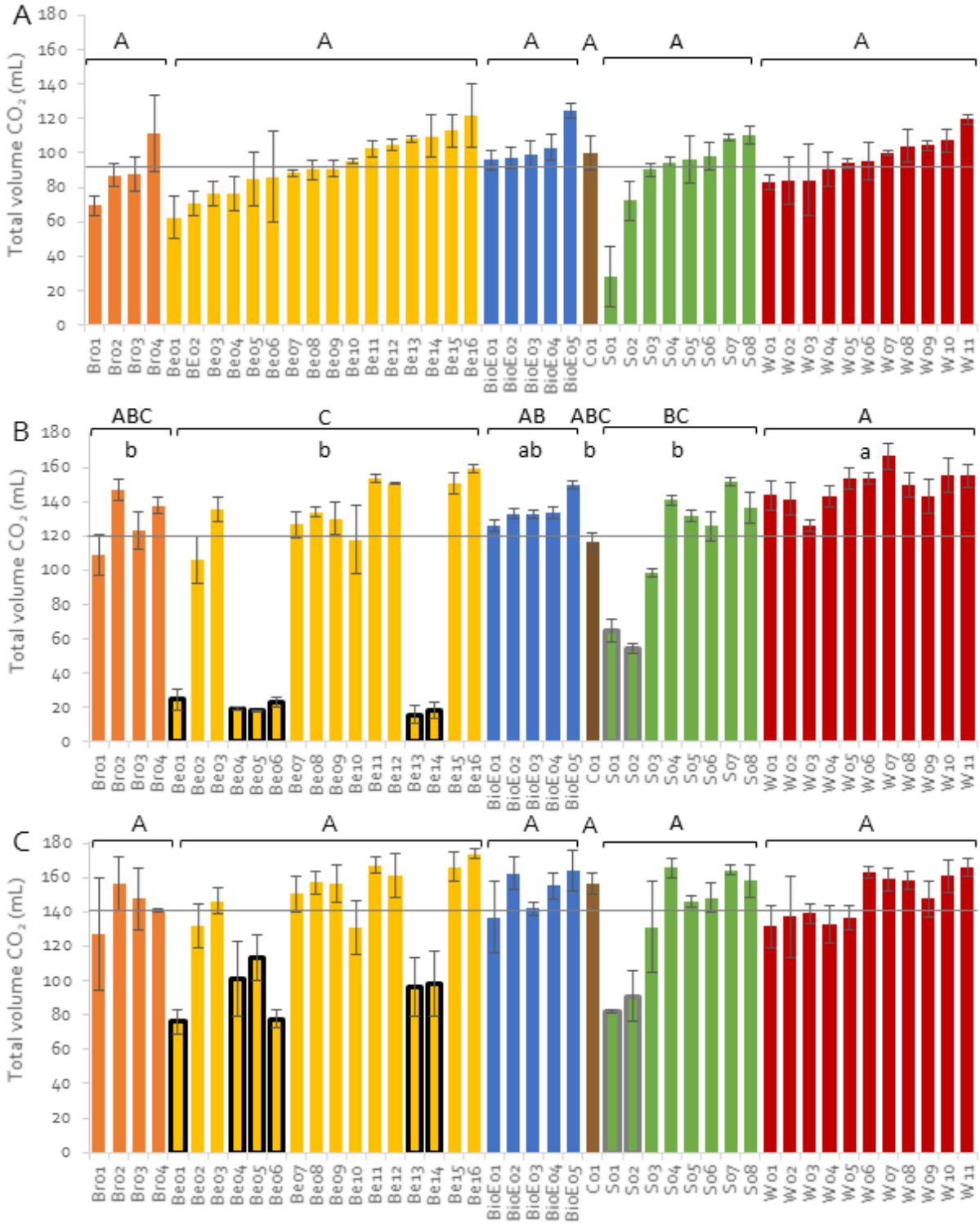

***Figure 2.1**: The total amount of CO_2 (mL) produced by 45 different yeasts after 3h in (A) liquid fermentation medium, (B) wheat flour dough and (C) gluten-starch dough. The yeasts are classified according to their industrial fermentation product: bread (orange), beer (yellow), bio-ethanol (blue), cocoa (brown), sake & spirits (green) and wine (red). Industrial categories with different letters differ significantly from each other (Tukey, $p<0.05$). Capital letters compare the whole industrial categories, small letters compare the industrial categories, excluding the weak and intermediate performers. Columns surrounded by black and gray lines represent resp. the weak and intermediate performers, significantly differing from each other (in wheat flour dough) and from the strong wheat flour dough fermenting yeast strains (Tukey, $p<0.05$). Vertical bars are standard deviations of biological triplicates. Horizontal lines indicate the overall averages (93 mL, 120 mL and 140 mL resp.).*

The weakly performing yeasts are all used in liquid fermentation applications such as beer and sake production. However, not all beer and spirits yeasts were weak or intermediate dough fermenters. Two-thirds of the tested strains from both categories belonged to the strong fermenters. The wine and bio-ethanol strains selected for this study appeared to be excellent dough fermenters as well. With an average CO_2 production of 148 mL, the former were even significantly better than the strong dough fermenters belonging to the bakery, beer, sake & spirits and cocoa category, with an overall average CO_2 volume of approximately 130 mL. It was also not surprising that bio-ethanol yeasts performed well in dough, because the biofuel industry was initially dominated by bakery yeasts (Jayus et al., 2016; Steensels et al., 2014b). Comparing the five industrial categories, the bakery strains did not differ significantly from any other category.

Interestingly, sake strain S01 was the weakest performer in the liquid model, but in dough it was classified among the intermediate performers. The amount of gas produced in wheat flour dough was more than twice as high as in the liquid system.

Rezaei and coworkers (2015a) already noticed the inability of some yeasts to ferment dough and ascribed this to the osmotic stress caused by sugar and salt, the semi-solid state of dough or the relatively low a_w in dough. Next to those stress-causing factors, constituents of wheat flour – sugars, peptides and proteins, vitamins and minerals – might also cause stress. To investigate this further, we examined the yeasts in a model dough system composed of gluten and starch.

2.3.3 Fermentation capacity of *S. cerevisiae* strains in gluten-starch dough

The fermentation capacity of the 45 strains in gluten-starch dough is shown in **Figure 2.1C**. Strains able to ferment in wheat flour dough were also capable of fermenting in gluten-starch dough, to the same extent or even better. For the weak and intermediate fermenters, the fermentation capacity in gluten-starch dough was significantly better compared to wheat flour dough. Fermentation profiles of strains Br04 and W07 as strong fermenters, Be05 and Be14 as weak fermenters and S01 and S02 as intermediate fermenters in liquid fermentation medium as well as in wheat flour and gluten-starch dough are compared in **Figure 2.2**. For the strong fermenting yeast strains, the CO_2 production rate in liquid medium was lower than in wheat flour dough (**Figure 2.2A and B**). In the former medium, it took the strong fermenting strains longer to reach their maximal fermentation rate, which was in most of the cases also lower than that in wheat flour dough. The fermentation profiles for these yeast strains were similar in wheat flour and gluten-starch dough.

More remarkable differences in fermentation profiles between three media could be noticed for the intermediate and weak fermenting strains. A possible explanation for the low volume of CO_2 produced by strain So1 after 3h in the liquid model system might be found in the fermentation profile (**Figure 2.2C**). Strain So1 shows an extremely slow fermentation rate in the first phase of the fermentation, and even at the end of the fermentation period the yeast strain did not seem to reach a maximum yet. In both dough systems the fermentation profiles resemble each other for the first 75 minutes. In gluten-starch dough the fermentation rate continued increasing at the same pace and did not reach a maximum within three hours. The fermentation rate of strain So1 in wheat flour dough stabilized after 75 minutes at plateau of 0.6 mL CO_2/min. Although strain So2 had a slightly longer lag phase in liquid medium than in gluten-starch dough, the fermentation profiles were rather similar and reached a maximal rate of approximately 0.7 mL CO_2/min towards the end of the fermentation period (**Figure 2.2D**). In contrast, a fermentation rate of 0.3 mL CO_2/min was already obtained after 45 minutes in wheat flour dough, and strain So2 continued to ferment at the same relatively slow pace throughout the complete fermentation. **Figures 2.2E and F** show the discrepancy between the fermentation profiles in gluten-starch and wheat flour dough for strains Be05 and Be14. For both strains, the fermentation rate in wheat flour dough was close to zero and much lower than in gluten-starch dough. In both liquid medium and gluten-starch dough, the fermentation rate exhibited by strain Be05 increased up to 0.8 mL CO_2/min. In gluten-starch dough a shorter lag phase and a faster increase was seen than in liquid medium. The fermentation profiles of strain Be14 in gluten-starch dough and in liquid medium were more analogous to each other and reached a maximal fermentation rate of at least 0.6 mL CO_2/min.

So, a notable difference between the behavior in wheat flour dough and in gluten-starch dough of the weak and intermediate fermenting strains was observed. The total CO_2 volume after 3h in gluten-starch dough of the six weak and two intermediate fermenting strains was, however, still significantly lower than that of all the other strong fermenting strains. Both observations taken together suggest that the semi-solid state plays a minor role in the reduced fermentation capacity of some strains, but, more striking, that constituents inherent to wheat flour have a key role in determining the ability of yeast strains to ferment in wheat flour dough. To confirm this, the water in the recipe of gluten-starch dough was replaced by wheat flour extract. The performance of the yeast strains was analyzed again, but now in this gluten-starch dough with wheat flour extract.

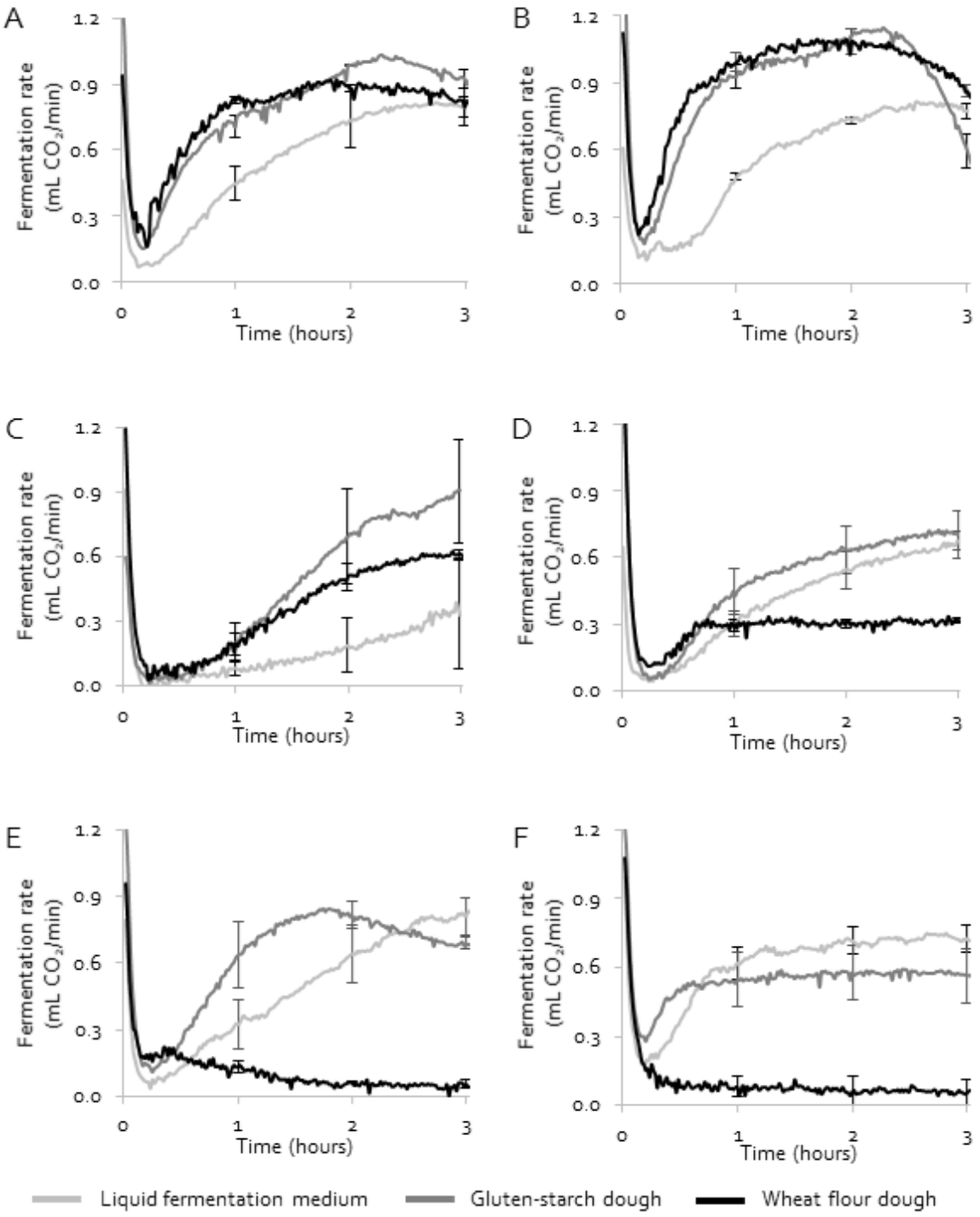

Figure 2.2: *Fermentation rate (mL CO_2/min) as a function of time in different media of strains showing strong fermentation (A, Br04 and B, W07); intermediate fermentation (C, S01 and D, S02) and weak fermentation (E, Be05 and F, Be14) in wheat flour dough. The fermentation rate was measured with a Risograph (30°C) in liquid fermentation medium (bright gray), wheat flour dough (black) and gluten-starch dough (dark gray). Vertical bars represent standard deviations of biological triplicates.*

As shown in **Figure 2.3,** the same distinction between strong and less performing strains could be made as in wheat flour dough. In contrast to wheat flour dough, the weak and intermediate strains did not differ significantly from each other in this model dough. This confirmed the existence of one or more water extractable components in wheat flour that are very harmful to certain yeast strains in dough and possibly less or not harmful for all others.

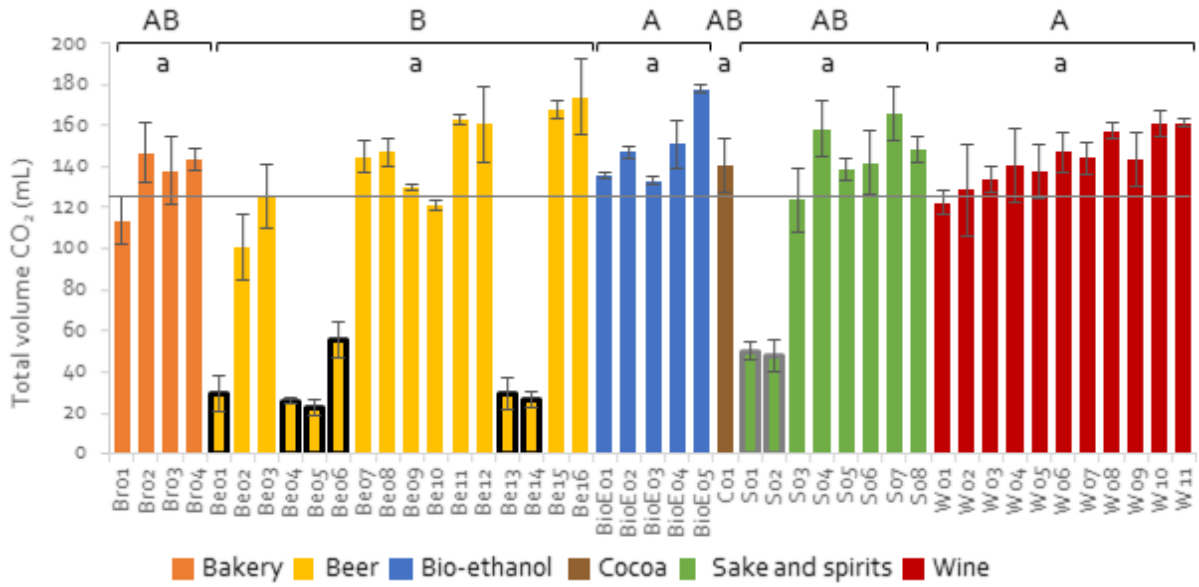

Figure 2.3: *Total amount of CO_2 (mL) produced by 45 S. cerevisiae strains after 3h in gluten-starch dough with wheat flour extract, measured with a Risograph (30°C). The yeast strains are classified according to their industrial category: bread (black), beer (dark gray), bio-ethanol (bright gray), cocoa (brown), sake & spirits (white) and wine (striped). Columns surrounded by black and gray lines represent resp. the weak and intermediate wheat flour dough fermenters, significantly differing from the strong wheat flour dough fermenting yeast strains, but not from each other ($p < 0.05$). Industrial categories with different letters are significantly different from each other ($p < 0.05$). Capital letters compare the whole industrial categories, small letters compare the industrial categories with exclusion of the weak and intermediate performers. Vertical bars represent standard deviations of biological triplicates. The horizontal line indicates the overall average CO_2 production, i.e. 123 mL.*

2.4 Conclusion

After screening 45 *S. cerevisiae* strains from different industrial categories for their fermentation capacity in a liquid model system, most of them seemed promising for bakery applications. However, when applied in wheat flour dough, part of the strains was not able to leaven wheat flour dough to any relevant extent. This observation led to the conclusion that liquid model systems not always sufficiently represent real life conditions. In the search for new yeast strains for bread production, wheat-based model systems are hence required. Experiments with a model dough, consisting of gluten and starch, showed that weak fermenting yeast strains did have the ability to ferment in semi-solid systems and simultaneously proved that one or more water extractable wheat flour components led to the inability of those yeast strains to ferment wheat flour dough. This yeast inhibition phenomenon is more extensively studied in the next chapter.

Chapter 3.

Characterization of the yeast strain specific fermentation inhibitor in wheat

This chapter is based on the following reference: Van der Maelen, E., Rezaei, M.N., Struyf, N., Proost, P., Verstrepen, K.J., Courtin, C.M. (2019). Identification of a wheat thaumatin-like protein that inhibits Saccharomyces cerevisiae. Journal of Agricultural and Food Chemistry, 67 (37): 10423-10431.

3.1 Introduction

Fermentation in wheat flour dough does not seem to be reserved for all *S. cerevisiae* strains. In the past, when less efficient fermentation abilities were noticed, it was often ascribed to the stress conditions dominating the semi-solid dough system. Rezaei and coworkers (2015c), for example, related the lack of fermentation of some yeast strains to the osmotic stress caused by sugar and salt, the semi-solid state of dough or the relatively low a_w in dough. However, we showed in Chapter 2 that wheat flour inhibition-sensitive yeast strains (i.e. the poor and intermediate performers) were capable of fermenting in a model dough. This is likewise a semi-solid system with a comparable low a_w, containing the same amounts of additional sugar and salt. These factors were consequently rather unlikely to cause the fermentation inhibition. Looking at their composition, wheat flour and the gluten-starch blend probably differ in their content of vitamins, minerals, non-starch carbohydrates and non-gluten proteins. Although vitamins and minerals could be important for the yeast's metabolism (Section 1.6.1.2), it would be illogical to assume that gluten-starch dough is more rich in these components. These constituents could therefore probably be eliminated as potential inhibitors. The same is true for non-starch carbohydrates. As in the formulation of both dough systems 6.0% sugar is added, the yeast strains should normally be independent of the fermentable sugar content. The only group of wheat constituents having inhibition potential, might be the peptides and non-gluten proteins (cfr. Section 1.6.2). Amongst this group of constituents are the enzymes releasing fermentable sugars or small peptides as nitrogen sources. However, these enzymes would rather have a positive impact on yeast during wheat flour dough fermentation than a negative one.

Taking the above in account, it is clear that the fermentation inhibition of *S. cerevisiae* in wheat flour dough needed further research. This chapter gives an overview of the characterization of the inhibitor(s) during wheat flour dough fermentation. To facilitate the characterization, this is carried out using only two yeast strains, being Bro4, representative of the inhibition-insensitive yeast strains and Be05, representative of the inhibition-sensitive yeast strains. Additionally, inhibition by different wheat varieties and cereal species is also investigated.

3.2 Materials and methods

3.2.1 Materials

Wheat grain was provided by EVD (Jodoigne, Belgium). The grains were milled into flour with a Bühler MLU-202 laboratory mill (Bühler, Uzwil, Germany) with a yield of 69.4%. Wheat varieties Atomic, Bergamo, Cellule and Mentor were obtained from the experimental site of the University of Liège (Agro-bio Tech, Gembloux, Belgium). Grains of all the wheat varieties were ground to whole meal with a Cyclotec 1093 sample mill (FOSS, Höganäs, Sweden). Rye (cultivar Dukato) and barley (cultivar Sebastian) grains were resp. obtained from AVEVE (Merksem, Belgium) and Cargill Malt (Herent, Belgium). Both were in-house milled to flour with the Bühler mill. Commercial oat flour was provided by Raisio Nutrition (Raisio, Finland), rice flour by Beneo-Remy (Wijgmaal, Belgium) and maize flour by Puratos (Groot-Bijgaarden, Belgium). Commercial wheat gluten (protein content: 78.6% dm; moisture content: 4.8%) and wheat starch (moisture content: 11.1%) were provided by Tereos Syral (Aalst, Belgium). Four industrial *S. cerevisiae* strains from four different industrial applications were obtained from the collection of the VIB Laboratory for Systems Biology (KU Leuven, Belgium). Bacteriological agar, yeast extract and balanced peptone were from Lab M (Brussels, Belgium), commercial sugar and salt from Tiense Suiker (Tienen, Belgium) and Everyday (Gellingen, Belgium), respectively. All other chemicals, reagents and solvents were of analytical grade and purchased from Sigma-Aldrich, unless specified otherwise.

3.2.2 Flour and whole meal characterization

The characteristics of the wheat flour used in this study were as described in Section 2.2.2. The protein and moisture content of the different wheat whole meal samples and other grain flours are given in **Table 3.1**. Because whole meal flour was only used to examine the potential presence of inhibition, a standardized water absorption and mixing time of 70.0% and 6 min 00 s, respectively, were applied. The optimal water absorption and mixing time of rye flour were determined using the Farinograph (Brabender, Duisburg, Germany) and the Mixograph (National Manufacturing, Lincoln, NE, USA) analyses according to AACC International Approved Methods 54-21.02 and 54-40.02, respectively (AACC International, 2000), and were 55.0% and 1 min 30 s, respectively. The other grain flours were used to make extracts. All measurements were carried out in triplicate.

3.2.3 Preparation of yeast cells for dough fermentation

Yeast cells were prepared as described in Section 2.2.3.

3.2.4 Preparation of flour extracts and composition of liquid fermentation and growth media

The extracts of wheat flour and flour of different grain varieties were prepared according to the procedure in Section 2.2.4. The composition of liquid fermentation medium was described in Section 2.2.5. Liquid fermentation medium with wheat flour extract had the same composition, except for the use of extract (Section 2.2.4) instead of deionized water. Growth medium was prepared similarly as liquid fermentation medium, but with deletion of sorbitol. Adjusting the a_w was not wanted for growth. Growth medium with wheat flour extract were done similarly, but also with the use of extract instead of deionized water.

Table 3.1: *Protein and moisture contents of wheat whole meals and flour of different grains.*

Species and variety	Protein content (% dm)	Moisture content (%)
Wheat – Atomic	9.6 (±0.28)	10.3 (±0.13)
Wheat – Bergamo	9.6 (±0.01)	10.7 (±0.08)
Wheat – Cellule	9.9 (±0.08)	10.2 (±0.16)
Wheat – Mentor	10.4 (±0.01)	10.7 (±0.09)
Rye – Dukato	4.0 (±0.02)	13.6 (±0.26)
Barley – Sebastian	6.3 (±0.05)	12.6 (±0.15)
Oat	13.3 (±0.04)	10.2 (±0.27)
Rice	4.8 (±0.1)	9.8 (±0.1)
Maize	6.7 (±0.05)	11.1 (±0.02)

3.2.5 Dough making

Dough of different wheat and grain varieties and gluten-starch dough with water or flour extracts were prepared according to the AACC International Approved Method 10-10.03, as described in Section 2.2.6. In case of the gluten-starch doughs with flour extracts, the 57.0% (v/w) of water was replaced by the extract.

3.2.6 Gas production analysis

The gas production during fermentation in dough and fermentation medium was measured using the Risograph as described in Section 2.2.7.

3.2.7 Cell growth measurement

Yeast precultures were prepared as previously described. After overnight incubation, the OD of the suspensions was measured at 595 nm with a microtiter plate reader (Bio-Rad Laboratories, Nazareth, Belgium) and adjusted to OD 1.0. Of this suspension 5 µL was pipetted into 145 µL YPD, growth medium or growth medium with WFE in a 96-well plate. The growth of different yeast strains in the three different media was determined using a Bioscreen C device (Thermo Fisher Scientific, Aalst, Belgium). The OD was measured every 15 minutes with continuous shaking. The experiments were performed in triplicate.

3.2.8 Thermal and enzymatic treatment of wheat flour extract

The heat sensitivity of the inhibiting components in the wheat flour extract was investigated. For this, the extract was heat treated at different temperatures. A falcon tube containing an aliquod of extract was incubated in a water bath at 40, 60, 70, 80 and 90°C. After one hour of incubation, the formed precipitate was removed by centrifugation (4000 g, 5 min). The sensitivity towards proteolysis was tested with *Bacillus lichenformis* Alcalase, a non-specific protease obtained from Novozymes (Bagsvaerd, Denmark). For this, the pH of the wheat flour extract was adjusted to pH 8.0 and it was overnight incubated with 25 µL enzyme per mL extract at 40°C. The inhibition activity of the treated extracts was examined in gluten-starch dough and liquid fermentation media.

3.2.9 Statistical analysis

Significant differences in total volume of CO_2 produced after 3h of fermentation were analyzed by a one-way ANOVA using JMP Pro software 13 (SAS Institute, Cary, NC, USA). Mean values were compared using a post-hoc Tukey HSD test with a significance level of 0.05.

3.3 Results and discussion

3.3.1 Impact of the inhibitor(s) on yeast growth

The yeast specific inhibition was in the previous chapter so far only observed during fermentation processes. As the fermentation and respiration metabolisms are closely related, the impact of the inhibitor(s) on yeast growth was examined. Because the concentration of the inhibiting component(s) in the wheat flour extract was already high enough to prevent certain yeast strains from fermenting in gluten-starch-extract dough, the impact of such an extract on growth was tested. This experiment was carried out with only four yeast strains instead of 45 to facilitate the analysis. Next to the two representatives of the inhibition-insensitive and inhibition-sensitive

strains, Bro4 and Be05, respectively, the growth of W10 (a strong fermenter) and So2 (an intermediate fermenter) was also analyzed in growth medium without (negative control) and with wheat flour extract. The purpose of using these four strains was to examine whether the fermentation behavior of the yeast strains in the presence of the inhibitor(s) could be extrapolated to the growth behavior or not. In the previous chapter, W10 turned out to be inhibition-insensitive during fermentation in wheat flour dough. The growth of this strain in both growth media was examined additional to Bro4, to analyze if yeasts with a similar fermentation behavior in the presence of the inhibitor(s) also showed similar growth in the presence of the inhibitor(s). It was expected that growth of Bro4 and W10 was not affected by the inhibitor, growth of So2 only slightly and of Be05 strongly. The basic composition of both growth media was the same as the liquid fermentation medium (Section 2.2.5), but without sorbitol. The concentration of yeast cells during growth was estimated by measuring the optical density (OD) at 600 nm. In **Figure 3.1**, the OD_{600} is displayed in function of time for the four yeasts. The growth curves of strains Bro4 and W10 (**Figure 3.1A** and **B**) in both media were overlapping, indicating that these yeasts were not influenced by the inhibitor(s). In contrast, the inhibitor(s) did indeed have an impact on the cell growth of the two other strains and most explicitly on Be05 (**Figure 3.1C**). In growth medium with wheat flour extract, the lag phase was longer and the exponential phase was less steep than in growth medium without the extract. However, the yeast still was able to grow and reached the stationary phase at the same OD as in the negative control, albeit almost 24 hours later. As for the intermediate fermenting strain (**Figure 3.1D**), a shift to the right of the growth curve in medium with wheat flour extract could also be noticed but to a much lesser extent, with only a three hour delay compared to the control. The question arose as to whether the shift of the growth curve could be caused by the occurrence of mutants dominating the yeast culture. To examine this, strains Bro4 (inhibition-insensitive) and Be05 (inhibition-sensitive, the growth delay of this yeast was the most outspoken) were first grown overnight in YPD medium in which the aqueous phase was replaced by a wheat flour extract, after which they were harvested and their fermentation capacity in wheat flour dough was analyzed. In case mutation of the inhibition-sensitive yeast strain had occurred, the mutated yeast could be assumed to have become resistant against the inhibition and consequently to be able to ferment in wheat flour dough. The fermentation rate in function of time of Bro4 and Be05, both grown in YPD and in YPD with inhibitor(s), in wheat flour dough is shown in **Figure 3.2**.

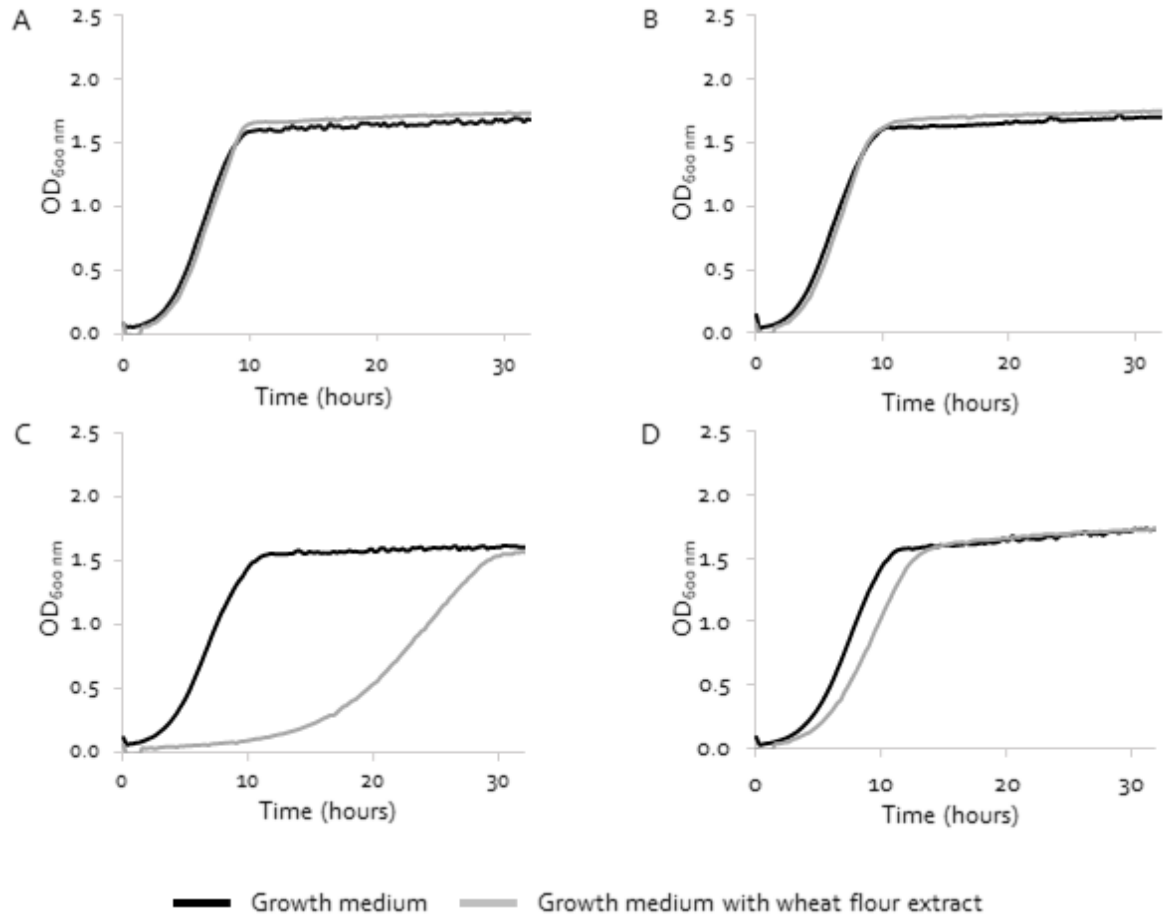

Figure 3.1: *Growth curves of (A, B) two strong (Bro4, W10), (C) a weak (Beo5) and (D) an intermediate (So2) wheat flour dough fermenting yeast in growth medium without (GM, black) and with (GM+WFE, gray) wheat flour extract (WFE). The curves are expressed as OD at 600 nm in function of time (h). Each curve is composed of a lag phase, exponential growth phase and stationary phase.*

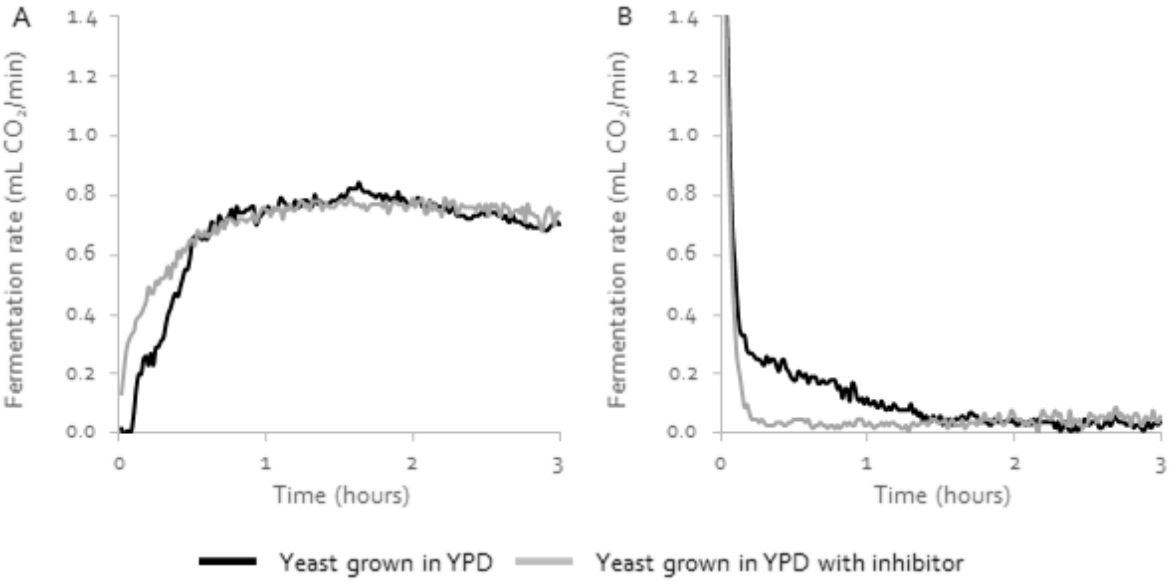

Figure 3.2*: The fermentation capacity (mL CO2/min) in function of time (hours) of (A) the inhibition-insensitive Bro4 and (B) the inhibition-sensitive Beo5 in wheat flour dough after overnight growth in YPD medium (black) and in YPD with inhibitor (gray).*

As expected, the presence of the inhibitor(s) in the growth medium did not impact the fermentation capacity of the inhibition-insensitive Bro4 yeast (**Figure 3.2A**). Comparing the fermentation curves of the inhibition-sensitive yeast strain grown in both YPD and YPD with inhibitor(s), proved that – although the yeast was able to grown overnight in YPD with inhibitor(s) – the presence of the inhibitor(s) did not affect the yeast's resistance during fermentation in wheat flour dough. Consequently, it could be concluded that the delay of the exponential growth phase of the inhibition-sensitive Be05 yeast was unlikely to be caused by the occurrence of yeast mutants.

Five decades ago, Okada and coworkers (1970) isolated a component from wheat and barley with antifungal activity against beer yeasts. When analyzing its mode of action, they concluded that a concentration of 0.4 ppm of the component inhibited sugar uptake of the yeast cell. Concentrations of 4 ppm caused cell death (Okada and Yoshizumi, 1973, 1970; Okada et al., 1970). This was in line with the research results of Stuart and Harris (1942), stating that wheat contained a protein slowing down cell growth of beer, bread and wine yeasts in low concentrations and killing yeast cells in high concentration. Analogously to these findings, the inhibitor(s) in this research slowed down the growth of *S. cerevisiae* strains. This was, however, not the case for the ones belonging to the industrial bread and wine categories. If the inhibitor(s) we are dealing with has(have) the same characteristics as the one described by Stuart and Harris (1942) or Okada and Yoshizumi (1973), we might conclude that the concentration in the crude wheat flour extract is not high enough to kill the yeast and is only able to slow down the yeast's metabolism.

Because the impact of the inhibitor(s) could be noticed both in fermentation and growth of yeast cells, we chose to focus on the fermentation behavior.

3.3.2 Sensitivity of the inhibitor(s) towards proteolytic activity and thermal treatment

As already stated in Section 1.6.2, a lot of plants and cereals contain PR proteins protecting them against pathogen attack during development and growing on the field. To check whether or not the inhibition-sensitive yeasts in this research are affected by such proteins, the sensitivity of the inhibitor(s) in wheat flour extract towards proteolysis and thermal treatment was examined. Strain Be05 was selected as representative of the inhibition-sensitive fermenters. As a faster screening method, a liquid fermentation medium based on wheat flour extract was used. In contrast to the liquid fermentation medium used in the previous chapter, inhibition could be noticed in the extract-based liquid medium (**Figure 3.3**) and was consequently representative for

wheat flour. The composition of the new liquid broth with wheat flour extract was comparable with the previous liquid medium. Only the water fraction was replaced by the extract and the sorbitol content was altered in order to keep the same water activity as in dough.

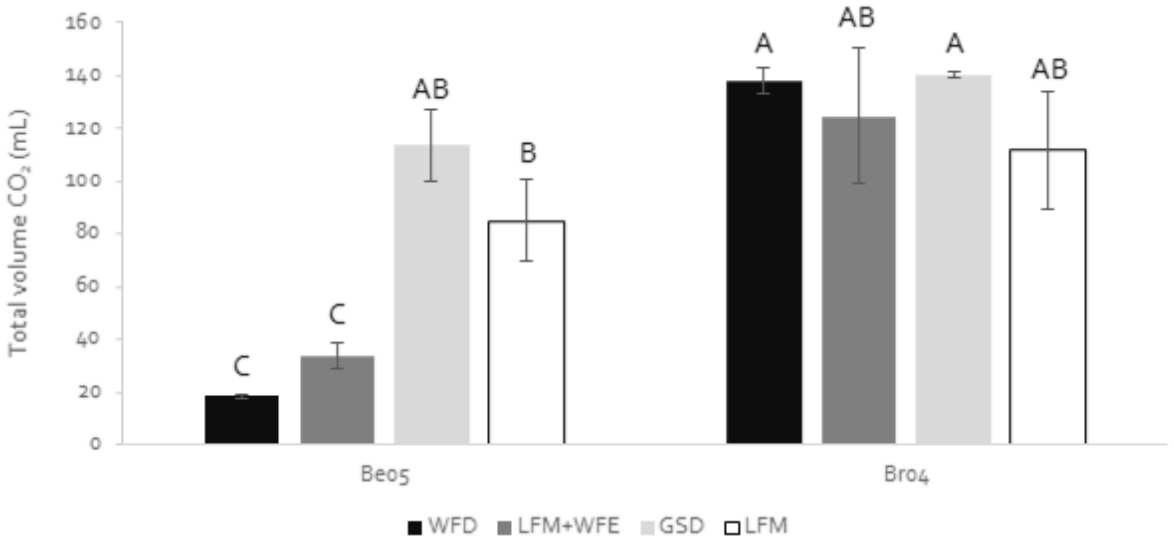

***Figure 3.3**: Comparison of the total CO_2 production (mL) of the inhibition-sensitive Beo5 (left) and inhibition-insensitive Bro4 (right) in wheat flour dough (WFD, black), liquid fermentation medium without (LFM, white) and with wheat flour extract (LFM+WFE, dark gray) and gluten-starch dough (GSD, bright gray). Strain Beo5 is similarly inhibited in LFM+WFE as in WFD. The vertical bars represent the standard deviations of three biological replicates. Columns with different letters are significantly different from each other (Tukey, $p < 0.05$).*

In order to degrade all proteins present in the wheat flour extract, a non-specific protease (*B. lichenformis* Alcalase) was added to the extract in a concentration of 25 µL/mL, after adjusting the pH to 8.0. The mixture was incubated overnight at 40°C. The precipitate was removed by centrifugation and the resulting supernatant was used in the liquid fermentation medium. The fermentation capacity of the inhibition-sensitive Beo5 strain was followed up in time and is represented in **Figure 3.4**. Compared to the positive control, i.e. the liquid fermentation medium with wheat flour extract, Beo5 was clearly able to ferment better in the presence of the protease treated extract and approached its fermentation profile in the negative control (absence of inhibitor). Based on this result, it could be assumed that the inhibitor(s) is(are) a protein(s) and thus potentially belonging to the PR proteins.

Next, a wheat flour extract was treated at different temperatures for 1h and used to formulate a liquid fermentation medium with extract. **Figure 3.5A** shows the fermentation profiles of strain Beo5 in the liquid fermentation medium without (negative control) and with untreated wheat

flour extract (positive control) and in liquid systems with wheat flour extracts treated at 40, 60, 70 and 90°C. When the extract had been treated at 40°C, the inhibitor(s) was(were) still as active as in the positive control. The fermentation capacity of strain Be05 in liquid systems to which extracts were added, increased with increasing treatment temperature and was completely restored when the wheat flour extract was treated at 70°C and higher. Based on these observations, it could be concluded that the inhibitor(s) was(were) heat sensitive. This is not in line with the observations of Balls and Harris (1944), who described the fermentation inhibition of a distiller's yeast strain in wheat mashes and found that heating the mashes to 80°C only partly destroyed the inhibitor.

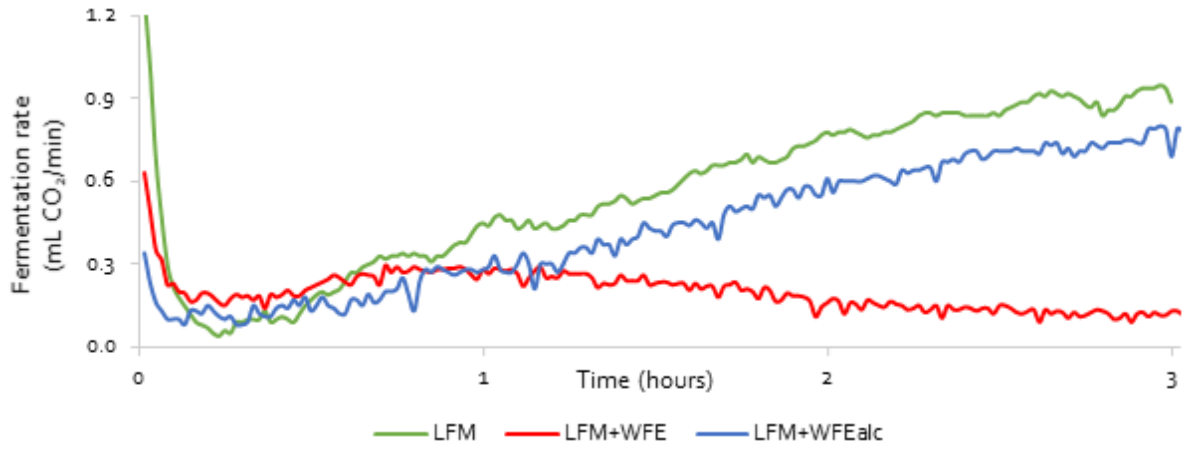

***Figure 3.4**: The fermentation capacity (mL CO_2/min) in function of time (hours) of the inhibition-sensitive Be05 in liquid fermentation medium with protease-treated wheat flour extract (LFM+WFEalc, blue) was tested. Treatment with the protease Alcalase made the inhibition activity disappear. The green and red curves resp. represent the negative (LFM, liquid fermentation medium) and positive (LFM+WFE, liquid fermentation medium + wheat flour extract) control.*

In another experiment, a wheat flour extract was also treated at 70°C but then added to gluten-starch dough. Although the extract had undergone the same treatment, the inhibition-sensitive yeast strain was still partially inhibited, as displayed in **Figure 3.5B**. This suggests that (i) the thermal treatment at 70°C might be less effective to inactivate the inhibitor(s) as initially thought based on the results in a liquid fermentation medium or that (ii) yeasts are more sensitive towards the inhibitor(s) in semi-solid systems compared to liquid systems. In the latter case, the impact of dough mixing on yeast performance might not be underestimated. Due to the continuous kneading, yeast undergoes mechanical stress and is constantly brought into contact with new

constituents. In this context, it might thus be likely that the exposure of the yeast cells to the inhibitor(s) in dough is higher compared to the liquid system without continuously stirring.

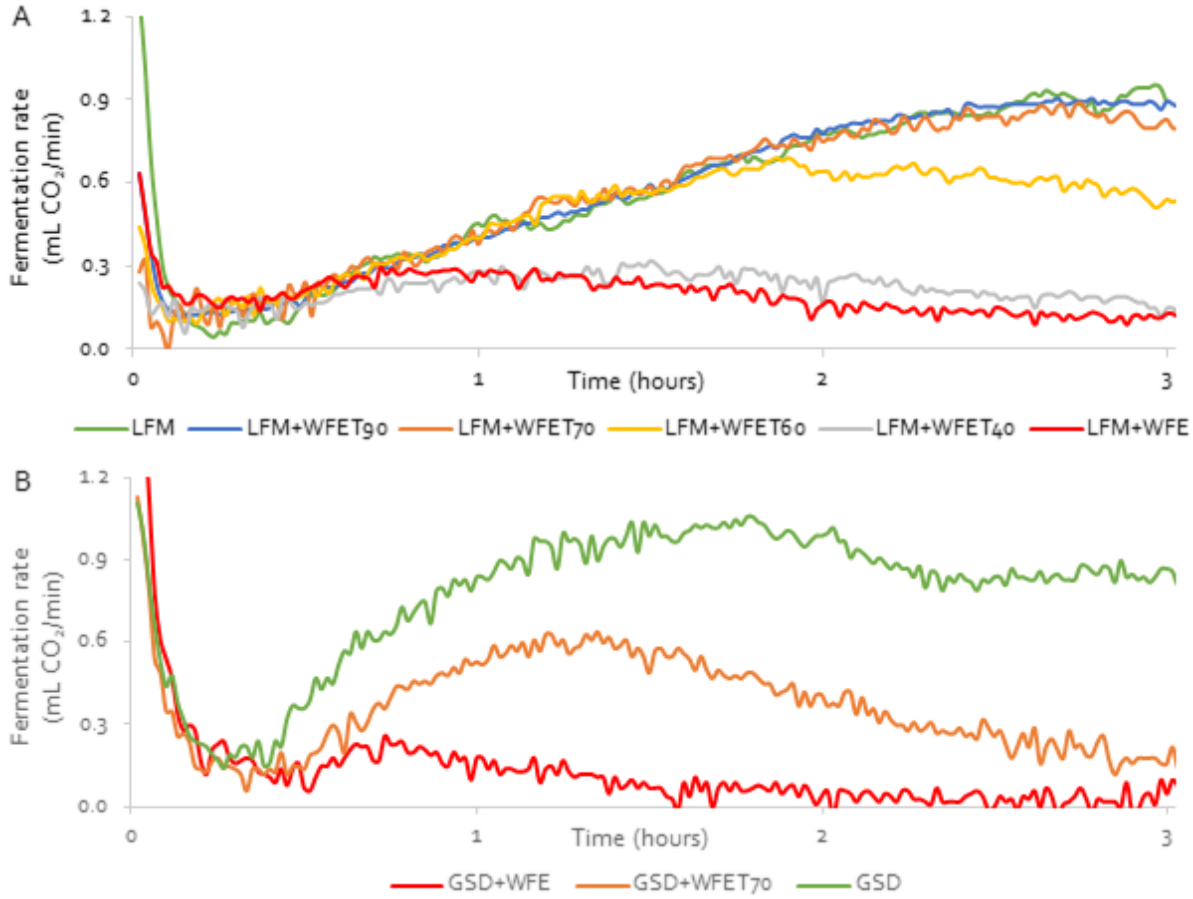

***Figure 3.5**: (A) The fermentation capacity (mL CO_2/min) in function of time (hours) of the inhibition-sensitive Be05 yeast strain in liquid fermentation medium with thermal treated wheat flour extracts (LFM+WFETx, with x the temperature of the treatment). The WFETx were incubated for 1h in a water bath at 40°C (gray), 60°C (yellow), 70°C (orange) and 90°C (blue). From 70°C on, the inhibition activity seemed lost. (B) The fermentation capacity of Be05 in gluten-starch dough with wheat flour extract treated at 70°C (GSD+WFET70, orange). In contrast to in liquid fermentation medium, 70°C did not seem high enough to eliminate the inhibition activity completely. In (A) and (B) the green and red curves resp. represent the negative (LFM of GSD) and positive (LFM+WFE or GSD+WFD) control.*

3.3.3 Concentration dependency of the inhibitor(s)

Because the activity of toxins is concentration-dependent, the question arose as to how sensitive the fermentation capacity of inhibition-sensitive yeasts would be to dilution of the inhibitor(s). Therefore, mixtures of wheat flour and gluten-starch blends were made and the fermentation capacity of the strongest inhibited yeast Be05.

The impact replacing quantities of gluten-starch blend by wheat flour dough on the fermentation capacity of the inhibition-sensitive Be05 was examined. The negative control was a gluten-starch dough with water, the positive control a wheat flour dough. A significant drop in fermentation rate was observed when replacing only 10% of the gluten-starch blend by wheat flour (**Figure 3.6A**). The inhibition-sensitive Be05 strain reached barely 30% of its fermentation capacity in the negative control after five hours of fermentation – the time needed by the yeast to consume all carbon sources in the negative control dough (**Figure 3.6B**). A further decreasing trend could be recognized until a plateau was reached when 40% and more of the gluten-starch blend was exchanged for wheat flour. From that concentration on, increasing amounts of the inhibitor(s) did not have any additional effect.

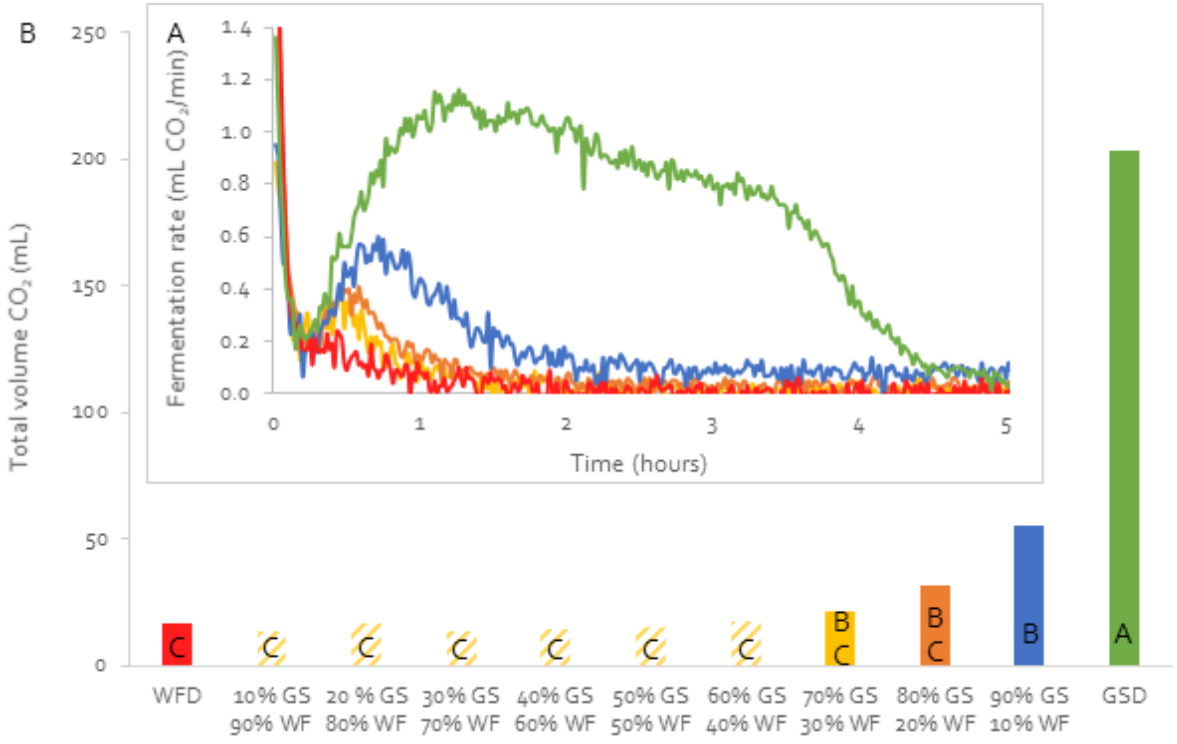

***Figure 3.6**: Fermentation capacity of the inhibition-sensitive Be05 strain in doughs made with a mixture of a gluten-starch blend (GS) and wheat flour (WF). (A) shows the fermentation profiles (mL CO_2/min) in function of time (hours) during 5 hours of fermentation, (B) displays the total amount (mL) of CO_2 after this period. Columns with different letters are significantly different from each other ($p < 0.05$).*

3.3.4 Presence in other wheat varieties and cereal grains

Pathogenesis-related proteins are by definition "encoded by the host plant but induced upon pathogen attack or related situations", like wounding or mechanical injury (Antoniw et al., 1980b; Vidhyasekaran, 2008). However, it should be noted that some of them can also be constitutively expressed or occur in healthy plant tissues, for example roots or seeds, when another part of the

plant, such as the leafs, has been attacked (Datta and Muthukrishnan, 1999; Sinha et al., 2014; Vidhyasekaran, 2008). As is also the case with premature yeast flocculation and gushing of the final beer product, some events occurring during the production process or in the final product, are direct results of accidental fungal infections in the field which might elevate antimicrobial activity in the grains (van Nierop et al., 2008). To make sure that the inhibition in this study was no happenstance, caused by fungal attack of this specific crop in the field, different wheat varieties were tested for their yeast inhibition activity. **Figure 3.7A** clearly shows that, no matter which wheat variety was used for dough making, the inhibition-sensitive Beo5 was not able to ferment properly. Compared to the inhibition-insensitive Bro4, the total volume of CO_2 produced by Beo5 was at least five times lower, indicating severe inhibition.

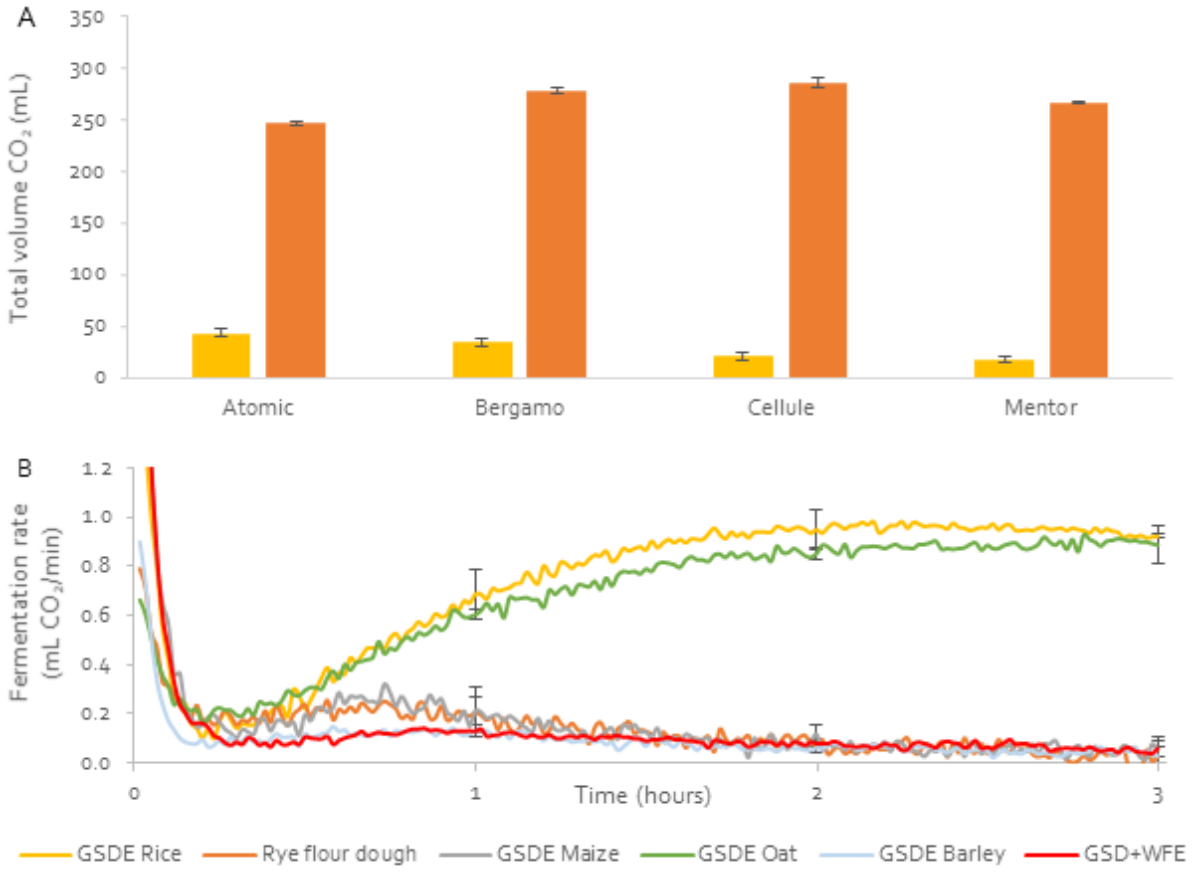

***Figure 3.7**: (A) Total volume of CO_2 (mL) produced after five hours of fermentation in dough with different wheat varieties (Atomic, Bergamo, Cellule, Mentor). The yellow columns represent the inhibition-sensitive Beo5, which is almost unable to ferment dough of different wheat varieties. The orange columns the inhibition-insensitive Bro4. (B) Fermentation capacities (mL CO_2/min) in function of time (hours) of the inhibition-sensitive Beo5 in gluten-starch dough with extracts (GSDE) made of rice (yellow), maize (gray), oat (green) and barley (bright blue) flour, in rye flour dough (orange) and in gluten-starch dough with wheat flour extract (GSD+WFE, red). Only rice and oat did not contain any inhibition activity. The vertical error bars represent the standard deviations of three biological replicates.*

Going back to the study of yeast fermentation inhibition by wheat proteins in the early seventies, Okada and coworkers (1970) only noticed toxicity for yeast in wheat and barley. The other cereals tested, amongst which maize, rice, oat and rye, did not seem to be harmful for yeast fermentation. However, in this study similar fermentation inhibition of strain Be05 was noticed in rye flour dough. Because it was impossible to make proper doughs with maize, rice, oat or barley flour, extracts were made and their inhibition activity on the inhibition-sensitive Be05 strain was tested in a model gluten-starch dough. In gluten-starch dough with maize flour extract, Be05 was inhibited to the same extent as it was in wheat flour dough (**Figure 3.7B**). Extracts of rice and oat flours did indeed not give any inhibition after adding it to gluten-starch dough, as observed by Okada and coworkers (1970). Barley inhibited the inhibition-sensitive Be05 to the same extent as wheat. The fermentation capacity of the inhibition-insensitive Br04 was also tested in the different dough systems, but – as expected – no inhibition could be noticed.

3.3.5 Cell wall binding

One of the major antifungal mechanisms of PR proteins is the permeabilization of the cell membrane or breakdown of the cell wall. In a next experiment, yeast cells of the inhibition-sensitive Be05 were inactivated (after harvesting, the yeast pellet was suspended in water, the suspension was kept at 80°C for 15 minutes, the supernatant was removed by centrifugation) and additionally mixed into a yeasted dough. The purpose was to examine substrate competition, meaning that the addition of yeast cell material would enable the active yeast to function properly while the majority of the inhibitor(s) would bind to the inactive yeast cell walls. Therefore, wheat flour was mixed with the inactivated yeast cells and half of the water for one minute, followed by a resting period of five minutes to allow interaction between the inhibitor(s) and the inactive yeast cell wall, after which the rest of the ingredients (half of the water, sugar, salt and active yeast) was added and mixed for two minutes. The different amounts of inactive yeast added were one (0.53 g), two (1.06 g), five (2.65 g) and ten (5.3 g) times the amount of active yeast normally added to dough. The two lowest concentrations were insufficient to notice potential cell wall binding capacity of the inhibitor(s) and showed no difference with the positive control (**Figure 3.8**). Addition of 2.65 g inactive yeast initially had no influence on the fermentation rate. Five hours after the start of the fermentation, however, the active yeast seemed to have regained its fermentation power and reached a maximum rate of 0.4 mL CO_2/min. A larger effect could be noticed when the highest concentration of inactive yeast was added. When 5.3 g of inactive yeast was mixed into the dough, the fermentation

capacity reached a higher initial rate (0.6 mL CO_2/min instead of less than 0.4 mL CO_2/min), followed by a fast drop down to 0.2 mL CO_2/min. After three hours of fermentation, the rate started to increase again until a maximum of 0.6 mL CO_2/min was reached after almost eight hours of fermentation. Although it could not unambiguously be concluded whether or not the inhibitor(s) was(were) bound to inactive yeast cells because of the inhibition activity that was still initially present in all of the cases, addition of an excess dead yeast cell material clearly had an impact on the fermentation capacity of the inhibition-sensitive yeast strain in wheat flour dough. It should be noted that thermal inactivation of yeast cells could cause leakage of internal cell material, such as vitamins, potentially having positive effects on the yeast fermentation metabolism. To take this into account, the supernatant obtained by harvesting the inactivated yeast pellet, was added to wheat flour dough instead of water (results not shown). Addition of this aqueous fraction did not have any positive effect on the fermentation capacity of the inhibition-sensitive Be05.

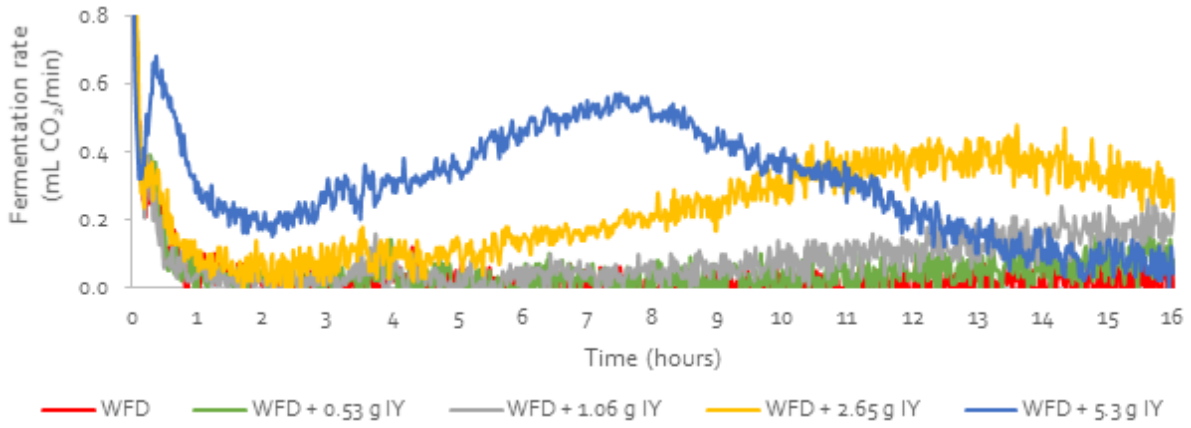

***Figure 3.8**: Fermentation rate (mL CO_2/min) in function of time (hours) of the inhibition-sensitive Be05 in wheat flour dough (WFD) with addition of inactive yeast cells (IY). The concentrations IY added to dough were 0.53 g (green), 1.06 g (gray), 2.65 g (yellow) and 5.3 g (blue), resp. one, two, five and ten times the amount of active yeast for dough preparation.*

The above described characteristics of the inhibitor(s) and the corresponding comparisons with scientific literature allow to deduce that the inhibitory component(s) is(are) dissimilar to the components previously described in this context. It is not clear either whether only the weak fermenters as identified in this study are affected or whether also strong fermenters are affected, be it to smaller extent.

3.4 Conclusion

The characteristics of yeast strain specific inhibition by a water extractable wheat flour constituent, described in the previous chapter, were studied in more detail. Not only the fermentation capacity of the weakly fermenting yeast strains was inhibited, also their growth encountered some hindrance. With a delay of almost 24 hours, however, the yeast cells were able to grow to the same $OD_{600\,nm}$ as in an inhibitor-free growth medium. This suggested that the metabolism of yeast was disabled, but apparently only temporary. After a while, the yeast seemed to gain a certain degree of tolerance towards the inhibitor(s). Breakdown with a non-specific protease confirmed the presumption that the inhibition was caused by a protein(s). Examination of the thermal treatment was, however, not unambiguous. In a liquid fermentation medium, the activity seemed to be lost from 70°C on whereas this temperature was not sufficient to eliminate the inhibition in a dough system. The susceptibility of the yeast for inhibition was higher in dough than in a liquid medium, making the latter unreliable for further inhibition-screening. Although no dose-response curves were determined, the dilution experiment showed that only small amounts of the inhibitor(s) (10% wheat flour in a gluten-starch blend or replacement of one fourth of the water by extract in gluten-starch dough) was enough to reduce the fermentation capacity with more than 70%. The inhibition was also not wheat variety-dependent and did not only occur in wheat. Of the five different cereals we screened, inhibition was established in rye, maize and barley, but not in rice and oat. Although all these results taught us more about the characteristics of the inhibitor(s), we still did not have an idea about the identity or working mechanism. This is investigated in the next chapter.

Chapter 4.

Activity-guided fractionation of a wheat extract and identification of a novel yeast strain specific fermentation inhibitor in wheat

This chapter is based on the following reference: Van der Maelen, E., Rezaei, M.N., Struyf, N., Proost, P., Verstrepen, K.J., Courtin, C.M. (2019). Identification of a wheat thaumatin-like protein that inhibits Saccharomyces cerevisiae. Journal of Agricultural and Food Chemistry, 67 (37): 10423-10431.

4.1 Introduction

The study presented in Chapter 2 revealed yeast specific fermentation inhibition by water extractable wheat flour components in dough. Characterizing the yeast strain specific fermentation inhibitors in the previous chapter showed that they also impacted yeast growth, were thermosensitive, were also present in other wheat varieties and cereals and – most importantly – were proteins.

The presence of pathogenesis-related (PR) proteins in plants and cereals, protecting them against invaders during growth on the field, is well-known and well-described in scientific literature (Ebrahim et al., 2011; Muthukrishnan et al., 2001; Sudisha et al., 2012). In general, PR proteins can defend a plant against pathogens through direct or indirect mechanisms. Direct mechanisms imply the breakdown of the fungal cell wall by β-1,3-glucanases and chitinases or permeabilization or disruption of fungal cell membranes (Ebrahim et al., 2011; Selitrennikoff, 2001). Indirect protection includes fortification of the host's cell wall by lignification and cross-linking of cell wall components, thereby complicating invasion by the unwanted visitor (Lagrimini et al., 1987; Vidhyasekaran, 2008). An overview of the different classes of PR proteins is provided in **Table 1.2** (Chapter 1).

Although these proteins are beneficial during development and growth of plants and cereals, they might hamper efficient processing of the raw materials or cause allergic reactions after consumption (Sinha et al., 2014). Thaumatin-like PR proteins and PR chitinases in grapes are for example known to cause a precipitate in white wine and champagne (Manteau et al., 2003; Van Sluyter et al., 2009; Waters et al., 2005, 1996). Lipid-transfer proteins from malting barley grain were found to decrease fermentation and respiration of *S. cerevisiae* brewer's yeast *in vitro*, but did not seem to have any effect during the brewing process (Gorjanović et al., 2004).

The inhibition of yeast by wheat components was already described in 1895, when Jago noticed the inability of brewer's yeast strains to ferment a wheat flour batter (Jago, 1895). Five decades later, Balls and coworkers (1940) observed that, while the baking quality of wheat flour was improved when it was extracted with petroleum ether, addition of the recovered extract lowered the quality of the final bread again, probably by affecting yeast fermentation. They identified a high sulfur-containing protein in the extract and were the first to call it *purothionin* (Balls and Hale, 1940; Balls et al., 1942a, 1942b). According to Stuart and Harris (1942), this protein had antibacterial and antifungal activity against gram-positive and gram-negative bacteria, *S.*

cerevisiae and other fungi. In the case of *S. cerevisiae*, growth of strains applied in the bakery, brewery and winery were examined. The antifungal compound slowed down cell growth at low concentrations and killed yeast cells at higher concentrations, including the bakery strain that was studied (Stuart and Harris, 1942). As already mentioned in Chapter 3, comparable research was done by Okada and Yoshizumi (1973) on *S. carlsbergensis*, revealing that the toxicity of an undefined toxic wheat protein depends on its concentration. Low concentrations of the protein (about 0.4 ppm) would only inhibit uptake of sugars, leaving the yeast cells alive, whereas high concentrations (4 ppm) would cause cell death within a few minutes. In a previous study, these authors, however, doubted that the toxic substance they investigated was a purothionin based on differences observed regarding the concentration of inhibitor and the amino acid composition (Okada and Yoshizumi, 1970). Hernandez-Lucas and coworkers (1974), on the other hand, started from the observations of Balls and coworkers (1944) that purothionins have antimicrobial properties, to investigate the impact of wheat purothionins on top and bottom fermenting brewery yeast strains.

In contrast to the study by Stuart and Harris (1942), the results discussed in the previous chapter pointed out a certain robustness of the tested bakery and winery yeasts against fermentation inhibition by water extractable wheat flour components. Although there are similarities between the studies of Balls and coworkers (1940; 1942a, 1942b) and Stuart and Harris (1942) on the one hand, and those of Okada and coworkers (1973, 1970; 1970) on the other hand, the latter research group has doubted some of the outcomes of the former.

Because of the inconsistencies within literature and between literature and our results, the inhibitor(s) we encountered in Chapters 2 and 3 was(were) purified, starting from a crude wheat flour extract, and identified. This chapter reports on the outcome of this work.

4.2 Materials and methods

4.2.1 Materials

Wheat grain was provided by EVD (Jodoigne, Belgium). The grains were milled into flour with a Bühler MLU-202 laboratory mill (Bühler, Uzwil, Germany) with a yield of 69.4%. Commercial wheat gluten (protein content: 78.6% dm; moisture content: 4.8%) and wheat starch (moisture content: 11.1%) were provided by Tereos Syral (Aalst, Belgium). Two industrial *S. cerevisiae* strains, one bakery and one brewery strain, were obtained from the collection of the VIB Laboratory for Systems Biology (KU Leuven, Belgium). Bacteriological agar, yeast extract,

balanced peptone and tryptone were from Lab M (Brussels, Belgium), kanamycin from MP Biomedicals (Brussels, Belgium) and commercial sugar and salt from Tiense Suiker (Tienen, Belgium) and Everyday (Gellingen, Belgium) respectively. All other chemicals, reagents and solvents were of analytical grade and purchased from Sigma-Aldrich, unless specified otherwise.

4.2.2 Flour characterization

The characteristics of the wheat flour used in this study were previously described in Section 2.2.2.

4.2.3 Preparation of yeast cells for dough fermentation

Yeast cells were prepared as described in Section 2.2.3.

4.2.4 Preparation of flour extracts

The extracts of wheat flour were prepared according to the procedure in Section 2.2.4.

4.2.5 Dough making

Wheat flour and gluten-starch dough with water or (fractions of) wheat flour extract were prepared according to the AACC International Approved Method 10-10.03, as described in Section 2.2.6. In case of the gluten-starch doughs with wheat flour extract, the 57.0% (v/w) of water was replaced by the extract.

4.2.6 Gas production analysis

The gas production during fermentation in dough and fermentation medium was measured using the Risograph as described in Section 2.2.7.

4.2.7 Purification of the inhibitor

4.2.7.1 Cation exchange chromatography

The first step in the purification procedure for the yeast inhibiting protein was performed by cation exchange chromatography (CEC). A crude wheat flour extract (Section 2.2.4) was diluted with an equal volume of a 50 mM sodium acetate buffer (pH 4.0) such that buffer conditions of the sample were the same as those of the starter buffer A (25 mM sodium acetate, pH 4.0). The extract was loaded on a column with a sulfopropyl sepharose fast flow (SP SFF) resin (GE Healthcare, Uppsala, Sweden). Positively charged proteins in the sample at pH 4.0 could bind to the negatively charged column matrix. After sample loading, the column was rinsed with buffer A. The bound proteins were subsequently eluted using an elution buffer B (25 mM sodium acetate, 1 M NaCl, pH 4.0) at a flow rate of 1 mL/min. Based on preliminary optimization of the

elution gradient, the bound proteins were either (i) eluted with a step gradient of 15%, 22%, 35%, 50% and 100% of elution buffer B, complemented with buffer A or (ii) gradually eluted from 0-22%, followed by a step at 100% of elution buffer. The stepwise elution was spread over 20 mL, the gradual elution over 160 mL. The eluted proteins were detected with a UV detector at 280 nm. The eluate was captured in fractions of 3 mL. The eluted fractions containing proteins with similar charges covered by one peak in the UV spectrum, were pooled per peak and the inhibition activity was tested in gluten-starch dough. This purification step and the subsequent inhibition activity test were performed three times on three different days.

4.2.7.2 Centrifugal filtration

Wheat flour extract or purified fractions thereof were concentrated using Amicon Ultra centrifugal filters (Merck Millipore, Darmstadt, Germany) with a volume of 0.5 mL or Vivacell 70 concentrator body (VWR, Leuven, Belgium) with a volume of 70 mL. The tested cut offs were 5 kDa, 10, kDa, 30 kDa and 50 kDa for the former type of filters and 10 kDa for the latter. On the small volume filters, 400 µL of sample was applied and centrifuged three times for 30 min at 12,000 g with an Eppendorf centrifuge 5415D (Eppendorf AG, Hamburg, Germany). With the high volume filters, 30 mL of sample was centrifuged twice for 25 min at 1,000 g. In both cases the sample was concentrated at least 5 times. The inhibition activity of both filtrate and retentate were tested in gluten-starch dough, the fermentation capacity was measured with a Risograph as described in Section 2.2.7.

4.2.7.3 Reversed phase-high performance liquid chromatography coupled to mass spectrometry

After concentration of the eluted CEC fractions with centrifugal filters, the retentate in which inhibition activity was tested, was further analyzed by means of reversed phase-high performance liquid chromatography (RP-HPLC) using a Waters 600 HPLC system (Waters Corporation, Milford, USA). Separation of the proteins in the sample was carried out with a Proto 300 C4 HPLC column (150 x 4.6 mm) (Higgins Analytical, USA) and gradually eluted with 0 to 80% (v/v) of acetonitrile, complemented with a buffer of 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.0 mL/min. Proteins in the eluted fractions were detected with a Waters 486 Absorbance Detector (Waters Corporation) at 214 nm and collected in amounts of 1.0 mL. Immediately after leaving the column, a small fragment of the eluate was sent through a splitter to a mass spectrometer (Amazon SL, Bruker Daltonics, Bremen, Germany) for mass analysis of the proteins in the sample. The eluted fractions were pooled (11 groups of 5 fractions) and their inhibition activity was tested in gluten-starch dough.

4.2.8 Identification of the inhibitor

4.2.8.1 Protein electrophoresis

Proteins present in the fractions were separated based on their molecular mass with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two types of gels and accompanying devices were used for separation: (i) a 12% Mini-Protean TGX Precast polyacrylamide gel (BioRad, Nazareth, Belgium) using a BioRad Mini-Protean separating device; (ii) a 20% homogeneous polyacrylamide Precast Phastgel (GE Healthcare, Uppsala, Sweden) using a Phast System (GE Healthcare). Five microliters of sample buffer (125 mM Tris-HCl pH 6.8, 4.0% (w/v) SDS, 30% (w/v) glycerol and 0.002% (w/v) bromophenol blue) was added to 20 µL of sample. This was boiled for 5 min and centrifuged for 3 min at 11,000 g with an Eppendorf centrifuge 5415D (Eppendorf AG). Of the resulting supernatant, 20 µL and 6 µL were loaded onto the Mini-Protean and the Phastgel, respectively. In both cases, 6 µL of a molecular marker mixture (Thermo Fisher, Merelbeke, Belgium) was applied. This marker mixture contained the following proteins: insulin B chain (4 kDa), aprotinin (6 kDa), lysozyme (16 kDa), myoglobin red (22 kDa), carbonic anhydrase (36 kDa), alcohol dehydrogenase (50 kDa), glutamic dehydrogenase (64 kDa), BSA (98 kDa), phosphorylase (148 kDa) and myosin (250 kDa). Electrophoresis using a Mini-Protean system (Biorad, Nazareth, Belgium) was performed at 160 V for approximately 90 min at room temperature. The electrode buffer contained 25 mM Tris, 0.1% w/v SDS and 182 mM glycine at pH 8.5. Electrophoresis using a Phast System was carried out in accordance with the operating instructions of the provider (GE Healthcare). Mini-Protean gels were stained with a Coomassie staining buffer (0.125 g Coomassie blue R 250, 200 mL methanol and 35 mL acetic acid adjusted to 500 ml with deionized water) and destained (7% (v/v) acetic acid and 5% (v/v) ethanol). The Phastgels were silver stained according to the instructions of the manufacturer described in Development Technique File no. 210 (GE Healthcare).

4.2.8.2 Protein sequencing

ELECTROBLOTTING AND EDMAN DEGRADATION

Electroblotting was performed with the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (BioRad). The proteins from within a non-stained precast polyacrylamide gel were transferred onto a polyvinylidene difluoride (PVDF) membrane. The gel was three times 10 min equilibrated in blot buffer (48 mM TRIS, 39 mM glycine and 20 % methanol). The PVDF membrane was consecutively equilibrated in methanol (2 sec), deionized water (2 min) and blot buffer (15 min) before application. The gel and the PVDF membrane were put into the device between two filter

papers, soaked in blot buffer. An electric potential difference of 15 V was applied for 40 min between the anode and cathode of the blot machine. Afterwards, the membrane was stained with Coomassie. Determination of the N-terminal amino acid sequence of the most abundant proteins present in the different bands separated on gel was performed with Edman degradation using a Procise cLC 491 Sequencer (Applied Biosystems, Foster City, California, USA).

LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Coomassie-stained bands of interest were excised from the gels and in gel digested with trypsin using the protocol developed by Shevchenko and coworkers (1996). The following LC-MS/MS analysis and data analysis was performed according to the procedure described by Holtappels and coworkers (2015). In short, the analysis was performed on an Easy-nLC 1000 liquid chromatograph (Thermo Scientific) on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Scientific) by a Nanospray Flex ion source (Thermo Scientific). The tryptic digested sample was first loaded, concentrated and desalted on a pre-column at a buffer A (0.1% v/v formic acid in Milli-Q water) flow rate of 5 µL/min for 5 min with subsequent separation on an Acclaim PepMap RSLC C_{18} column (Thermo Scientific). The elution was done at a flow rate of 250 nL/min using a linear gradient going from 0% to 70% buffer B (0.1% v/v formic acid in acetonitrile) over a time span of 40 min. The MS data were obtained with Xcalibur software (version 2.2.SP1.48). Data analysis was done using Proteome Discoverer software v.1.2 (Thermo Scientific) with build-in Sequest v.1.4.0288 and interfaced with an in-house Mascot v.2.5 server. The obtained MS/MS spectra were searched against protein collections extracted from NCBI database with query *Triticum aestivum*. The results were automatically evaluated in Scaffold v.4.8.9 (Holtappels et al., 2015).

4.2.9 Recombinant protein expression in *E. coli* and preparation of cell lysates

The gene coding for the suspected inhibiting wheat flour protein was synthesized, introduced in a pET28a(+) plasmid expression vector and expressed in *E. coli* (BL21). This was done by Qinglan (Yixing, Jiangsu, China). Single colonies of *E. coli* cells were grown in 5 mL Lysogeny Broth (LB), composed of 1.0% (w/v) tryptone, 0.50% (w/v) yeast extract and 1.0% (w/v) sodium chloride in deionized water. Because the pET28a(+) vector contains a gene for kanamycin resistance, kanamycin was added in a final concentration of 100 µg/mL to the LB to avoid growth of *E. coli* cells that did not take up the plasmid successfully. These precultures were grown overnight (37°C, 250 rpm) and transferred to Erlenmeyers at a ratio of 1:100. The OD of this suspension was followed up at 600 nm until an OD of 0.5-0.6 was reached. At the desired OD of 0.5-0.6, IPTG was

added to induce protein expression and the suspension was further incubated at 18°C and 250 rpm up until harvesting (3,700 rpm, 20 min, 4°C) the next day. The resulting pellets, derived from 50 mL of cell culture, were kept on ice and resuspended in 2 mL lysis buffer (50 mM sodium dihydrogen phosphate dehydrate and 300 mM sodium chloride, pH 7.4). Subsequently, 50 µL of a 40 mM protease inhibitor solution (47.92 mg Pefablock in 5.0 mL deionized water) and 80 µL of the 25 mg/mL lysozyme solution were added and incubated on ice for 30 min. The enzymatic lysis of the cells was followed by three freeze-thaw-cycles and sonication of the cells (4 x 15 sec). The sonicated cell suspension was distributed over 2 mL Eppendorf tubes and centrifuged at 13,000 rpm for 20 min at 4°C. The protein of interest is located in the supernatant. To test the inhibition activity of the recombinant protein, the lysis buffer was first mostly removed from the supernatant by centrifugal filtration (3 kDa cut off). The retained fraction (ten times concentrated) was then diluted five times with deionized water and added to gluten-starch dough as the aqueous phase. The fermentation capacity was examined using a Risograph.

4.3 Results and discussion

4.3.1 Purification of the inhibitor

As the yeast inhibiting wheat flour protein we were looking for was so far unknown, an activity-guided fractionation was performed starting from a crude wheat flour extract. A series of chromatographic techniques was applied and in between, the inhibition activity of the obtained fractions on both the inhibition-sensitive Be05 and the inhibition-insensitive Br04 was examined with the Risograph in gluten-starch dough. The fermentation capacity of Br04 was measured and considered as a control.

4.3.1.1 Cation exchange chromatography

Crude wheat flour extract was loaded on a CEC column and the bound components were stepwise eluted as described in Section 4.2.7.1. The elution profile is displayed in **Figure 4.1A**. The eluted fractions were pooled into four groups based on the peaks in the elution profile (represented by the numbers on the graph in **Figure 4.1A**). Because the high salt levels could be harmful for the yeast cells when the eluted fraction were tested as such, the eluates were first filtered by centrifugal filtration with a cut off of 3 kDa to remove the majority of this buffer. The inhibiting protein was assumed to be retained by the filter. The retained fractions were 1.2 times diluted with water, compared to the original grouped CEC fractions. The activity of these fractions was tested in gluten-starch dough with the two representative yeast strains, Br04 and

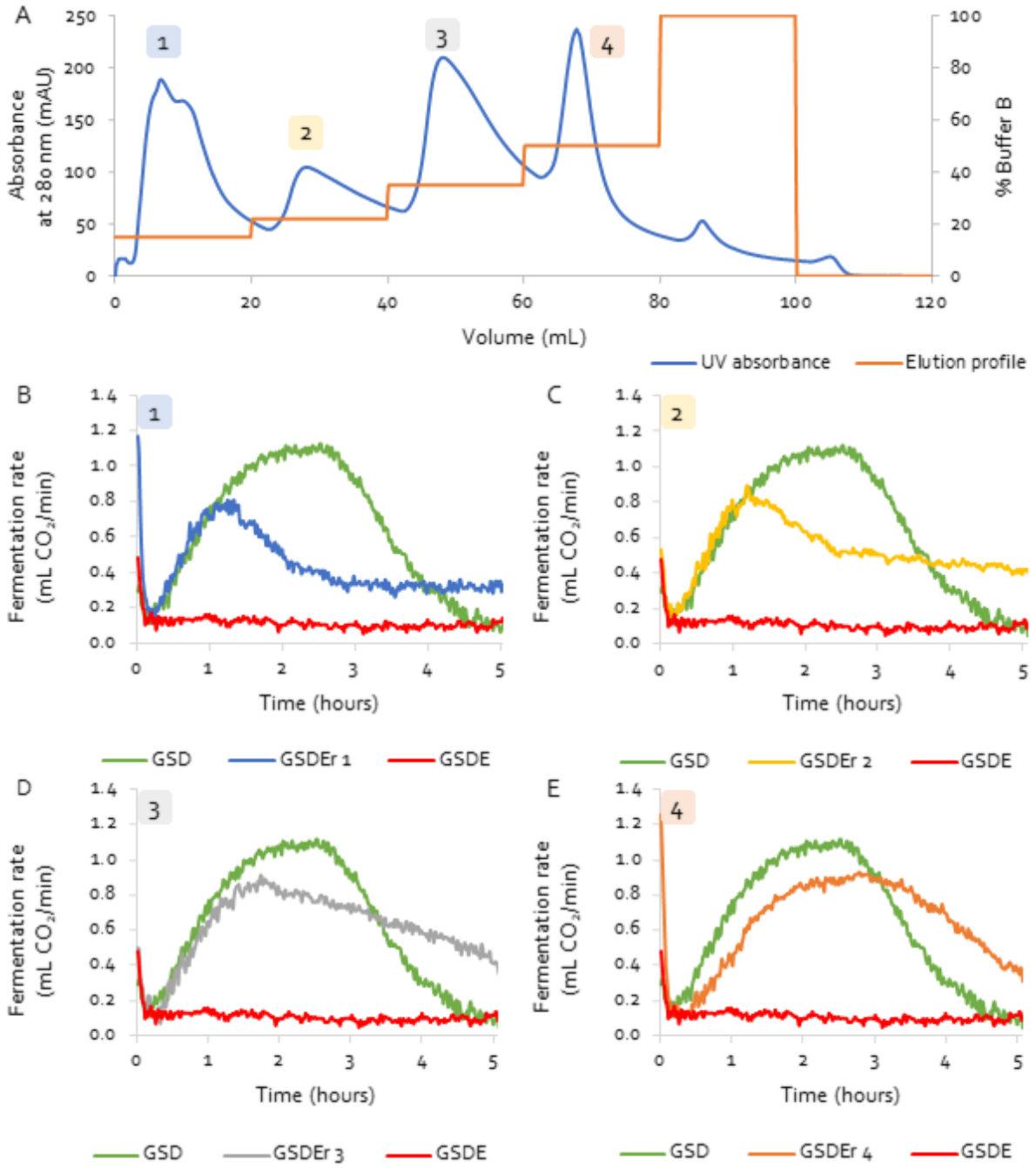

***Figure 4.1**: (A) Chromatogram of the elution of the CEC with stepwise increasing concentration of elution buffer B. The percentage of buffer B in the eluent is represented by the orange curve, with steps at 15%, 22%, 35%, 50% and 100% and can be read from the right vertical axis. The absorbance at 280 nm (mAU) is represented by the blue curve and can be read from the left vertical axis. Each step was spread over 20 mL and matched one peak of proteins eluted with a certain concentration of buffer B. The numbers resemble the pooled fractions of which the inhibition activity was tested in gluten-starch dough. (B-E) Fermentation rate (mL CO_2/min) in function of time (hours) of Be05 in gluten-starch dough without extract (GSD, green) as positive control, with extract (GSDE, red) as negative control, with the retained fraction (GSDEr x, blue, yellow, gray and orange) after centrifugal filtration of the CEC protein fractions, with x the respective pool of fractions coinciding with the protein peaks in the elution chromatogram.*

Be05. The fermentation profiles of the inhibition-sensitive Be05 are presented in **Figures 4.1B-E**. Because the bakery strain Br04 experienced inhibition with none of the fractions and had comparable fermentation profiles in the positive and negative control (gluten-starch dough with and without wheat flour extract resp.) and in the retained CEC protein fractions, the Br04 results are not shown.

More interesting were the gluten-starch doughs to which the retained fractions were added, especially the ones coming from the first and second CEC protein peak (**Figures 4.1B** and **C**). Although the fermentation rates were not coinciding with the positive control, there was definitely a difference with the negative control. The total volume of CO_2 produced after 5 hours in the doughs with retained fractions of CEC protein peaks 1 and 2 were significantly different from the negative control, whereas the CO_2 production of Be05 in ones with retained fractions of CEC protein peaks 3 and 4 did not differ from that control. This suggested that the inhibitor was eluted from the negatively charged SP SFF column with a NaCl concentration of maximally 0.22 M. However, it was remarkable that the fermentation profile of the inhibition-sensitive yeast in gluten-starch dough with partially purified wheat flour extract was completely different from the one with the crude wheat flour extract. This could be ascribed to dilution of the tested samples compared to the crude extract (**Figure S1** in Appendix). It was also presumed that part of the inhibitor was lost after the first purification step because chromatographic separations often do not result in a 100% yield. Beside this, another possible reason could be that the inhibitor was separated from other non-toxic synergistically acting wheat flour components (Hernandez-Lucas et al., 1974). From now on during the purification process of the inhibitor, fermentation profiles comparable with those of Be05 in gluten-starch dough with the retained fractions of CEC protein peaks 1 and 2 are assumed as profiles showing inhibition.

As could be deduced from the elution chromatogram (**Figure 4.1A**), the four protein peaks were not completely separated from each other as the absorbance between two peaks did not drop down to zero. Therefore, an attempt was made to separate them by adapting the elution profile. Unfortunately, the elongation of the elution did not have the desired effect (**Figure 4.2A**). The eluted fractions were however pooled into 5 groups, and filtered with 10 kDa cut off centrifugal filters. It had been proven that the inhibitor was also retained by this cut off (results not shown). The higher the cut off, the more we could get rid of unneeded components. The inhibition activity on Be05 of the retained fractions was tested in gluten-starch dough (**Figure 4.2B-F**). Only in the fifth CEC protein peak there was almost no inhibition noticed (**Figure 4.2F**). All the other

fermentation profiles, especially the ones with the retained fractions of peaks 1, 3 and 4, were typical inhibition profiles. The dilution scheme can be found in **Figure S2** in Appendix.

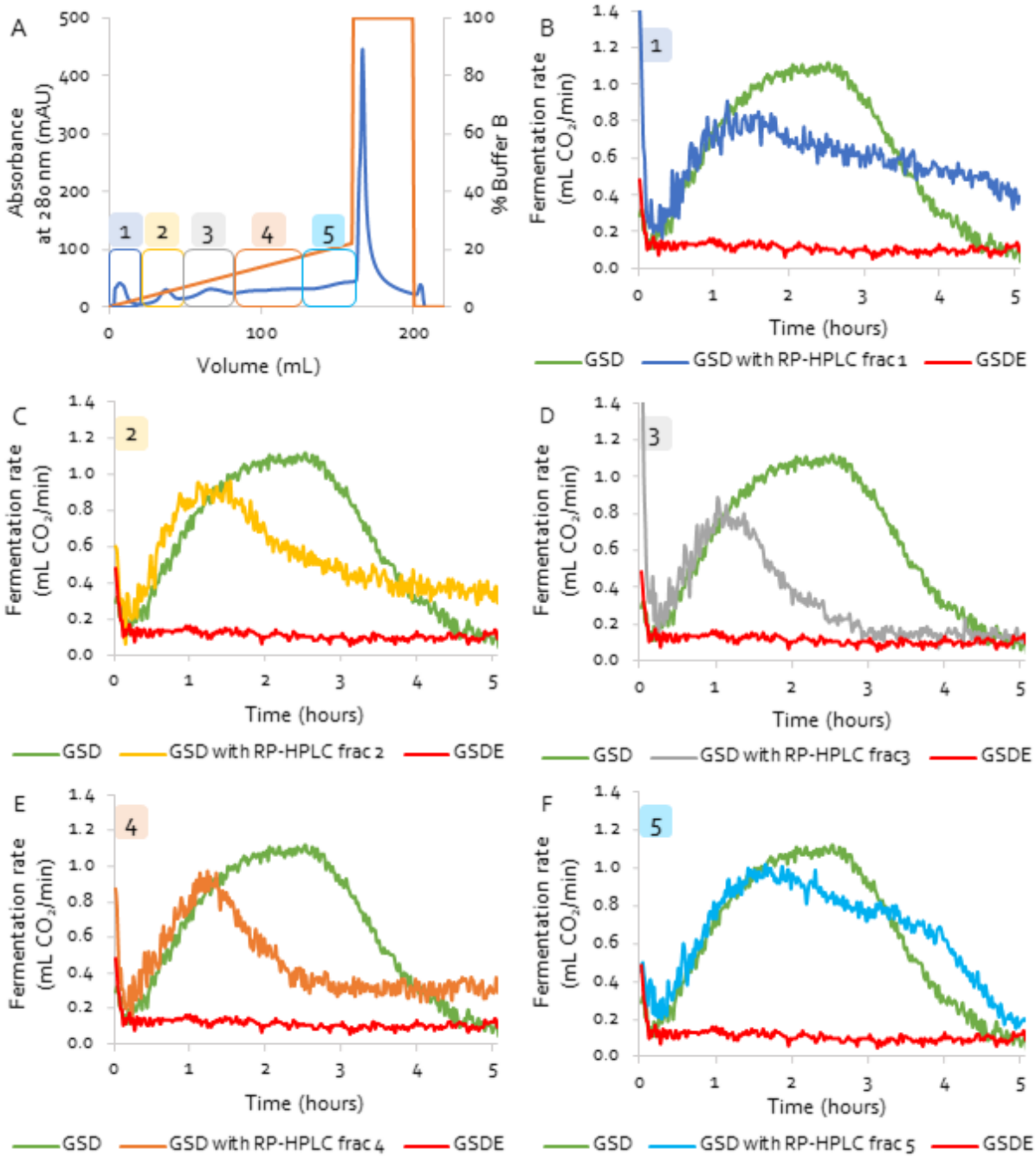

***Figure 4.2**: (A) Chromatogram of the elution of the CEC with gradually increasing concentration of the elution buffer B from 0% to 22%. The elution profile (% buffer B) is represented in orange and can be read from the right vertical axis. The absorbance at 280 nm (mAU) is given by the blue curve and can be read from the left vertical axis. The gradual elution was spread over 160 mL. The eluted fractions were pooled into five groups (1 to 5 on the graph) of which the inhibition activity was tested in gluten-starch dough. (B-F) Fermentation rate (mL CO_2/min) in function of time (hours) of Be05 in gluten-starch dough without extract (GSD, green), with extract (GSDE, red), and with the retained fraction (GSDEr x, dark blue, yellow, gray, orange and bright blue) after centrifugal filtration of the CEC protein fractions, with x the respective pool of fractions (indicated on the elution chromatogram).*

4.3.1.2 Reversed phase-HPLC

No single peak or pool of eluates could be singled out as inhibitor containing fractions after cation exchange chromatography. To proceed, the complete eluate (160 mL) of the gradual elution was taken together and ten times concentrated with a 10 kDa centrifugal filter. Subsequently, the concentrated protein fraction was loaded on a C_4 column for RP-HPLC analysis. The bound proteins were eluted with a gradient of 0% to 80% acetonitrile in deionized water with 0.1% trifluoroacetic acid (pH of approximately 2.1) and collected in fractions of 1.0 mL. Because the proteins were highly concentrated, the chromatogram did not show clearly aligned protein peaks. The eluted fractions (0.5 mL of each) were consequently pooled five by five into eleven groups of 2.5 mL. The solvent was evaporated with a rotational vacuum concentrator (1 mbar, 35°C, 4 hours). The residues were then redissolved in 6.0 mL deionized water and tested for inhibition activity on the Be05 yeast in gluten-starch dough. Of the ten times concentrated fraction (as loaded on the column), 2.5 mL was also diluted with deionized water to a total volume of 6.0 mL. Compared to the original CEC eluate, this sample was thus 1.2 times concentrated (**Figure S3** in Appendix). The inhibition activity was tested on Be05 in gluten-starch dough as the positive control. The fermentation profiles of the eleven groups, together with the positive and negative control are displayed in **Figure 4.3**.

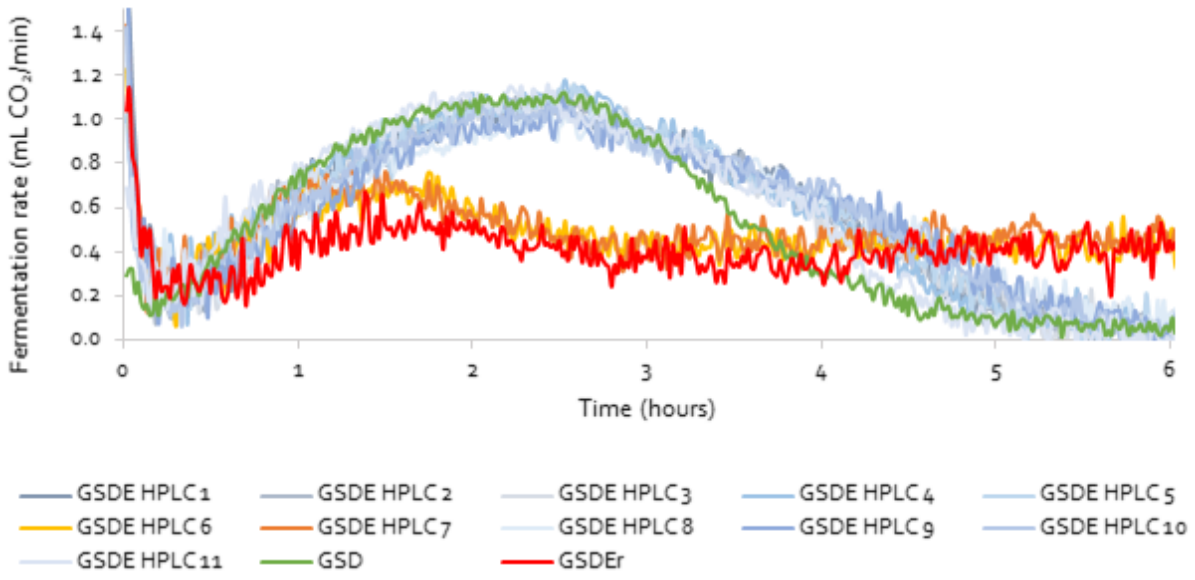

***Figure 4.3**: Fermentation capacity (mL CO_2/min) in function of time (hours) of Be05 in gluten-starch dough without wheat flour extract (GSD, green), with the concentrated elution (GSDEr, red), with non-inhibiting RP-HPLC fractions (GSDE HPLC 1-5; 8-11, shades of blue) and with inhibiting RP-HPLC fractions (GSDE HPLC 6, yellow and GSDE HPLC 7, orange).*

For nine out of the eleven groups the inhibition-sensitive Beo5 displayed a fermentation capacity completely comparable with the negative control, indicating the absence of inhibition. Use of pools 6 and 7, however, resulted in a fermentation profile similar to the positive control. As each pool was a combination of five fractions, each fraction was tested individually in order to get the most pure fractions containing the inhibitor. Similarly to the previous experiment, 0.4 mL of each fraction was dried and the residue was dissolved in 6 mL deionized water after which it was added to dough for the inhibition activity test (**Figure 4.4**).

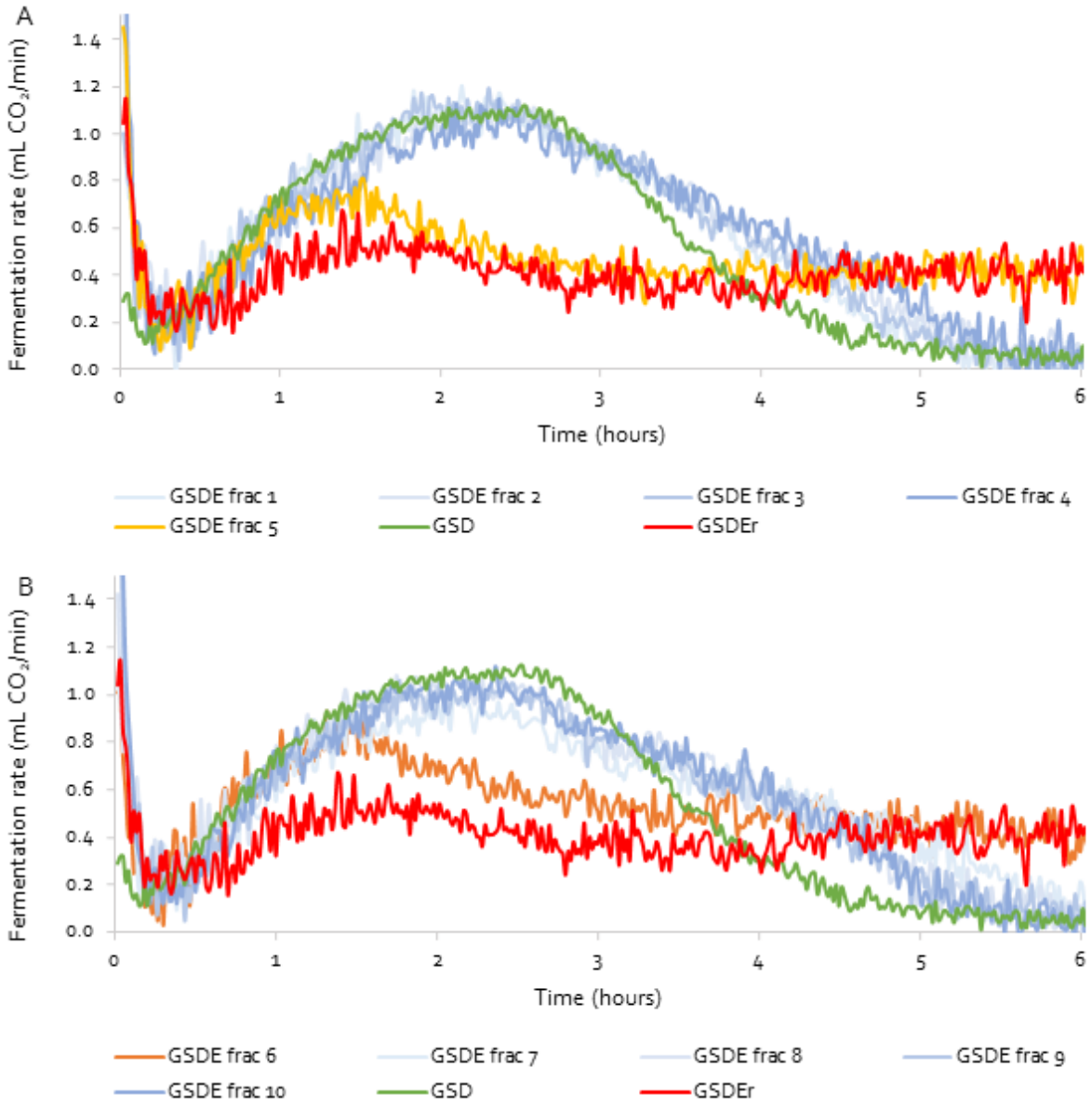

***Figure 4.4**: Fermentation capacity (mL CO_2/min) in function of time (hours) of Beo5 in (A) gluten-starch dough with the individual fractions of pool 6 (GSDE frac 1-5), the negative (green) and positive (red) control and in (B) gluten-starch dough with the individual fractions of pool 7 (GSDE frac 6-10), the negative (green) and positive (red) control. In both series of fractions, 4 out of the five (shades of blue) did not show any inhibition; one fraction, resp. fraction 5 (yellow) and fraction 6 (orange), inhibited the Beo5 to the same extent as the positive control.*

Pool 6 and 7 contained one fraction each with inhibition activity. This activity coincided entirely with the impaired fermentation by the positive control. As the two fractions with inhibition activity were consecutive fractions, we presumed that one inhibitor was spread over the two fractions rather than that there were two or more inhibitors acting. However, this presumption needed to be verified. The inhibition activity tests with the RP-HPLC fractions using the inhibition-insensitive Bro4, showed no impact on its fermentation capacity, as expected.

4.3.2 Identification of the inhibitor

4.3.2.1 Mass spectrometry

The two inhibiting RP-HPLC fractions were analyzed with a mass spectrometer, coupled to the RP-HPLC system. In **Figure 4.5A** and **B** the mass spectrum of the most abundant protein in fraction 5 is displayed, together with the deconvoluted mass spectrum. The most abundant protein in this fraction had a molecular mass of approximately 31 kDa. Exactly the same result was obtained after mass spectrometric analysis of the other inhibiting fraction (**Figure 4.5C** and **D**). This confirmed the presumption that there was only one inhibiting component present in both fractions.

4.3.2.2 SDS-PAGE, LC-MS/MS and N-terminal amino acid sequencing

The proteins present in the two inhibiting RP-HPLC fractions were separated based on molecular mass by SDS-PAGE and silver stained (**Figure 4.6**). Both fractions showed two clear bands of the same size each, being one around the 22 kDa marker and one right below the 36 kDa marker. Analysis of all the proteins present in fraction 6 was done with LC-MS/MS. As this resulted in a list of more than thirty different proteins of all kinds, the two bands present in both fractions (marked on the gel in **Figure 4.6**) were analyzed separately. The list of proteins present in both bands is given in **Table 4.1**.

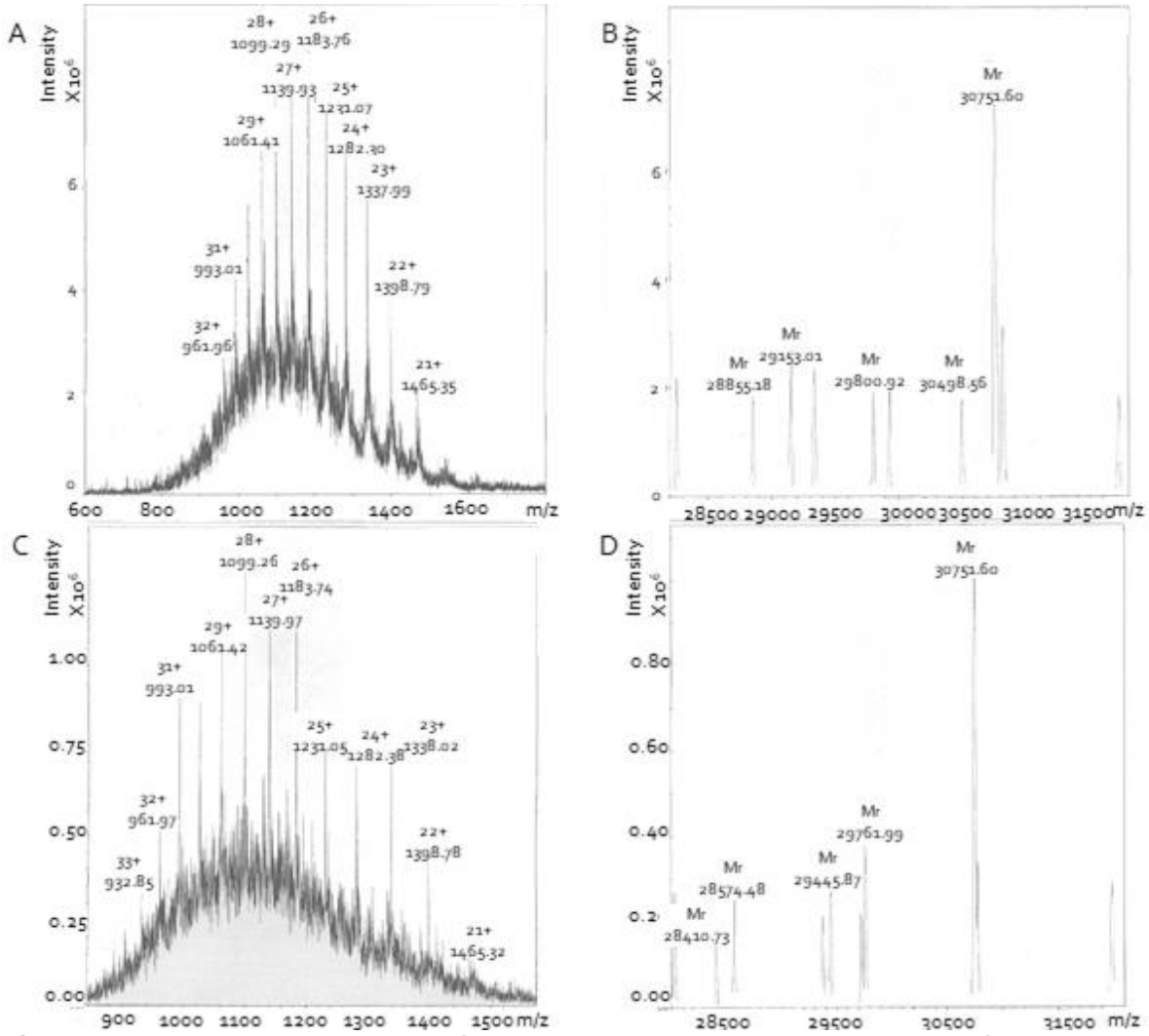

Figure 4.5*: (A), (C) The mass spectra of the most abundant protein present in resp. fractions 5 and 6 obtained through RP-HPLC. The peak intensity is represented in function of the m/z value of the ions coming from this protein. Above each peak, the charge of the respective ions and their m/z value, with m the mass of the ion and z the charge, are given. (B), (D) The mass spectra after deconvolution in which the molecular mass of the original peptide or protein is calculated.*

Most of the proteins of the upper band (around 36 kDa), being an avenin-like protein, a LMW glutenin subunit and gliadins, were related to gluten proteins which should also be present in the gluten-starch blend. As the inhibition-sensitive yeast strains were able to ferment in gluten-starch dough, it is very unlikely that those proteins would cause inhibition. Next to these proteins, also subunits of grain softness protein 1 were detected. Grain softness protein is structurally related to puroindolines (Elmorjani et al., 2013). These puroindolines are suggested to have in vitro antifungal activity based on their capacity to bind with polar lipids, as part of fungal membranes (Dubreil et al., 1998; Pauly et al., 2013). However, Elmorjani and coworkers (2013) showed that grain softness protein lacks the ability to bind lipids. So it can be assumed that this protein does not possess antifungal activity.

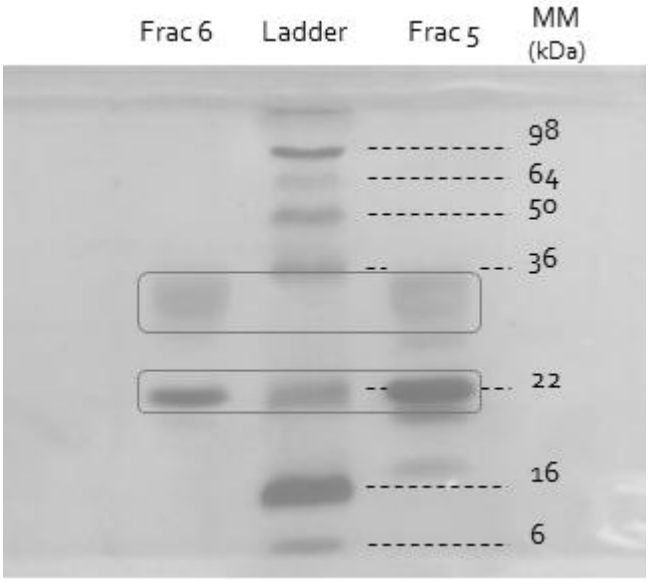

Figure 4.6*: SDS-PAGE profiles of the two inhibiting RP-HPLC fractions. In the middle lane, the ladder is displayed; left from the ladder is the profile of fraction 6 and right the profile of fraction 5. The molecular weights (kDa) of the proteins in the ladder are shown on the right of the figure.*

The last protein present in the upper band was a *thaumatin-like protein,* belonging to the fifth class of PR proteins. The same *thaumatin-like protein* was found in the lower band in higher abundance, next to an *alpha-amylase/trypsine inhibitor* – to which also antifungal characteristics are ascribed –, and *avenin-like* and *grain softness proteins*. As already explained, the latter two proteins could be excluded as potential inhibitor. As the antifungal activity of *alpha-amylase/trypsine inhibitor* lies in the inhibition of alpha-amylase and trypsine (Vidhyasekaran, 2008), this protein could be neglected as potential inhibitor of yeast, leaving only the *thaumatin-like protein*.

However, this analysis so far only led to a suspected protein by elimination, but did not give an unambiguous answer regarding the identity of the inhibitor. In the next step, fraction 6 was electroblotted and the N-terminal amino acid sequence of the upper protein band and the protein band around 22 kDa were analyzed with Edman degradation. While the result for the upper band was inconclusive, analysis revealed the following sequence of fifteen amino acids derived from the most abundant protein in that band:

A T I T V V N R (X) S Y T V (X) P

An X means that a weak signal for more than one amino acids at that place was obtained. The search for proteins of wheat (*Triticum aestivum*) containing this sequence in the NCBI database (National Centre for Biotechnology Information, Bethesda MD, USA) yielded two proteins. One, named *antifungal protein* (Accession number AAB21823.1), existed of a partial sequence of 23 amino acids beginning at the N-terminus with the identified sequence.

***Table 4.1**: Overview of the proteins present in the two bands visible on gel of the inhibiting fractions obtained by RP-HPLC.*

Identified proteins (lower band)	**MW (kDa)**		**Identified proteins (upper band)**	**MW (kDa)**
Thaumatin-like protein	24		Avenin-like protein	32
Alpha-amylase/trypsine inhibitor	18		Grain softness protein 1D, partial	17
Avenin-like a1	18		Grain softness protein 1A, partial	17
Grain softness protein 1A, partial	17		LMW glutenin subunit, partial	24
			Alpha-gliadin	34
			Alpha/beta-gliadin	34
			Thaumatin-like protein	24

The other protein containing the above sequence was the *thaumatin-like protein* (Accession number AAM15877.1) previously found with LC-MS/MS. The complete amino acid sequence is shown in **Figure 4.7**. The protein is made up of 225 amino acids and its molecular mass is 24 kDa, which approximated the size estimated based on the gel. However, there was a big discrepancy between this mass (24 kDa) and the one found with mass spectrometric analysis (31 kDa). As could be seen on the gel, both fractions 5 and 6 also had a similar large protein band with a molecular size smaller than 36 kDa. It might also be possible that the 31 kDa molecule found with mass spectrometry referred to a protein in this band. The fifteen lettered N-terminal amino acid sequence found with Edman degradation, was positioned in the sequence of this protein from amino acid 23 to 37 (underlined in the sequence in **Figure 4.7**). According to the UniProt database (The UniProt Consortium, 2018) the first 22 amino acids formed the signal peptide and were removed from the mature protein.

1	mastrvlhli	alvlavataa	daatitvvnr	csytvwpgal	pgggvrldpg
51	qswalnmpag	tagarvwprt	gctfdgsgrg	rcitgdcggt	lacrvsgqqp
101	ttlaeytlgq	ggnkdffdls	vidgfnvpmn	fepvggscra	arcatditke
151	clkelqvpgg	casacgkfgg	dtyccrgqfe	hncpptnysk	ffkgkcpday
201	syakddqtst	ftcpagtnyq	ivlcp		

***Figure 4.7**: Amino acid sequence of the wheat thaumatin-like protein. The fifteen-lettered amino acid sequence found by Edman degradation is underlined.*

Taking all results together, the *thaumatin-like protein* – from now on referred to as TLP (AAM15877.1) – was pinpointed as the most plausible candidate to be the inhibitor of certain yeast strains during wheat flour dough fermentation.

4.3.3 Recombinant expression of the thaumatin-like protein in *Escherichia coli*

To confirm the identity of the inhibitor, the gene associated with the above mentioned amino acid sequence of the TLP (AAM15877.1) was inserted into a vector and brought to expression in *E. coli.* As the protein was intracellularly produced, the cells were lysed to release the recombinant protein. The lysate was centrifuged with 3 kDa filters to remove the majority of the lysis buffer. The retained fraction was then dissolved again in deionized water, such that the final lysate sample was two times more concentrated compared to the original lysate. This sample (from now on called 'recombinant TLP (rTLP) fraction') was added to gluten-starch dough to test the inhibition activity on both Be05 and Br04. The fermentation profiles of both yeasts in gluten-starch doughs with different samples is given in **Figure 4.8**. Compared to gluten-starch dough with water, the lysis buffer in itself had an impact on the fermentation capacity of both Br04 and Be05. Since this fermentation profile coincided with the fermentation profile in gluten-starch dough with the filtrate (after centrifugal filtration of the lysate) for both yeasts, it could be concluded that the salt concentration in the formulation of this buffer (50 mM sodium dihydrogen phosphate dehydrate and 300 mM sodium chloride, pH 7.4) most probably has been the major cause of delayed and reduced fermentations, probably due to osmotic stress (results not shown).

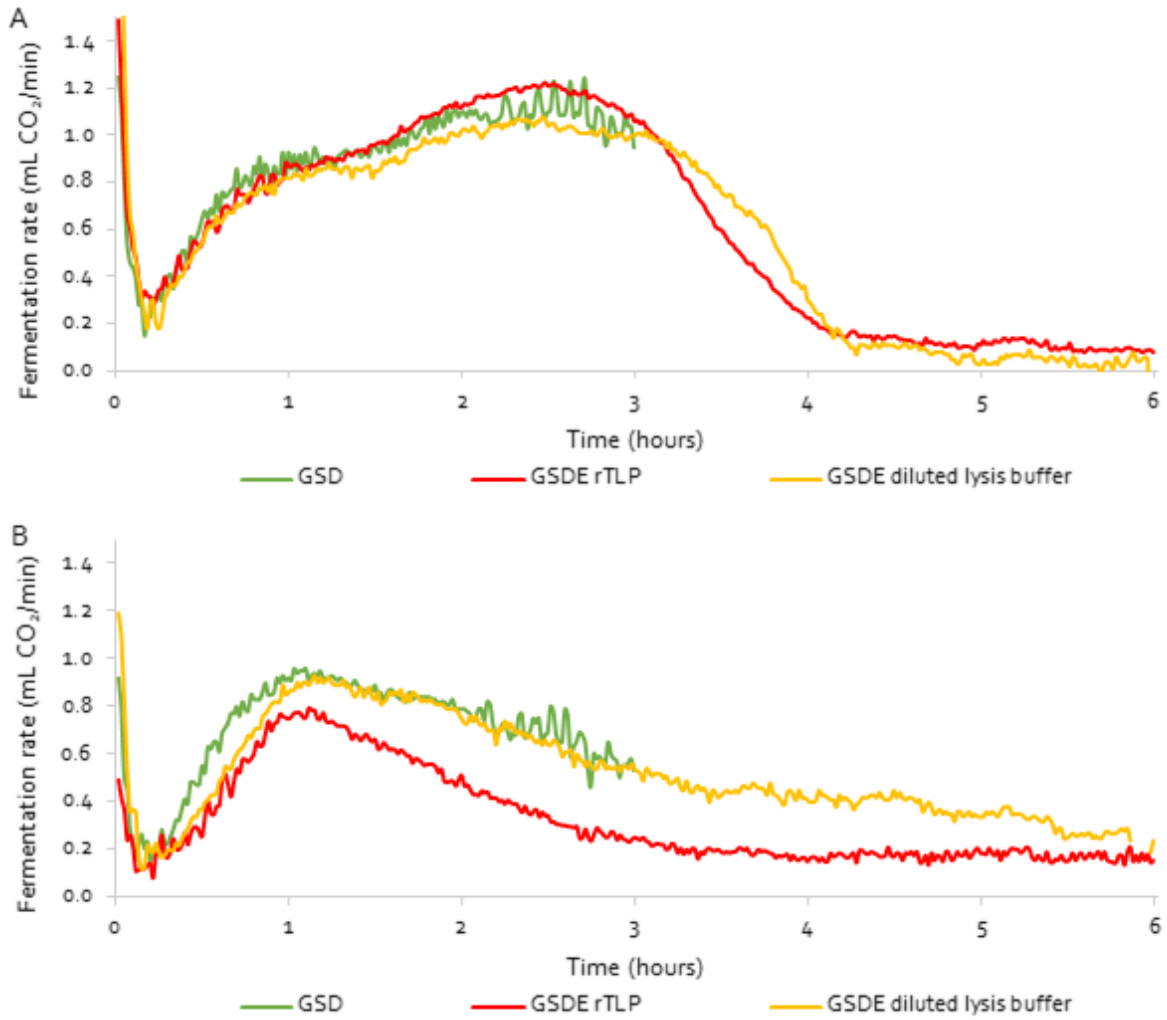

***Figure 4.8**: Fermentation capacity (mL CO_2/min) in function of time (hours) of (A) the inhibition-insensitive Bro4 and (B) the inhibition-sensitive Beo5 in gluten-starch dough with water (GSD, green), with five times diluted lysis buffer (GSDE diluted lysis buffer, yellow) and with the recombinant TLP fraction (GSDE rTLP, red).*

To mimic the conditions in the rTLP fraction, the lysis buffer was diluted five times prior to use in the dough. The behavior of both yeasts in gluten-starch dough with water and in gluten-starch dough with the diluted lysis buffer was similar. The diluted lysis buffer as present in the rTLP fraction should hence not have an impact on the yeast's fermentation capacity.

When adding the rTLP fraction to a gluten-starch dough with the control yeast Bro4, the fermentation profile coincided with the ones in gluten-starch dough with water and diluted lysis buffer. In contrast, the presence of the the rTLP fraction did have an impact on the fermentation capacity of the inhibition-sensitive Beo5. The maximal fermentation rate reached in dough with the rTLP fraction was approx. 0.2 mLCO_2/min lower than in the samples without inhibitor, followed by a faster drop. When comparing this with the fermentation profile of the inhibiting

RP-HPLC fractions, exactly the same inhibiting effect was noticed. The profile with the rTLP even dropped to lower fermentation rates after three hours of fermentation (0.2 mL CO_2/min versus 0.4 mL CO_2/min). This provided evidence that the inhibitor we were looking for was indeed the TLP (AAM15877.1) of wheat with the above mentioned amino acid sequence.

4.3.4 The wheat thaumatin-like protein as fermentation inhibitor

It is plausible that the identified wheat TLP (AAM15877.1) causes inhibition of *S. cerevisiae* yeasts as thaumatin-like proteins form the fifth family of PR proteins. The wheat TLP (AAM15877.1) was first described by Kuwabara and coworkers (2002). They found that it was induced by cold stress and the subsequent production of abscisic acid in winter wheat and that it showed antifungal activity against snow mould (Kuwabara et al., 2002). Furthermore, antimicrobial activity of a barley malt thaumatin-like protein was shown by Gorjanovic and coworkers (2007). The activity of the TLP (AAM15877.1) described in this study against *S. cerevisiae* and during bread making, however, has not yet been described in literature.

The identification of the TLP (AAM15877.1) seems to contradict what several research groups suggested in the past, being that the inhibition of some *S. cerevisiae* strains during bread making is caused by purothionins (Balls and Harris, 1944; Hernandez-Lucas et al., 1974). While it is beyond dispute that purothionins do have antifungal activities as they form a separate class of PR proteins (PR-13) (Datta and Muthukrishnan, 1999; Epple et al., 1995), there are at least three arguments to discard purothionins as the causal agent responsible for yeast inhibition in this study. First, the inhibitor in this study was extracted with pure deionized water instead of petroleum ether as was done in the past. Secondly, there is a size difference. Purothionins are known to be small molecules with a molecular weight of about 5 kDa (Datta and Muthukrishnan, 1999), whereas the component here was in the range of 22 kDa. Thirdly, the addition of divalent ions in this research did not lead to the loss of inhibition activity (results not shown), while inactivation of the antifungal activity of purothionins by 2 to 5 mM Ca^{2+} has been described (Gorjanović et al., 2007; Oard and Karki, 2006).

4.3.5 Thaumatin-like proteins in other cereals

As investigated in Section 3.3.4, the inhibition-sensitive yeast strain was not inhibited in a model dough with flour extracts from oat and rice. However, according to literature, all five of the tested cereals – oat and rice included – contain thaumatin-like proteins (TLPs) (Muthukrishnan et al., 2001; Shi et al., 2015). When aligning the amino acid sequences of TLPs of barley, oat, rice, maize and rye with high similarity to TLP (AAM15877.1), together with thaumatin (sweet tasting protein

from *Thaumatococcus daniellii*), it is clear that a lot of domains are conserved (**Figure 4.9**). When looking into more detail (not illustrated in **Figure 4.9**), all 16 cysteine residues are present in the TLPs (with exception of rye TLP, containing only 10 cysteine residues), which is of importance for the protein's stability. Based on this alignment, it is difficult to explain why the inhibition-sensitive yeast is not inhibited in model doughs with rice and oat flour extract, whereas it is in rye flour dough and model dough with maize and barley flour extracts. Possible explanations could be that the TLPs in rice and oat are not expressed or only expressed in low amounts or that they could not be extracted in the same way as the TLPs in the other cereals tested in this experiment. Optimization of the extraction method, purification and identification of the TLPs of other cereals is requested.



***Figure 4.9**: Alignment of the amino acid sequences of TLPs, present in barley, oat, rice, maize and rye, with high similarity to wheat TLP (AAM15877.1), together with the amino acid sequence of thaumatin from Thaumatococcus daniellii. Red represents highly conserved domains, blue less conserved domains and gray domains with a high degree of variability. The picture is adapted from the Contraint-based Multiple Alignment Tool (COBALT) from NCBI (National Centre for Biotechnology Information).*

4.3.6 Differences in sensitivity of *S. cerevisiae* towards the wheat thaumatin-like protein

The question remains as to why some yeasts are sensitive towards the inhibitor, while others are not. As this dissertation does not include an analysis of the mode of action of the inhibitor, further research on this topic is highly recommended. The differences, that were in the past observed in the sensitivity of *S. cerevisiae* strains towards osmotin (Accession number AAA34089.1), a tobacco TLP and member of the fifth class of PR proteins were noticed (Ibeas et al., 2001), and the mode of action of osmotin could offer a possible lead in this respect. Osmotin is thought to permeabilize the plasma membrane of its target cells (Abad et al., 1996). As yeast cells are surrounded by a cell wall, the antifungal protein first has to cross this wall before it reaches the cell membrane. In the study of Ibeas and coworkers (2001), the susceptibility of yeast cells to osmotin has been shown to depend on the presence or absence of PIR proteins (Proteins with Internal Repeats). These stress-related proteins fortify the cell wall, making it more difficult to

be crossed (Yun et al., 1997). The deposition of PIR proteins in the cell wall is determined by the SSD1 gene. Deletion of this gene results in the absence of PIR proteins in the cell wall, making the yeast more sensitive to osmotin (Ibeas et al., 2001). In addition, deletion of this gene also includes weakening of the cell wall by reduction of glucan cross-linking within the inner layer of the cell wall, thereby increasing the susceptibility of the yeast cell for the antifungal action of osmotin (Ibeas et al., 2001; Van Der Weerden et al., 2013). Once entered into the cell, although the mechanism still remains unclear, osmotin has also been shown to stimulate a mitogen-activated protein (MAP) kinase signal pathway inducing ROS and consequent programmed cell death or apoptosis (Narasimhan et al., 2001; Van Der Weerden et al., 2013). Hypothesizing that the TLP (AAM15877.1) functions analogously to osmotin, combined with the results of the growth experiment in the previous chapter (**Section 3.3.1**), one could suggest that the induction of ROS might be the most important feature of the inhibitor.

4.4 Conclusion

After fractionating a crude wheat flour extract with cation exchange chromatography and RP-HPLC and identifying the proteins present in an inhibition-active fraction with SDS-PAGE, Edman degradation and LC-MS/MS a TLP (AAM15877.1) of wheat was pointed out as the most possible candidate causing inhibition of fermentation by yeasts in dough. To prove the inhibitory activity of the identified protein, it was recombinant expressed in *E. coli* and the cell lysate was tested for its inhibitory activity. As this test turned out to be positive, it was concluded that this specific TLP (AAM15877.1) is indeed the causal factor of the observed inhibition. Although this protein was already identified in the early 2000s and proven to be active against snow mould and barley malt thaumatin-like proteins had already been shown to be antifungal against some *S. cerevisiae* strains, it is to our knowledge the first time that the antifungal activity of this particular TLP (AAM15877.1) against *S. cerevisiae* during wheat flour dough fermentation has been described. Concerning the selectivity for some and not all *S. cerevisiae* strains, the composition of the cell wall might be of importance for the susceptibility of the different yeast cells towards the inhibitor, determining whether the cells are subjected to the accumulation of ROS and apoptosis.

Chapter 5.

Impact of different non-bakery *Saccharomyces cerevisiae* strains on dough rheology and bread quality

5.1 Introduction

The enormous diversity within the *S. cerevisiae* species is reflected in a wide variation in fermentation properties. Not only CO_2 and ethanol (as evidenced in Chapter 2) but also the other fermentation metabolites, like glycerol, organic acids (Rezaei et al., 2015c), and aroma compounds (Birch et al., 2013a; Steensels et al., 2014a) are produced in variable concentrations. These differences potentially lead to a different impact on dough manageability and rheology, and on bread volume, texture, crumb structure and aroma. As discussed in Section 1.2.2, preparation of dough and bread with different *S. cerevisiae* yeast strains revealed large differences in metabolite profiles during fermentation, accompanied by a divergent impact on dough rheology and bread quality (Heitmann et al., 2015; Jayaram et al., 2014; Meerts et al., 2018b; Rezaei et al., 2015c).

To our knowledge, the combined analysis of metabolite and aroma production of *S. cerevisiae* strains from different industrial applications (different from bakery) in wheat bread together with their potential impact on dough and bread quality parameters has not yet been described. In this chapter we first analyzed the fermentation metabolites and aroma components produced by the 45 yeast strains in the model dough system. Based on these results, we made a comparison between the different categories and the bakery strains in order to gain insight in the potential of non-bakery strains for bread making. Then, we selected 10 yeast strains with divergent behavior out of the 45 strains for further analysis. Studying rheological properties of doughs and volume, aroma, texture and crumb structure of breads made with the selected yeasts, together with their fermentation capacity presented in Chapter 2, gave us a holistic view on differences in yeasts performance in bread making.

5.2 Materials and methods

5.2.1 Materials

Wheat grain was provided by EVD (Jodoigne, Belgium). The grains were milled into flour with a Bühler MLU-202 laboratory mill with a yield of 69.4%. Commercial wheat gluten and wheat starch was provided by Tereos Syral (Aalst, Belgium). Forty-five *S. cerevisiae* strains from various industrial applications were obtained from the collection of the VIB Laboratory for Systems Biology (KU Leuven, Belgium). Bacteriological agar, yeast extract and balanced peptone were provided by Lab M (Brussels, Belgium), commercial sugar and salt by Tiense Suiker (Tienen,

Belgium) and Everyday (Gellingen, Belgium), respectively. All other chemicals, reagents and solvents were purchased from Sigma-Aldrich and were of analytical grade.

5.2.2 Flour and whole meal characterization

The characteristics of the wheat flour used in this study were as described in Section 2.2.2.

5.2.3 Preparation of yeast cells for dough fermentation

Yeast cells were prepared as described in Section 2.2.3.

5.2.4 Dough and bread making

Dough at 10 g scale was prepared according to AACC International Approved Method 10-10.03 as described in Section 2.2.6. Dough at 100 g scale was prepared analogously, starting from 100.0 g flour. Bread making both at 10 g and 100 g scale was done according to the same AACC International approved method. Dough was fermented for 90 min in a fermentation cabinet (National Manufacturing) at 32°C and 95% relative humidity, with intermediate punching at minutes 52, 77 and 90 min, using a dough sheeter (National Manufacturing). After the third punch, doughs were molded and proofed for 36 min in the fermentation cabinet (32°C, 95% relative humidity), followed by baking in a rotary oven (National Manufacturing) at 232°C for 13 min (10 g doughs) or 215°C for 24 min (100 g doughs). Immediately after baking, breads were weighed and allowed to cool down for 1 or 2 hours (10 g or 100 g dough resp.) before determination of the bread volume with a Volscan Profiler (Stable Micro Systems, Godalming, Surrey, UK).

5.2.5 Dough pH measurement

Doughs were prepared as described in Section 2.2.6 and fermented in the Risograph at 30°C. The pH of the doughs was measured after mixing and after 126 min (duration of fermentation and proofing during the bread making process) of fermentation using a pH probe (HI 9126, Hanna Instruments, Temse, Belgium) that was put directly into the dough.

5.2.6 Analysis of fermentation metabolites

Extracts of dough samples were prepared by blending dough with a volume of deionized H_2O equal to two times the final weight of the dough for 30 s with a Waring 8011E blender (Warring Products, Torrington, CT, USA). The resulting batter was transferred to Eppendorf tubes (2 mL) and centrifuged with an Eppendorf centrifuge 5415D (Eppendorf AG, Hamburg, Germany) at 11,000 *g* for 3 min. After filtration of the supernatant with a Millex-HP 0.22 µm polyethersulfone

membrane (Merck Millipore, Carrigtwohill, Ireland), the samples were stored at -20°C until further analysis. Quantification of ethanol, glycerol, acetic and succinic acid in the fermented dough extracts was done with ion-exclusion HPLC using an LC-20AT modular HPLC system (Shimadzu, Kyoto, Japan). Separation of the metabolites was carried out with an ion-exclusion ROA-organic acids guard (50 x 7.8 mm) and analytical (300 x 7.8 mm) column (Phenomenex, Torrance, CA, USA) eluted with a 2.50 mM H_2SO_4 solution at a flow rate of 0.60 mL/min. The guard and analytical column were kept at 60°C. Three biological replicates of each yeast strain were used for dough preparation in triplicate.

5.2.7 Dough spread test

An adapted version of the spread test described by Hoseney and coworkers (1979) was performed to examine the impact of fermentation metabolites on dough rheology. Doughs were prepared as described in Section 2.2.6, put in a lightly greased covered bowl and fermented in the fermentation cabinet (National Manufacturing) at 32°C, with 95% relative humidity for 0, 60, 120 and 180 minutes. In line with the bread making process, doughs were punched at 52, 77 and 90 min. After the fermentation period, doughs were rounded and put on a Plexiglass plate. Height (H) and orthogonal widths (W_1 and W_2) of the dough balls were measured immediately after rounding and after a proofing period of 60 minutes in the fermentation cabinet. Measuring was done with a ruler and the dimensions were measured at the highest or widest points. **Figure 5.0** gives a schematic overview of the parameters measured in the spread test.

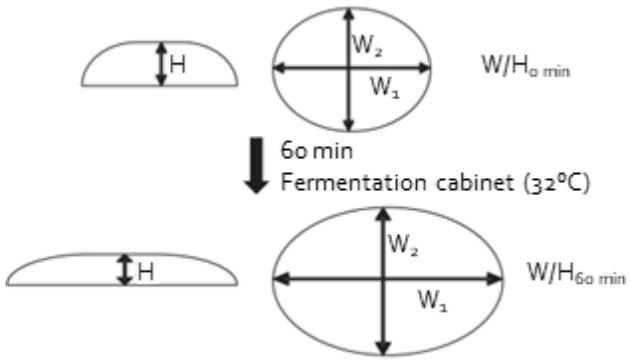

***Figure 5.0**: Schematic representation of the spread test. Left: side views, right: top views of the dough. Parameters measured are height (H) and widths (W_1 and W_2) (adapted from Decamps et al, 2012).*

The spread ratio $\Delta (W/H)_x$ at time point x, being the fermentation time of 0, 60 or 120 minutes, was measured according to the formula described by Decamps and coworkers (2012):

$\Delta (W/H)_x = (\text{average } W/H)_{x+60\ min} - (\text{average } W/H)_{x\ min}$

Because the dough balls after rounding were not spherical, the average of W_1 and W_2 (=average W) was used to calculate the spread ratio.

At each time point, ΔH_x and ΔW_x are defined as:

$\Delta H_x = H_{x+60\ min} - H_x$

$\Delta W_x = W_{x+60\ min} - W_x$

With H_x and W_x resp. the height and width before proofing at time point x and $H_{x+60\ min}$ and $W_{x+60\ min}$ resp. the height and width after proofing at that time point.

The experiment was carried out in duplicate.

5.2.8 Texture profile analysis of bread

Texture profile analyses were performed on breads (100 g) prepared in triplicate (according to Section 5.2.4) using a selection of 10 different *S. cerevisiae* yeast strains. Firmness and resilience of the bread crumbs were measured by compression with an Instron 3342 (Norwood, MA, USA). To this end, each bread was sliced at the center with an electric slicer (Affettatrice Slicer 30N, Galesecca, Italy), yielding three slices of 3.0 cm thickness (and two slices of 1.0 cm thickness for crumb image analysis). Hence, for testing the impact of the different yeast strains on bread texture, nine samples were analyzed for each strain. A cylinder with a height of 25 mm and diameter of 30 mm was cut from each slice with a cylindrical borer. The bread samples were compressed twice with a cylindrical probe (diameter 75 mm) at a constant speed of 100 mm/min. The crumb firmness was determined from the force-time curve and equaled the force required to compress samples for the first time by 30%. Resilience of the bread crumb shows the extent of the sample recovery after compression. It was obtained from the force-time curve and calculated as the ratio of the area under the decompression curve to the area under the compression curve of the first compression peak.

5.2.9 Bread crumb image analysis

Crumb image analyses were performed on breads (100 g) prepared in triplicate (according to Section 5.2.4) using a selection of 10 different *S. cerevisiae* yeast strains. Two slices (1.0 cm thickness) were cut from the center of each bread with an electric slicer (Affettatrice Slicer 30N). Hence, 6 slices were analyzed for each yeast strain. The slices were scanned with a flatbed

scanner (HP Scanjet 3800, Hewlett-Packard, Beijing, China) in 256 gray levels at 300 dots per inch and saved in bitmap format. A square of 40 x 40 mm at the center of each slice was selected and evaluated. Image J (Bethesda, MD, USA) was used to convert the gray scale images to binary images according to the Otsu thresholding algorithm (Otsu, 1979). The binary images were analyzed with Matlab R2018b (Mathworks, Natick, MA, USA), enabling the calculation of the total cell count, the total and mean cell area (mm^2), the number of cells/cm^2 and the 2D porosity.

5.2.10 Bread aroma analysis

The aroma compounds of bread fermented using different *S. cerevisiae* yeast strains were analyzed and compared with those of bread fermented with a commercial bakery strain (Bro4). Aroma analysis was performed using headspace solid-phase micro extraction (HS-SPME) followed by gas chromatography–mass spectrometry (GC–MS) as described by Aslankoohi and coworkers (2016). Per yeast strain, six samples, i.e. three biological replicates each inoculated in two different bread samples (two technical replicates), were analyzed with GC-MS. The relative amounts of the volatiles were quantified by the ratio of the peak area of the volatile to the sum of peak areas of all volatiles produced in the sample.

5.2.11 Statistical analysis

One-way ANOVA using JMP Pro software 13 (SAS Institute) was performed to detect significant differences between the mean values of data groups. For evaluation of the metabolite production, comparison of all means was executed using the Tukey test ($p<0.05$). Correlations between all variables were detected using Principal Component Analysis (PCA) with JMP Pro software 14 (SAS Institute). The variables are linearly transformed to create new independent variables, called the Principal Components. The correlations between the original variables are presented in loading plots, formed with the two main principle components. Arrows pointing in the same direction describe a positive correlation. Arrows pointing in the opposite direction describe a negative correlation. Orthogonal arrows indicate the absence of a correlation. A pairwise correlation analysis between the variables in Section 5.3.2 was done using JMP Pro software 14 (SAS Institute). Coefficients with a probability of $p<0.05$ pointed out significant correlations.

5.3 Results and discussion

5.3.1 Screening of 45 *S. cerevisiae* strains in gluten-starch dough

Experiments in Chapter 2 showed a wide variation in fermentation capacity between 45 different *S. cerevisiae* strains. Because the production of CO_2 is not the only determinant of fermentation, the production of metabolites and aroma production during this process was also analyzed in this chapter. As previously shown, some yeast strains were inhibited by the TLP (AAM15877.1) (Chapter 2 and 3) during fermentation in wheat flour dough. To make sure that all strains could perform to their best ability and to generate insight in their full potential in conditions without inhibition, the gluten-starch model dough system was used for the analysis of metabolites and aroma compounds of the 45 *S. cerevisiae* strains. As only one cocoa strain was included in the selection of yeast strain, it was not taken into account for the comparison between the different industrial categories.

5.3.1.1 Metabolite analyses

Next to CO_2, the fermentation metabolites of interest were ethanol, glycerol, succinic and acetic acid. The amount of these fermentation metabolites produced by the different yeast strains was determined with ion-exclusion HPLC. As this analysis was part of a screening of the strains before making a selection for further analysis of their impact on dough and bread properties, only two technical replicates were analyzed.

Principle component analysis (PCA) was performed on the metabolites produced by all yeast strains. The resulting score and loading plots are presented in **Figure 5.1**. It could clearly be concluded from the score plot in **Figure 5.1A** that there was no clustering of the yeast strains according to the industrial category they belong to. In Chapter 2, we grouped yeast strains based on their fermentation capacity in wheat flour dough into weak, intermediate and strong fermenters. Although the weak and intermediate yeast strains could ferment significantly better in gluten-starch dough than in wheat flour dough, the inhibition-sensitive yeast strains seemed to distinguish themselves as a group from all the other strains as encircled in **Figure 5.1A**. This could be explained based on the individual concentrations of ethanol and fermentation metabolites they produced (results not shown). Indeed, their CO_2 production in gluten-starch dough was still significantly lower than that of the strong fermenters. As expected, this was also reflected in the ethanol production data. Likewise, the weakly and intermediately fermenting yeast strains produced significantly less succinic acid, whereas no statistically significant difference was noticed for the amounts of acetic acid.

Correlations between the concentrations of the different metabolites are shown in the PCA loading plot in **Figure 5.1B**. Acetic acid concentration was not correlated with the concentration of succinic acid, ethanol and CO_2. There was only a slightly positive correlation with glycerol, which could be expected based on the yeast's fermentation metabolism (**Figure 1.2**). Indeed, during glycolysis, glucose is metabolized to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, precursors of ethanol and glycerol, respectively. This explanation was definitely also applicable for the positive correlation between glycerol on the one hand and ethanol and CO_2 on the other hand. At the onset of fermentation, yeast is subjected to high (osmotic) stress levels because it is transferred from a liquid growth medium to a semi-solid dough system with low water activity and high sugar and salt concentrations, resulting in an increased production of the osmolyte glycerol (Aslankoohi et al., 2013). As this increase makes the yeast cell more stress resistant, it might lead to a quicker start of the fermentation (Aslankoohi et al., 2015), resulting in a positive correlation between glycerol and ethanol. Aslankoohi and coworkers (2015) investigated the impact of deletion and overexpression of the glyceraldehyde-3-phosphate dehydrogenase (GDP1) gene in a lab strain and a bakery strain on glycerol production. They noticed that an increase in glycerol production resulted in higher acetic acid and lower succinic acid concentrations, meaning that glycerol would be positively correlated with acetic acid and negatively with succinic acid. The latter could, however, not be confirmed based on our results.

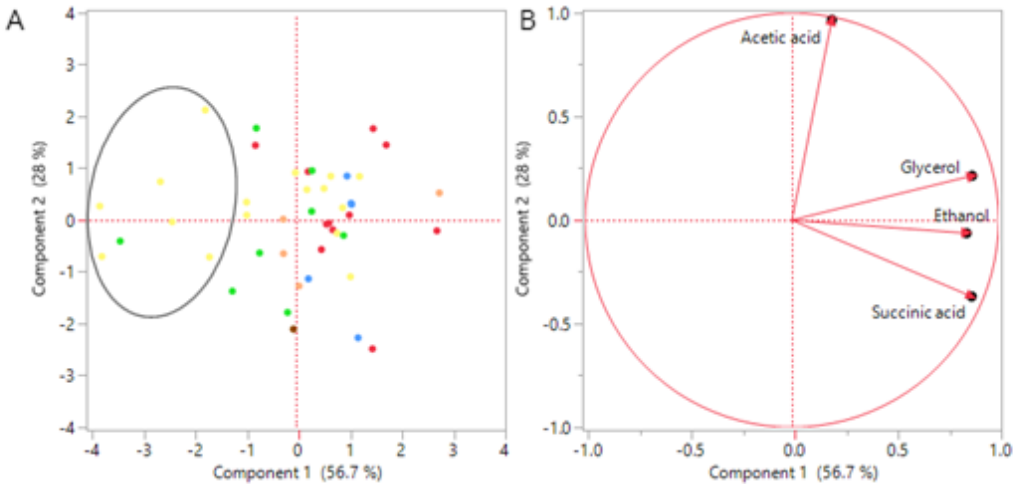

Figure 5.1: (A) PCA score plot performed with the concentrations of acetic acid, succinic acid, glycerol, and ethanol produced by all yeast strains in gluten-starch dough. The colors of the dots represent the industrial category: beer, yellow; bioethanol, blue; bread, orange; brown, cocoa; sake & spirits, green; wine, red. The encircled dots represent inhibition-sensitive yeast strains. (B) PCA Loading plot. Metabolites represented by vectors pointing in the same or opposite direction are positively or negatively correlated, respectively. Metabolites with perpendicular vectors are not correlated. The first and second principal components explained 56.7% and 28% of the variance, respectively.

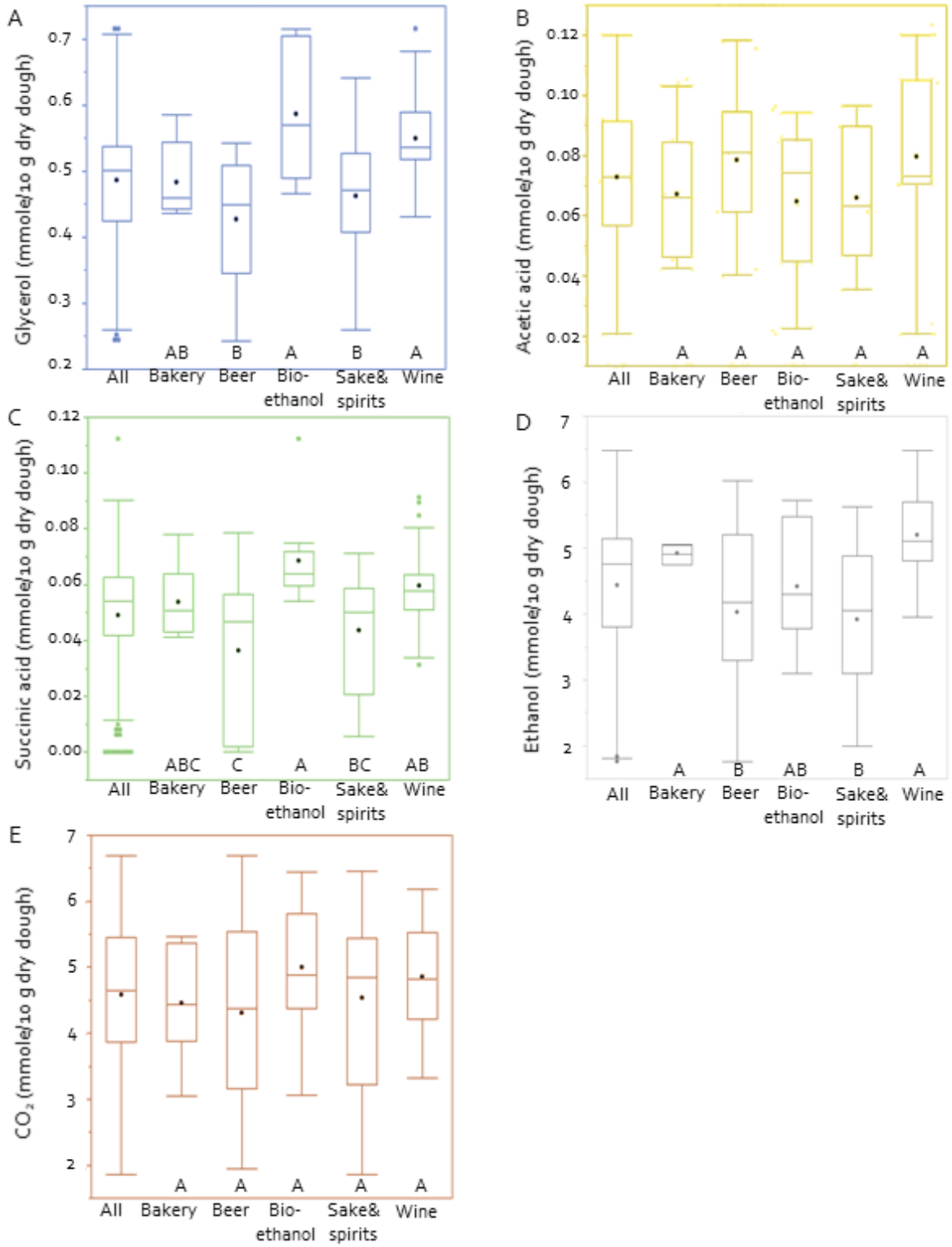

***Figure 5.2**: Distribution of the concentrations (mmole/ 10 g dry dough) of (A) glycerol, (B) acetic acid, (C) succinic acid, (D) ethanol and (E) CO_2 produced by 45 S. cerevisiae strains, originating from different industrial categories. The overall scattering (All) and scattering within each industrial category (Bakery, Beer, Bioethanol, Sake & spirits, Wine) of the components are represented by boxplots. Black dots indicate means, colored dots indicate outliers. For each component, categories with different letters are significantly different from each other (Tukey, $p<0.05$).*

Because we wanted to compare the performance of yeast strains from different industrial categories with the performance of bakery yeasts in the first place, the industrial category of bakery yeasts was taken as the control group. Compared to this group, no statistical differences in glycerol production could be noticed in the other industrial categories. Boxplots in **Figure 5.2** represent the distribution of the concentrations of glycerol, organic acids, ethanol and CO_2 produced by the 45 yeasts in gluten-starch dough after 126 minutes of resting in the Risograph. This period equaled the fermentation and proofing period of the bread making process used in this PhD-study. An overview of the medians, means and standard deviations accompanying the boxplots is given in **Table S1** in Appendix. Comparable to the results in Chapter 2, a lot of variability in concentrations of fermentation metabolites produced by the different yeast strains could be noticed. The amounts of glycerol varied between 0.25 mmole/10 g dry dough (Be01) and 0.71 mmole/10 g dry dough (BioE03) (**Figure 5.2A**). The lowest values were typically for the beer and sake & spirits yeasts, whereas the highest values were mainly found for the bioethanol and wine yeasts.

The concentrations of glycerol produced by beer and sake & spirits yeasts were significantly lower than those of the bioethanol and wine yeasts. Glycerol is produced when the cell undergoes osmotic stress and subsequently functions as an osmolyte. Higher glycerol levels result in a higher stress resistance and hence a better fermentation capacity in stressful environments. Indeed, it was shown by Aslankoohi and coworkers (2015) that an increased glycerol production leads to a quick start of the fermentation. In our study, this was expressed as a positive correlation with ethanol. An exception here was e.g. strain S02. For this strain rather high glycerol concentrations are observed, whereas its CO_2 production is significantly below the average. Probably, a high level of glycerol in the cell was in first instance needed to protect the cell against osmotic stress, resulting in a retarded onset of the fermentation.

The production of acetic acid by the 45 yeasts was normally distributed without outliers, with a lowest and a highest concentration of 0.02 mmole/10 g dry dough (W06) and 0.12mmole/10 g dry dough (W07), respectively (**Figure 5.2B**). Because of the large scattering of acetic acid concentrations in all industrial categories, none of them were significantly different from the control group or from each other. The concentrations of succinic acid, excluding outliers, varied between 0.01 mmole/10 g dry dough (S01) and 0.09 mmole/10 g dry dough (W06). The succinic acid production of yeast strains Be01, Be04, Be13 and Be14 was too low to be detected during the analysis, represented by the outliers with value zero (**Figure 5.2C**). Remarkably, all these

outliers belonged to the inhibition-sensitive yeast strains (as defined in Chapter 2). Although they were able to ferment in gluten-starch dough, they seemed to lack the ability to produce succinic acid during wheat dough fermentation. Also here, no significant differences were observed between the different industrial categories and the control group (bakery strains). The bioethanol strains had, however, a significantly higher ability to produce succinic acid compared to beer and sake & spirits yeast strains.

The ethanol production varied between 1.80 mmole/10 g dry dough (Be13) and 6.39 mmole/10 g dry dough (W09) (**Figure 5.2D**). The industrial categories of beer and sake & spirits yeasts had a significantly lower production of ethanol than the bakery and the wine yeasts. Although the ethanol and CO_2 production were strongly and positively correlated (**Figure 5.1B**), we noticed no significant differences amongst the five industrial categories for CO_2 production (cfr. **Figure 2.1C**). Remarkable for the ethanol concentrations was the extremely narrow range between the different bakery strains. As the ethanol production is a measure for the fermentation capacity, this possibly reflected the evolution of bakery yeasts to leaven dough as fast as possible and to large extents. However, this trend was not outspoken in the concentrations of CO_2 produced by the bakery strains (**Figure 5.2E**). This could probably be explained by the lower accuracy of the CO_2 measurement with the Risograph (pressure changes as a measure of CO_2) compared to the metabolite analysis with HPLC.

5.3.1.2 Volatile compound analysis

The fermentation metabolites determined in the above section also potentially contribute to bread flavor and aroma. Although glycerol is odorless, it might influence bread flavor because of its sweet taste (Morrison, 2000). Succinic acid is known to give a bitter taste to wine and sake (Arikawa et al., 1999; Bartowsky and Pretorius, 2009). Acetic acid and ethanol have been reported to cause a sour and alcoholic aroma, respectively (Heitmann et al., 2017; Pico et al., 2015). An overview of the most important aroma compounds identified in all crumbs is given in **Table S2**. **Figure 5.3 r**epresents a heatmap of the relative amounts of 10 aroma compounds detected in the crumb of breads with the 45 different yeast strains. These compounds were of importance – according to literature – for bread aroma or showed large differences for certain aromas, noticed for some strains relatively to the bakery strains. Although the aroma compounds were produced by all yeast strains, the relative production of these volatile molecules strongly differed amongst the strains. In the heat map, yeast strains with similar aroma profiles were clustered. It was clear that all yeast strains were diffused and not grouped

according to their category, meaning that the aroma production is really strain dependent and probably independent of the industrial application. For all yeasts, the heatmap shows the z-scores for each component, expressed against yeast strain Bro4 as control. These z-scores are calculated from the average log2 peak areas and express how many standard deviations any value differs from the value of the control yeast for that specific aroma compound. Red boxes mean a higher production compared to the control strain, blue boxes indicate a lower relative production. According to literature, phenylacetaldehyde, phenethyl ethanol, benzaldehyde, nonanal, hexanal and 1-octen-3-ol are important compounds determining bread aroma and were also found in gluten-starch breads in this experiment.

Volatile compounds in bread crumb and crust originate from (i) yeast activity, (ii) lipid oxidation during mixing and fermentation and (iii) Maillard reactions during baking (Pico et al., 2015).
Isoamyl alcohol is a product of yeast activity in bread. Because isoamyl alcohol is positively correlated with bread aroma, variation amongst the strains would potentially impact the appreciation of the final bread aroma. Yeasts BioE02, S02, Be05, Be06 and Be14 produced less of this compound compared to Bro4. Remarkably, these strains (except BioE02) are inhibition-sensitive yeasts. This could probably be explained by the lower fermentation capacity of these strains. Although this experiment was performed in gluten-starch dough in which all yeast strains were able to ferment, results in Chapter 2 showed that the weak and intermediate strains still had a significant lower CO_2 production in this model compared to the strongly fermenting strains. On the other hand, the crumbs of Be12, W01 and W04 contained more of this volatile alcohol compared to Bro4, increasing their potential for usage in bread. The other bakery strains, Bro1, Bro2 and Bro3, differed for their isoamyl alcohol production less than one standard deviation from Bro4. Most of the alcohols and aldehydes are products of lipid oxidations by alcohol dehydrogenase and *in se* independent of the performance of yeast (Birch et al., 2014). Because lipid oxidation components with their green odors are often denoted as off-aromas (Birch et al., 2014), equal or lower concentrations in comparison with the control strain Bro4 would be desirable.
Phenylacetaldehyde and phenethyl ethanol are formed as a result of both yeast activity and Maillard reactions; benzaldehyde is formed by yeast activity and lipid oxidation (Birch et al., 2014; Pico et al., 2015). Phenylacetaldehyde and phenethyl alcohol belong to the most aroma-active aldehydes and alcohols of bread crumb and also showed here lots of variation between the different strains. The yeasts grouped at the left side of the heatmap, going from S03 to W08 and with exception of Bro1, gave higher concentrations of phenylacetaldehyde to the bread

crumb. This might be positive for the final product as a sweet and honey-like odor is ascribed to it. Poor fermenters Be01, Be04, Be06, Be13, Be14, S01 and S02 added less phenylacetaldehyde to the crumbs. A similar trend was noticed for phenethyl alcohol.

S01, Be13, W01 and Br01 were characterized by high amounts of benzaldehyde, while Be05 produced relatively much less of this component than the control. To a certain extent, a higher production of this compound might be beneficial because of its almond and caramel odor. D-limonene, another compound associated with pleasant odors, was produced in high amounts by BioE03 and much less by W03.

The fact that gluten-starch dough was used for this screening instead of wheat flour dough could partly explain the differences in volatile aroma compounds detected compared to what was found in literature. The absence of wheat-inherent components in the gluten-starch mixture, functioning as precursors of volatiles, e.g. ferulic acid (converted to 4-vinylguaiacol) (Coghe et al., 2007) and flour lipids (converted to several aldehydes, ketones and furans) (Birch et al., 2014), probably resulted in a dissimilar aroma profile. Overall, when looking at the individual yeast strains, differences in relative concentrations of the volatile compounds with the control bakery strains could be noticed. However, there were no clear trends within one industrial category.

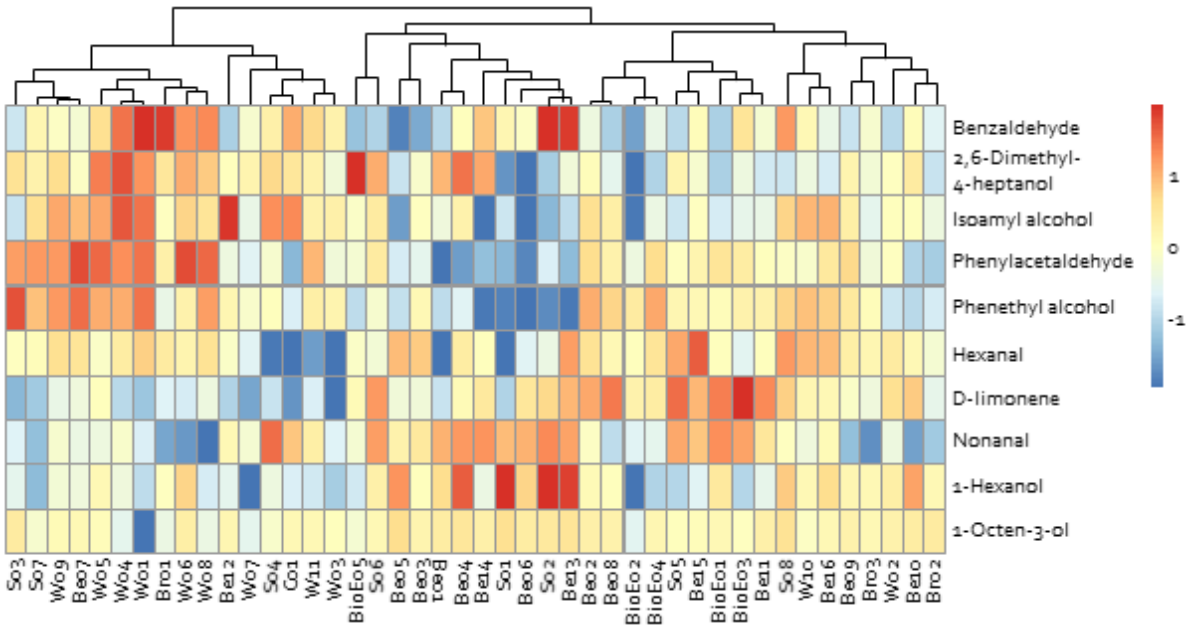

***Figure 5.3**: Heatmap representing the differences in relative concentrations of the volatile compounds (listed on the right) in the crumb of gluten-starch breads produced with 45 different S. cerevisiae strains (names on the x-axis). The values shown in the heatmap are z-scores, representing how many standard deviations the average log2 relative peak area of each component, produced by a certain strain is lower (blue tainted boxes) or higher (red tainted boxes) than the respective value of the control Br04 strain. The values are the average of two technical replicates.*

5.3.1.3 Selection and comparison of 10 S. cerevisiae strains for further analyses

Based on the results of the metabolite and aroma analyses, as no category was outspokenly better than the bakery strains, two yeast strains from each of the five industrial categories were selected for investigating the impact of their differences in fermentation capacity on bread quality aspects. Their performance was compared with bakery strain Bro4. A summary of the metabolite and aroma production of each strain is given in **Figure 5.4**. For the comparison of the metabolites produced by the strains, the glycerol and the organic acid concentrations were expressed relative to the ethanol concentration of each strain to compensate for the varying fermentation ability.

Figure 5.4C again shows a lot of variation in fermentation capacity, expressed as ethanol and CO_2 production. The control strain Bro4 is an average fermenter. Compared to this strain, two of the other strains (Bro1 and W10) had a comparable ethanol production, three of them a higher one (Be11, BioE05 and W06) and the remaining four strains (Be05, BioE03, S02 and S03) a lower one. The relative glycerol production (**Figure 5.4A**) was for the majority of the strains equal to that of Bro4, except for strains BioE03 and S02, having a significantly higher production. Concerning the organic acids (**Figure 5.4C**), Bro4 was again situated in the middle. Because of the influence yeast metabolites exert on the properties of dough, we would expect variation in dough and possible also bread characteristics when the different yeast strains are applied. Concerning the volatile compounds, we made sure that the selected strains all had aroma profiles divergent from Bro4 (**Figure 5.4D**).

5.3.2 Impact of ten different *S. cerevisiae* strains on wheat flour dough properties

5.3.2.1 Metabolite analysis

After selecting 10 out of 45 yeast strains, we investigated their potentially different impact on dough properties. Therefore, the metabolite production was now analyzed in wheat flour dough instead of gluten-starch dough. This was done to make the results as representative as possible for the bread making industry. Results are presented in **Figure 5.5**. To take fermentation ability of the yeasts into account, the concentration of all metabolites was put against the ethanol concentration. Correlation coefficients between the different metabolites are presented in **Table 5.1**. The two inhibition-sensitive yeasts included in this selection of 10, Be05 and S02, are discussed at the end of this section because of their divergent metabolite profile and were, for the same reason, left out of the correlation analysis in Table 5.1.

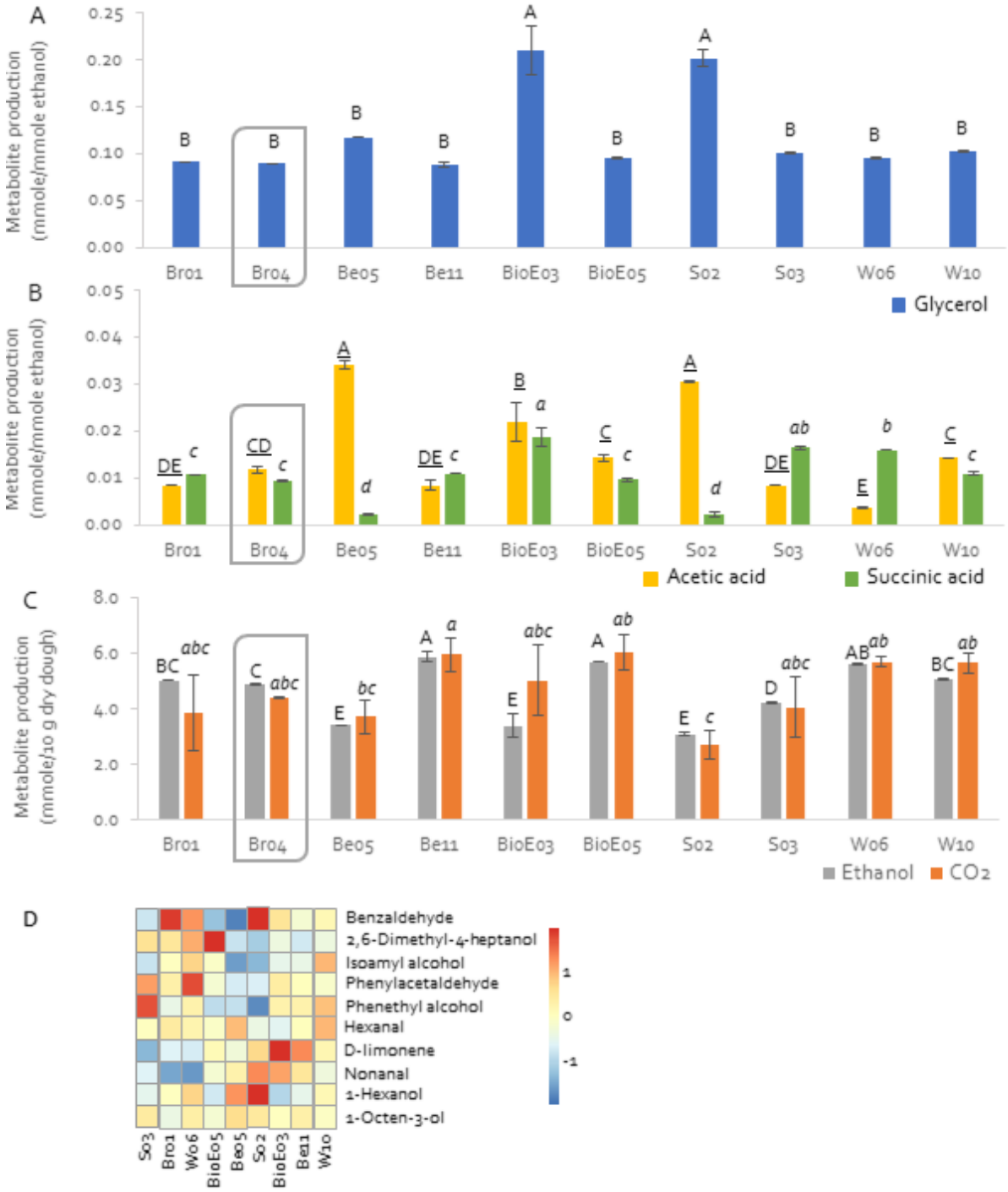

***Figure 5.4**: (A) The relative production of glycerol, (B) acetic acid (yellow) and succinic acid (green) expressed against the ethanol concentration (mmole metabolite/mmole ethanol) and (C) the ethanol (gray) and CO_2 production (mmole/10 g dry dough) of ten selected yeast strains in gluten-starch dough. The vertical error bars represent the standard deviations of two technical replicates. Bars indicated with different letters are significantly different from each other (Tukey, $p<0.05$). In (B), capital underlined and small letters are respectively used for comparison of the concentrations of acetic acid and succinic acid relative to ethanol. In (C), capital letters are used for the comparison of the ethanol production, small letters for the comparison of CO_2 production. (D) Heatmap representing the profiles of the selected yeast strains for 10 most relevant aroma components.*

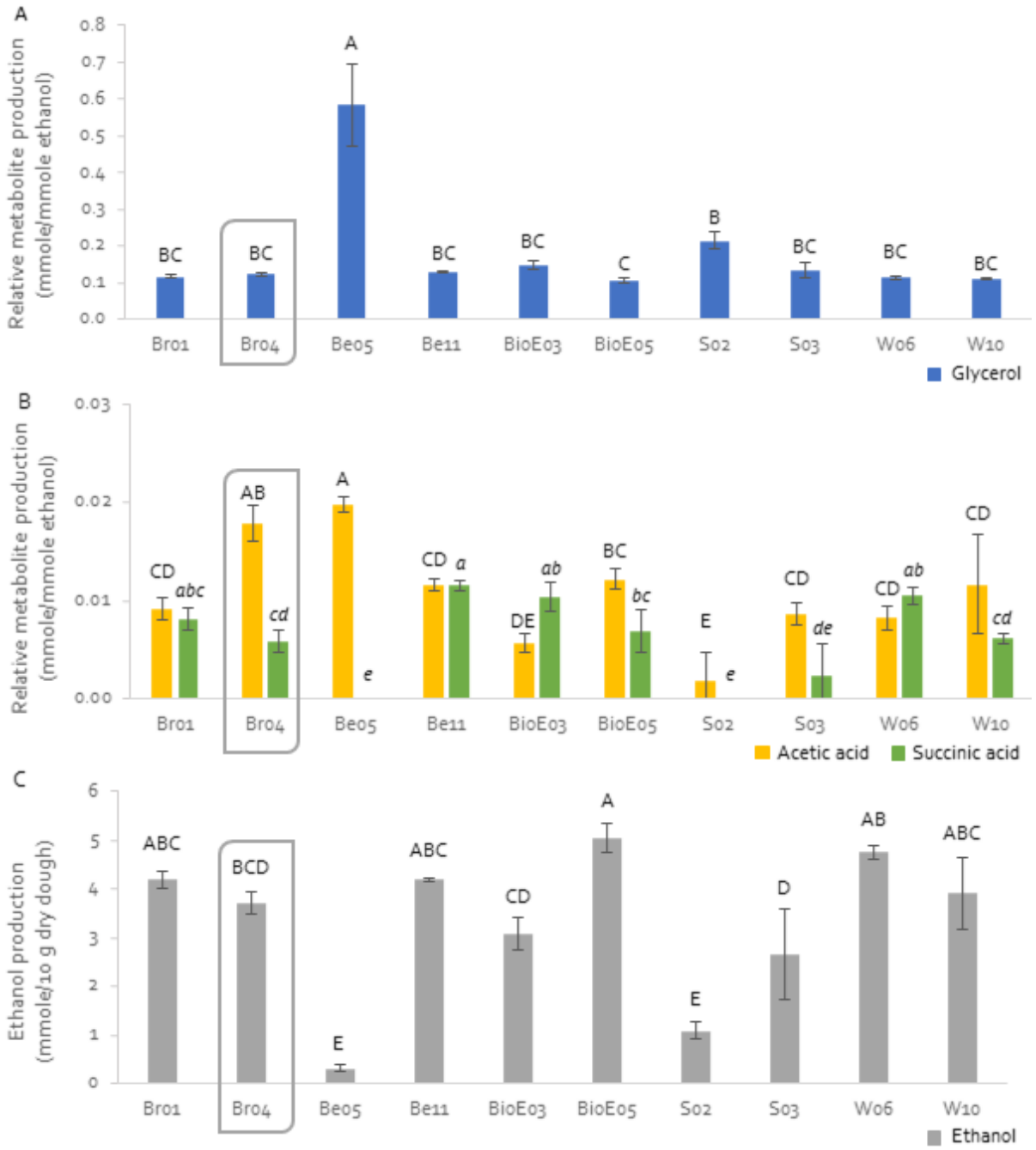

***Figure 5.5**: Metabolite production of (A) glycerol (blue) (mmole/mmole ethanol), (B) acetic acid (yellow) and succinic acid (green) (mmole/mmole ethanol)and (C) ethanol (gray) (mmole/10 g dry dough) of the ten selected yeast strains in wheat flour dough. The framed yeast strain Bro4 is the control bakery strain. Vertical error bars represent the standard deviations of three biological replicates. Columns with different capital (glycerol, acetic acid and ethanol) or small (succinic acid) letters are significantly different from each other (Tukey, p<0.05).*

INHIBITION-INSENSITIVE YEASTS

The relative glycerol production – disregarding Be05 and So2 – was similar for all yeast strains (**Figure 5.5A**). Glycerol production hence seemed to go hand in hand with ethanol production (r=0.874). This was also observed in gluten-starch dough (**Figure 5.2F**).

The relative concentrations of the organic acids fluctuated more between the yeast strains as well as within the industrial categories (**Figure 5.5B**). Be05 set aside, the bakery strain Br04 produced – relative to its fermentation capacity – the highest amount of acetic acid and differed significantly from all other strains, except from BioE05. Even Br01 produced significantly less acetic acid, despite the similar ethanol production (**Figure 5.5C**). Because of the broad range in acetic acid production within the industrial category of bakery strains, there were no strains from other categories producing significantly more or less acetic acid than the bakery strains. However, with the impact of organic acids on dough rheology in mind, variation in rheological properties as a result of individual differences could be expected.

Acetic acid and ethanol, both products of the alcoholic fermentation, were slightly positively correlated (r=0.564). However in theory, based on yeast metabolism, a negative correlation would have been expected between acetic acid and ethanol concentrations, as they both have acetaldehyde as precursor. A positive correlation between these two metabolites should imply rather similar relative acetic acid concentrations for all strains, which was not the case. The bakery strains, for example, had a comparable ethanol production, whereas their relative acetic acid production differed significantly. The wine yeasts on the other hand, produced similar amounts of both ethanol and acetic acid. Consequently, the production of this organic acid could not directly be linked to or estimated based on the fermentation capacity.

The control bakery strain Br04 differed significantly from Be11, BioE03 and W06 concerning the relative succinic acid production (inhibition-sensitive yeasts not taken into account). Furthermore, comparable relations between ethanol and succinic acid could be detected as for ethanol and acetic acid, also with in a positive correlation between both fermentation metabolites (r=0.655). Similarly as suggested for the differences in acetic acid production, doughs with varying rheological properties could be expected.

INHIBITION-SENSITIVE YEASTS

Despite its poor fermentation capacity and although the absolute concentration of glycerol was lower compared to the other strains, Be05 was able to produce significantly more glycerol per mmole of ethanol than the others (**Figure 5.5A**). The same was true for acetic acid (**Figure 5.5B**). The relative amounts of glycerol and acetic acid produced by S02 were average and low, respectively, compared to the other strains.

Remarkably, the amounts of succinic acid produced by both inhibition-sensitive yeasts seemed to be too low to be detected during the analysis or were even absent (**Figure 5.5B**). When looking back at the results in gluten-starch dough, the succinic acid production of these two strains was extremely low as well, despite their significantly higher fermentation capacity compared to in wheat flour dough. Although the phenomena behind these observations remain unclear, it might be suggested that the inhibition-sensitive yeast strains were more sensitive towards low oxygen concentrations in both dough systems compared to the other strains. Indeed, the presence of oxygen is needed for the (residual) functionality of the TCA cycle and glyoxylate shunt, two pathways producing succinic acid during fermentation. As the glyoxylate shunt makes use of acetate for the production of succinic acid, this might explain the accompanying high acetic acid concentrations in gluten-starch dough. Moreover, ROS intracellularly produced during respiration (in case of gluten-starch dough) or in response to the inhibitor (in wheat flour dough), might also impact the TCA cycle. Aconitase, reversibly catalyzing the isomerization of isocitrate to citrate, is known to be affected by ROS (Lushchak et al., 2014). This might hamper the production of subsequent intermediates amongst which succinic acid.

In line with the poor CO_2 production, established in Chapter 2, the ethanol production of Be05 and S02 was significantly lower than that of the insensitive strains (**Figure 5.5C**).

A comparison between the metabolite production of the selected yeast strains in gluten-starch versus wheat flour dough, is presented in **Figure S5** in the Appendix. As expected, the biggest differences could be found in the fermentation capacity and metabolite production of the poor and intermediate fermenter Be05 and S02.

5.3.2.2 Dough pH

The pH values of doughs made with different yeast strains varied between 5.14 and 5.54 at the beginning of the fermentation and decreased on average 0.69 (± 0.16) units after 126 minutes of fermentation. The start pH of Be05 and S02 was comparable with the other strains. At the end

of the fermentation period, however, the pH of dough with So2 only slightly decreased with 0.21 units. For dough with Be05, the pH stayed approximately the same. This proved the importance of the fermentation ability of yeast for dough pH, Be05 and So2 are, after all, inhibition-sensitive yeast strains. Correlations between pH and fermentation metabolites are included in **Table 5.1**.

Correlation analysis established a negative correlation only between pH and succinic acid concentrations (**Table 5.1**). This was in line with the findings of Jayaram and coworkers (2013). Although similar amounts of both organic acids were produced, not even a slightly negative correlation between acetic acid and the pH could be noticed (r=-0.041), which was also in conflict with one of the conclusions of Rezaei and coworkers (2015c), showing that the pH in dough was dependent on the concentration of both organic acids.

5.3.2.3 Dough rheological properties

During fermentation, dough is subjected to three forces: (i) gravity, the vertically downward force, (ii) pressure due to gas production, forcing dough to expand in all directions and (iii) cohesion of dough, limiting expansion (Hoseney et al., 1979). The metabolites yeast produces during fermentation are known to impact the rheological properties of dough and consequently have part in the extent to which the above forces act on dough. As we wanted to investigate the final impact of yeast strains on bread properties, we started with the examination of the influence of the diverse metabolite profiles of the ten strains on dough rheology. To this end a dough spread test was performed. First, dough was fermented for 0, 60 and 120 minutes, rounded and proofed during 60 minutes. Before and after proofing, dough height and both orthogonal widths were measured. With these parameters, the spread ratio of a dough ball at each time point (x), defined as $\Delta(W/H)_x$, was determined (Section 5.2.7). Because dough was not spherically shaped after rounding, the average of both widths was used to calculate the spread ratios. The standard deviation of the measurements on the replicates, was on average 0.1.

***Table 5.1**: Overview of the correlations between the concentration of fermentation metabolites ethanol, CO_2, glycerol, acetic acid, succinic acid and the spread ratio Δ(W/H) x at time point x (0, 60 and 120 minutes). Negative values (between -1 and 0) indicate an inverse correlation, positive values (between 0 and 1) a direct correlation. The closer to ± 1, the stronger the correlation, the closer to 0, the weaker. The inhibition-sensitive strains were left out of the analysis. Values indicated with an asterisk (*) have a corresponding probability of p<0.05, showing a significant correlation.*

	Ethanol	CO_2	Glycerol	Acetic acid	Succinic acid	$\Delta (W/H)_0$	$\Delta (W/H)_{60}$	$\Delta (W/H)_{120}$	pH
Ethanol	1	**0.560***	**0.874***	**0.564***	**0.655***	-0.381	-0.233	**-0.498***	-0.364
CO_2	**0.560***	1	**0.550***	**0.454***	**0.521***	0.029	-0.158	-0.389	-0.287
Glycerol	**0.874***	**0.550***	1	**0.405***	**0.869***	-0.070	-0.232	**-0.469***	-0.442
Acetic acid	**0.564***	**0.454***	**0.405***	1	0.139	-0.261	-0.134	**-0.570***	-0.041
Succinic acid	**0.655***	**0.521***	**0.869***	0.139	1	0.092	-0.343	-0.308	**-0.546***
$\Delta (W/H)_0$	-0.381	0.029	-0.070	-0.261	0.092	1	0.256	0.121	0.075
$\Delta (W/H)_{60}$	-0.233	-0.158	-0.232	-0.134	-0.343	0.256	1	-0.025	**0.721***
$\Delta (W/H)_{120}$	**-0.498***	-0.389	**-0.469***	**-0.570***	-0.308	0.121	-0.025	1	0.282
pH	-0.364	-0.287	-0.442	-0.041	**-0.546***	0.075	**0.721***	0.282	1

No correlations could be established between the fermentation metabolites and spread ratios after 0 and 60 minutes of fermentation. This suggested that, during the first 60 minutes of a fermentation process, the strength and coherence of the gluten-starch network was not significantly influenced by the yeast metabolites. However, $\Delta(W/H)_{60}$ was positively correlated with the pH (r=0.721), assuming that the decrease of the pH along the fermentation period of one hour resulted in a lower spread ratio, involving a stiffer and less extendable dough. Because the dough pH was inversely correlated to the succinic acid concentration, a correlation between succinic acid and $\Delta(W/H)_{60}$ could be expected. However, there was no such correlation noticed.

When dough was fermented for 120 minutes, $\Delta(W/H)_{120}$ was negatively correlated to ethanol (r=-0.498), glycerol (r=-0.469) and acetic acid (r=-0.570), pinpointing these metabolites as determining for dough spread at that time point. However, based on literature, a positive correlation was expected between dough spread and the glycerol concentration. Indeed, Aslankoohi and coworkers (2015) found that glycerol increased the extensibility of dough.

So far, general correlations between rheological parameters and yeast metabolites were examined. When looking at the individual yeast strains, differences in impacts on dough rheology could be noticed. **Figure 5.6** represents the change in ΔW_{120-0} between 0 and 120 minutes of fermentation – calculated as $\Delta W_{120} - \Delta W_0$ – in function of the change in ΔH_{120-0} between 0 and 120 of fermentation – calculated as $\Delta H_{120} - \Delta H_0$. As was expected, 120 minutes of fermentation did almost not change the height and width of doughs made with the inhibition-sensitive S02 and Be05 strains, as their dimensions were still close to those at onset. Remarkable was that all the other yeast strains, except for S03 and BioE03, had a negative ΔW_{120-0} after 120 minutes fermentation, meaning that the spreading of dough at the end of the fermentation period was smaller compared to the dough spread observed after at 0 minutes fermentation. It was suggested that this could be a result of the increasing organic acid concentration with consequent pH decrease. The lower the dough pH, the lesser its extensibility. The opposite could be true for strains S03 and BioE03 having a positive ΔW_{120-0}. However, this theory could not be established based on the organic acid concentrations measured previously. Dough with the BioE03 strain had by far the highest ΔH after 120 minutes, which was thought to be linked to an excellent fermentation capacity. Although the yeast strain belonged to the top 5 best CO_2 producers of the selection, it was certainly not the best fermenting strain. So, the results of the fermentation experiment could not directly be extrapolated to dough height during fermentation.

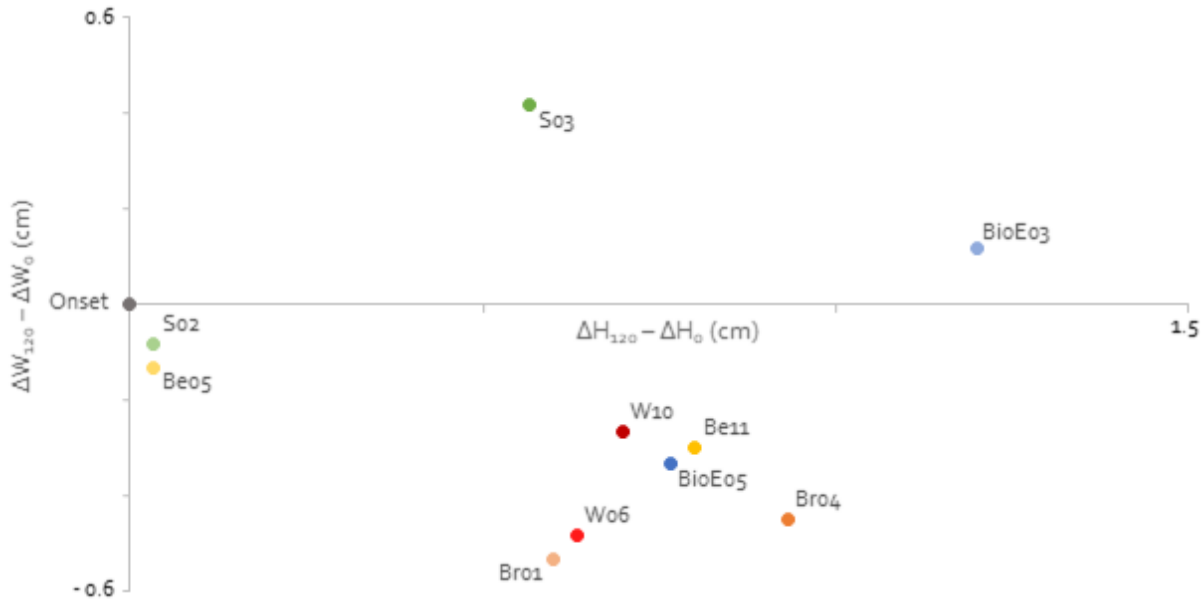

***Figure 5.6**: The overall change in $\Delta W_{120\text{-}0}$ ($\Delta W_{120} - \Delta W_0$) (cm) after 120 minutes of fermentation in function of the overall change in $\Delta H_{120\text{-}0}$ ($\Delta H_{120} - \Delta H_0$) (cm) after 120 of fermentation in doughs made with the 10 different yeast strains. Each point represents one yeast strain. The onset of fermentation for each strain was reduced to the zero point of the graph.*

5.3.3 Impact of ten different *S. cerevisiae* strains on wheat bread properties

5.3.3.1 Bread volume

When a bakery strain is replaced by another (non-bakery) yeast, it is important that the bread volumes obtained are at least comparable. Higher bread volumes might be beneficial because this could imply a reduction of the amount of yeast necessary to obtain similar bread volumes. **Figure 5.7** shows the bread volumes (100 g scale) obtained with the ten selected yeast strains. As the fermentation capacity of yeast is assumed to be directly correlated to the volume of the bread produced with it, it was not unexpected that breads with the inhibition-sensitive Beo5 and intermediate fermenting So2 yeast had significant lower volumes (206 and 361 mL, respectively) than the Bro4 bread yeast (538 mL). The W10 (614 mL) performed significantly better than the control bakery strain. As no significant differences were observed between the fermentation metabolites produced by W10 and Bro4, it was assumed that the trend of higher CO_2 production of W10 was the cause of this bigger bread volume. Breads made with the other good performing yeasts had comparable volumes, varying between 497 and 592 mL.

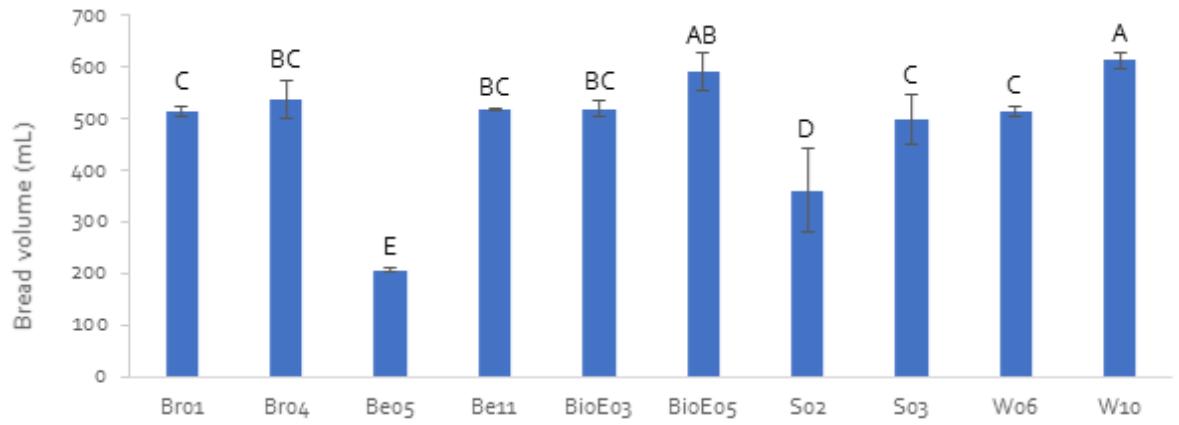

***Figure 5.7**: Wheat flour bread volumes (mL) obtained when baking with each of the ten selected yeast strains. The vertical error bars represent standard deviations of three biological replicates. Columns with different letters are significantly different from each other (Tukey, p<0.05).*

5.3.3.2 Crumb texture and structure

Next to the bread volume, bread quality is also determined by the structure and the textural properties of its crumb. Bread parameters that can be derived from the texture profile analysis are the hardness of the crumb and its cohesiveness, springiness, chewiness and resilience. In the following, we focus on the crumb hardness and resilience. Crumb hardness equals the force needed to compress a crumb cylinder over 30% of its height. Resilience is a measure for the work performed by the crumb to recover from compression. High firmness and low resilience are associated with lower bread quality or staling (Goesaert et al., 2009). As the volume of gas cells is related to the fermentation capacity of the yeast strain and a light structure with gas cells of uniform size is desired, analysis of the crumb structure (the amount of cells/cm² and the mean cell area (mm²)) also shows us more about the impact of the different strains on bread quality. Here, the hardness, resilience and crumb structure of breads made with the selected yeast strains were measured 24 hours after baking to determine whether or not the differences in fermentation capacity could be linked to these bread quality parameters. The results are presented in **Table 5.2**.

***Table 5.2**: Overview of the hardness (N), resilience (%), amount of cells/cm² and mean cell area (mm²) of bread crumbs made with the ten selected strains. The values are averages of three biological replicates, together with their standard deviations. For each column, (i) values indicated with different capital letters, differ significantly from each other (p<0.05) in a Tukey test including all yeast strains; (ii) values indicated with different small letters, differ significantly (p<0.05) in a Tukey test without Be05 and S02.*

Yeast	Hardness (N)	Resilience (%)	Cells/cm²	Mean cell area (mm²)
Br01	4.24 ± 0.39 (C, a)	38.8 ± 1.3 (ABC, ab)	78.5 ± 10.4 (AB, ab)	0.35 ± 0.03 (AB, a)
Br04	3.41 ± 0.88 (C, ab)	39.2 ± 1.2 (ABC, ab)	104.3 ± 12.1 (AB, ab)	0.30 ± 0.02 (B, a)
Be05	40.0 ± 0.1 (A, -)	/	51.4 ± 13.8 (B, -)	0.48 ± 0.05 (AB, -)
Be11	3.47 ± 0.21 (C, ab)	40.3 ± 0.7 (AB, a)	92.9 ± 9.9 (AB, ab)	0.30 ± 0.01 (B, a)
BioE03	4.27 ± 0.17 (C, a)	41.1 ± 0.4 (A, a)	75.1 ± 11.4 (AB, ab)	0.33 ± 0.03 (AB, a)
BioE05	3.02 ± 0.62 (C, ab)	39.0 ± 1.3 (ABC, ab)	87.0 ± 21.6 (AB, ab)	0.35 ± 0.04 (AB, a)
S02	10.1 ± 2.0 (B, -)	36.9 ± 1.0 (BC, -)	66.7 ± 27.0 (B, -)	0.37 ± 0.07 (AB, -)
S03	4.26 ± 1.23 (C, a)	39.4 ± 1.5 (ABC, ab)	123.4 ± 8.6 (A, a)	0.27 ± 0.03 (B, a)
W06	4.68 ± 0.40 (C, a)	36.8 ± 1.1 (C, b)	68.1 ± 13.0 (AB, b)	0.41 ± 0.13 (AB, a)
W10	2.39 ± 0.24 (C, b)	40.0 ± 0.8 (ABC, a)	79.1 ± 42.4 (AB, b)	0.54 ± 0.29 (A, -)

When comparing the normal performing strains with the control bakery strain Br04, values varied between 2.39 N and 4.68 N and no differences in hardness were noticed. As the use of yeast W10 gave bread the highest volume, it could be expected that it would also result in bread with a lowest hardness. While, indeed, it reached the lowest hardness of all strains but, in contrast to the volumes, it did not differ significantly from the control bakery strain. The hardness of strains Be05 and S02 deviated significantly from the other, good fermenting yeast strains. This was as expected based on the results from previous section. Indeed, the hardness of bread crumb and bread volume were inversely correlated to each other, meaning that a higher volume resulted in a less dense and consequently less firm crumb. As both yeast strains were not able to ferment dough properly, less air was incorporated in the matrix, resulting in a low volume bread with an extremely firm crumb (40.0 N for Be05 and 10.1 N for S02). Texture analysis of bread crumb with Be05 was even interrupted because the force needed to compress the sample was too high.

Concerning the resilience, none of the yeast strain (Be05 and S02 excluded) were statistically any different from the control strain Br04 (39.2%). Differences could, however, be noticed amongst other yeasts. Bread with W06 (36.8%) had the lowest resilience, significantly differing from Be11 (40.3%), BioE03 (41.1%) and W10 (40.0%). No explanation, however, could be found for this general lack of difference in spite of the diverse fermentation performances. Resilience was not correlated to the bread volume, nor to the amounts of fermentation metabolites produced. It

could be suggested that other yeast-inherent factors, like enzyme activity, might influence the final bread quality.

The amount of cells/cm² was the lowest for the poor and intermediate fermenting yeast strains Be05 and S02, implying a crumb with fewer but larger gas cells. This was indeed visible when comparing pictures of the crumb structure of inhibition-insensitive and inhibition-sensitive yeasts (**Figure 5.8**). The crumbs of bread with the control bread strain Br04 and the good fermenting Be11 were characterized by small and evenly distributed gas cells (**Figure 5.8A and B**). The crumb of bread made with the poor fermenting Be05, on the other hand, consisted of both small and large, irregularly distributed gas cells (**Figure 5.8C**). Likewise, a mixture of large and small gas cells made up the crumb structure of bread with the intermediate fermenting S02 (**Figure 5.8D**).

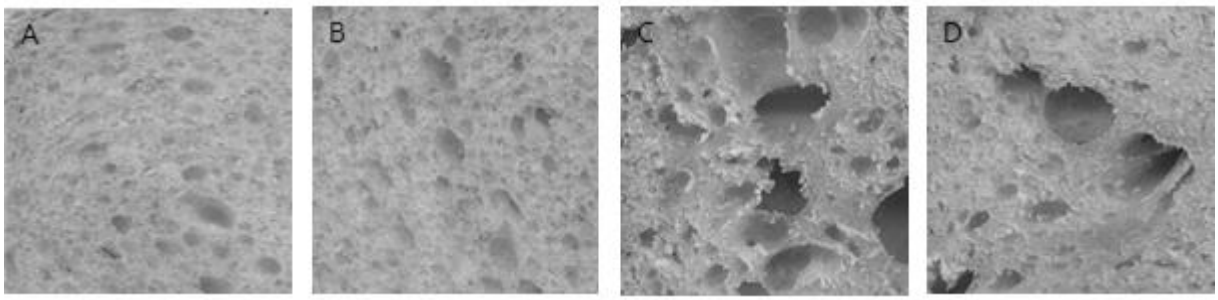

Figure 5.8*: Crumb structure of breads made with (A) control bakery strain Br04 and (B) strain Be11, both strong fermenting, (C) the weak fermenting Be05 and (D) the intermediate fermenting S02 yeast strains.*

The amount of cells/cm² was negatively correlated with the mean cell area, implying that the more cells there are present in a certain area of a bread slice, the smaller they would be. Consequently, Be05 and S02 were expected to produce cells with the largest mean cell area compared with the other well performing strains. However, the highest mean cell area was reached by W10. According to the large standard deviation, this could probably be ascribed to an incorrect measurement. As this result could confound the outcome of the significance test, it was removed from of a second statistical analysis together with the results for Be05 and S02 (small letters in the mean cell area column of **Table 5.3**). Amongst the other yeast strains, no significant differences in mean cell area were observed.

Although metabolite and fermentation analysis showed variation amongst the yeast strains, the differences could not be clearly extrapolated to final bread characteristics. This implied that the

application of each of the yeast strains, with exception of Be05 and S02, resulted in equally high-quality breads as the control bakery strain. The analysis of their aroma profiles in bread should point out the potential applicability of the selected strains for bread making purposes.

5.3.3.3 Volatile compound analysis

Analogously to Section 5.3.1.2, the aroma profiles of the ten selected yeast strains were determined, albeit in wheat flour dough instead of gluten-starch dough. As already stated in that section, bread aroma compounds are derived from (i) yeast activity, (ii) lipid oxidation and/or (iii) Maillard reactions. The main components resulting from the yeast's metabolism in wheat flour bread were phenethyl alcohol, 2,3-butanediol, phenylacetaldehyde, benzaldehyde, phenethyl acetate and 2-pentyl furan (Birch et al., 2014; Pico et al., 2015). Most of them have a honey, fruity or floral odor, giving a rather pleasant aroma to bread. Compounds, such as 1-hexanol, 1-octen-3-ol, heptanal and isobutyric acid are to be avoided because of their undesirable odor (Pico et al., 2015).

Use of the different yeast strains resulted in unique aroma profiles in bread. The heatmap presented in **Figure 5.9** enabled us to compare the volatile compound profiles in bread made with the selected yeast strains with a control bakery strain Br04. Striking, again, was the distinction between the strains with low fermentation ability (S02 and Be05) on the one hand and the strongly fermenting yeast strains on the other hand. Relative amounts of the pleasant aroma components benzaldehyde, ethyl octanoate, phenylacetaldehyde, phenethyl alcohol and phenethyl acetate were evidently far below the average level for Be05 and S02, as most of them are products of the yeast metabolism. In addition, breads made with S02 and Be05 contained relatively more compounds leading to off-flavors (e.g. 1-hexanol, 1-octen-3-ol, heptanal and isobutyric acid) compared to the other strains. These components are mainly derived from lipid oxidations during fermentation and are able to overrule the aroma of bread due to the absence of fermentation dependent aromas. This clearly indicated that a proper fermentation ability of the applied yeast strain is also of great importance for a desirable bread aroma.

When comparing the good performing yeast strains with the bakery strain Br04, differences in the relative production of certain components could be noticed. For example, the BioE03 strain produced relatively more phenylacetaldehyde, phenethyl alcohol and phenethyl acetate and less hexanal, isobutyric acid and 1-hexen-3-ol compared to Br04. This might benefit the final bread aroma as the former three molecules have a pleasant odor and the latter three an undesired odor.

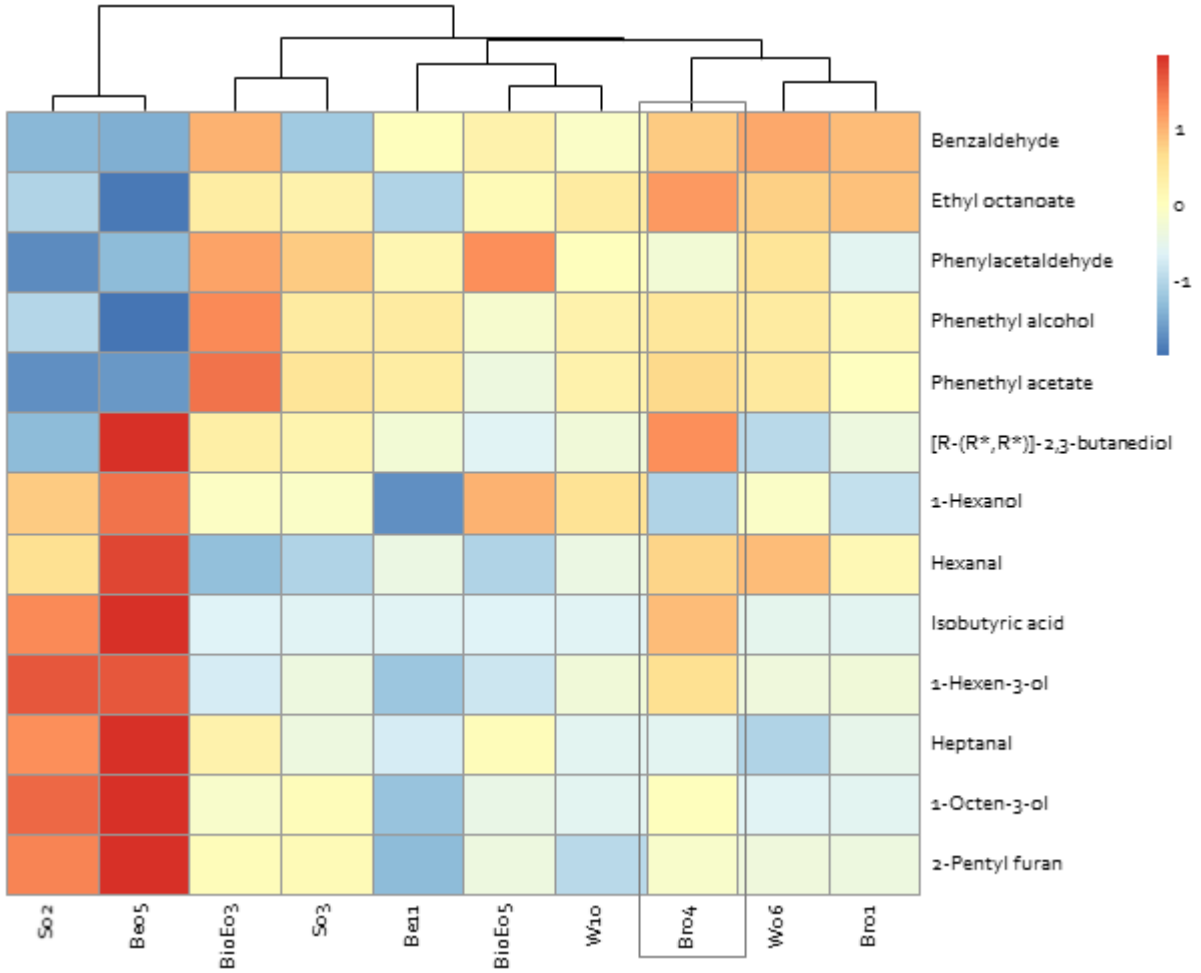

***Figure 5.9**: Heatmap representing the differences in relative concentrations of the volatile compounds (listed on the right) in wheat flour bread crumbs produced with the ten selected S. cerevisiae strains (names on the x-axis). The values shown in the heatmap are z-score, representing how many standard deviations the average log2 relative peak area of each component, produced by a certain strain is lower (blue tainted boxes) or higher (red tainted boxes) than the average production of a certain component. The values are the average of three biological and two technical replicates. The control strain Bro4 is framed.*

Because we could not determine significant differences between the bread quality parameters (Sections 5.3.3.1 and 5.3.3.2) of BioE03 and Bro4, the promising aroma profile could lead to the industrial applicability of BioE03 for bread making. Similarly, in bread with Be11, less lipid oxidation compounds resulting in off-flavors (e.g. 1-hexanol, 1-hexen-3-ol and 1-octen-3-ol) were detected. However, the lower production of ethyl octanoate and 2-pentyl furan, two desired components, could have a negative impact on the final perception of the bread aroma. Likewise, the remaining strains could be compared with Bro4, each individually differing from the control for several compounds. However, the technique used for volatile compound analysis was not

quantitative. Hence, based on these results only, it was difficult to predict which yeast strain would lead to improvement of bread aroma.

5.4 Conclusion

The aim of this chapter was (i) to compare the metabolite and aroma production of bakery and non-bakery strains during bread making and (ii) to get a holistic view of the applicability of non-bakery *S. cerevisiae* strains in the bread making process. For studying the first part, the gluten-starch model dough was used to make sure that all yeast strains could perform to their best ability and to generate insight in their full potential in conditions without inhibition. Despite the wide variation in the concentrations of acetic acid, succinic acid and glycerol amongst all 45 *S. cerevisiae* strains individually, no significant differences were established between the control group of bakery strains and the other 4 industrial categories (beer, bioethanol, wine and sake & spirits). Only the ethanol production of the bakery strains was significantly higher than that of the beer and sake & spirits strains, which was in line with the observations in Chapter 2. Concerning the aroma production, strains of the 5 industrial categories were all mixed up in the heatmap, which clusters strains with similar aroma profiles, making it difficult – if not impossible – to make a comparison between the 5 categories. Based on the fermentation metabolite production, wine and bioethanol strains were suggested to be good candidates for bread making.

Ten yeast strains (2 from each industrial category), including a control bakery strain, were selected for investigating their applicability in bread making in more depth. To this end, their impact on metabolite production, dough rheology and pH, bread volume, texture, crumb structure and aroma production were examined. The selection included two inhibition-sensitive yeasts which proved themselves to be inferior in most aspects of bread making. Despite the variability in fermentation metabolites produced by the 8 remaining and well fermenting strains, no clear correlations could be drawn between the metabolites, dough spread parameters and bread characteristics. For example, a significantly higher CO_2 production or a large increase in dough height during fermentation did not necessarily result in a bread with a significantly higher volume. Consequently, it was difficult to point out one yeast strain as being superior to the bakery strain. However, because none of the strains were significantly worse than the bakery strain, this implies they all have potential for bread making purposes.

PART 3 GENERAL DISCUSSION AND PERSPECTIVES FOR FUTURE WORK

The yeast species dominating the majority of the industrial fermentation processes is *Saccharomyces cerevisiae*. For each process specific yeast strains with divergent characteristics, mostly historically developed for that particular process, are available. For bread making purposes, some of the most desired yeast characteristics are a fast adaptation to the semi-solid environment, a high CO_2 production rate and high osmotic stress tolerance. When trying to improve bread quality, be it from an organoleptic or nutritional point of view, yeast as an essential ingredient mostly remains out of scope. Given the wide variety of *S. cerevisiae* strains, this means, however, that a lot of potential remains largely unexplored.

In this doctoral research, we set out to investigate this potential by studying the fermentation behavior of a broad set of *S. cerevisiae* strains from different industrial applications in wheat dough and their impact on dough rheology and bread quality. However, when analyzing the fermentation capacity of 45 yeast strains, yeast specific fermentation inhibition in wheat flour dough was brought to light. This made the goal of this thesis two-fold:

(i) Achieve better understanding of the yeast fermentation inhibition occurring during wheat flour dough fermentation and identification of the potential inhibitor

(ii) Gain insight in the impact and evaluate the potential of non-bakery *S. cerevisiae* strains on dough and bread quality

In the following, the outcome of this research is discussed and general conclusions are drawn. The relevance of the present findings is shown and perspectives for future work are given. A scheme summarizing the research is presented in **Figure A**.

Characterization and identification of the fermentation inhibitor

Fermentation experiments performed in Chapter 2 pointed towards yeast specific inhibition. Out of the 45 strains tested, six – all beer yeasts – produced up to 85% less CO_2 in wheat flour dough than the best bakery strain and were called the weak performers. Two yeasts – industrially applied in sake & spirits production – had a CO_2 production that was at least 50% lower and were called the intermediate performers. The other strains were identified as good performers. All yeast strains, however, fermented significantly better in liquid fermentation medium and in a gluten-starch model dough. Extracting wheat flour with water and adding the obtained extract to the model dough instead of water, resulted in a comparable weak and intermediate performance of the yeast strains as in wheat flour dough. Based on these results, it was hypothesized that wheat flour contains a water-extractable fermentation inhibiting component.

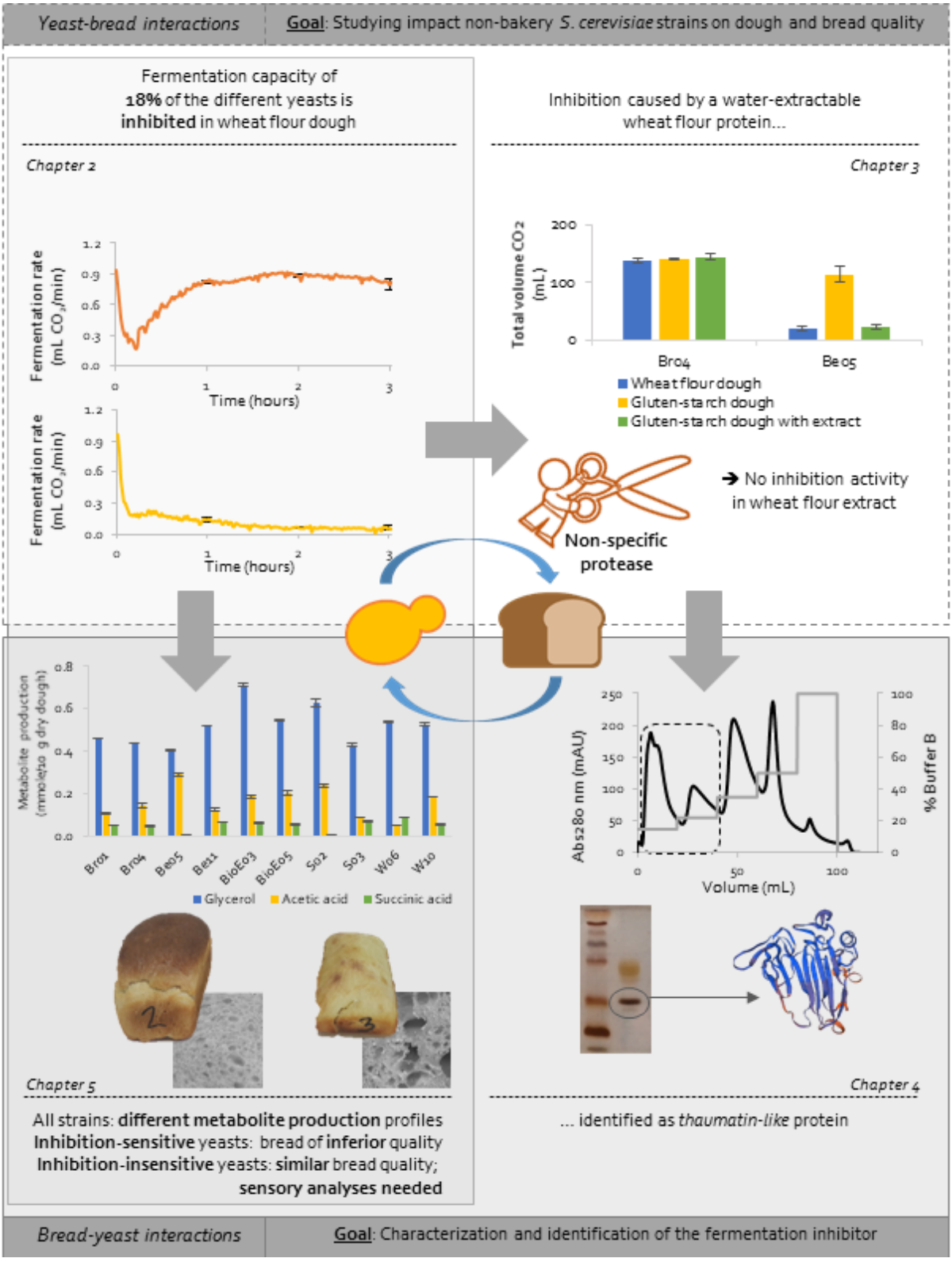

***Figure A**: A summarizing overview of the results in this doctoral dissertation. The research can be divided in two parts: (i) the yeast-bread interactions and (ii) the bread-yeast interactions. The yeast-bread interactions include the search for the applicability of different non-bakery S. cerevisiae strains for bread making, by testing their fermentation capacity on the one hand, and analyzing their metabolite and aroma production together with the impact on bread quality characteristics, on the other hand. The part of the bread-yeast interactions consists of the characterization and identification of the exposed yeast-inhibiting wheat flour protein.*

Characterization of the inhibition phenomenon was done in Chapter 3, mostly using one strain for each performance group (strong, intermediate and weak). It was observed that, next to inhibition of the fermentation metabolism of a number of yeast strains, also the yeast cell growth was retarded. A weak performer reached the stationary growth phase at the same OD as a strongly performing baking strain, albeit almost 24 hours later. The delay was less outspoken for an intermediate performer with a delay in growth of only three hours. Inhibition thus not only affected the yeast's fermentation, but probably the whole yeast metabolism. Inhibition was not strong enough, however, to completely inhibit yeast growth or kill the yeast.

The wheat flour used for most research was not exceptional in leading to yeast inhibition. Indeed, a weak performing yeast strain also showed an inhibited fermentation capacity in four other wheat varieties. Moreover, rye, maize and barley had the same effect on the yeast's fermentation ability as wheat.

Further analyses led to the conclusion that yeast inhibition in wheat flour dough was caused by a protein. In Chapter 4, starting from a crude wheat flour extract, the inhibitor was isolated through activity-guided fractionation with subsequent cation exchange chromatography and reversed phase-HPLC. Identification of the proteins present in the purest inhibition active fraction by means of LC/MS-MS and Edman degradation pinpointed the TLP (AAM15877.1) as the causing factor of inhibition. Recombinant expression of this TLP (AAM15877.1) confirmed this.

Wheat grains, together with other cereals, are known to contain proteins to defend themselves against pathogen attack and several stresses on the field. These pathogenesis-related (PR) proteins, showing antimicrobial activity, are divided into 17 classes, the 5th being the one of the thaumatin-like proteins. So, it was likely that the inhibition phenomenon could be ascribed to the identified TLP (AAM15877.1). As most of the PR proteins act upon conserved cell structures, such as cell walls or membranes, the remaining question was as to why some yeast strains were sensitive towards the inhibitor, while others were not. Suggestions could be made based on the comparison with the thaumatin-like tobacco antifungal protein osmotin. However, as this is only speculation, the search for the mode of action is included in the Suggestions for future work-section.

In the past, several researches pinpointed purothionins as yeast specific fermentation inhibitors. These proteins were, however, not found in this study, putting into question the relative

importance of purothionins and the TLP (AAM15877.1) in fermentation inhibition. The fact that purothionins did not show up in our research, could possibly be attributed to the solvent used for making extracts of flour. Flour was extracted with deionized water, whereas the past studies made use of petroleum ether extract for the purification of purothionins. In wheat flour dough, the fermentation inhibition of the sensitive yeasts could be the result of a combined effect of different antifungal wheat flour components, causing a very strong reduction in CO_2 production. Testing the fermentation capacity of the sensitive strains in gluten-starch dough with the aqueous wheat flour extract, however, gave similar results. This suggests that the water extractable components alone also causes complete fermentation inhibition, albeit probably in the absence of purothionins. As the activity-guided fractionation progressed, everything pointed in the direction of the identified TLP (AAM15877.1), assuming that this was the main inhibiting component of the wheat flour extract. However, throughout the fractionation, the extent to which the sensitive yeast was inhibited, seemed to decrease (**Figure B**). Although considered as inhibition containing fraction, the maximal fermentation rate of the weakly performing Be05 in gluten-starch dough with the RP-HPLC fraction 6 was at least 4 times higher compared to gluten-starch dough with the crude wheat flour extract. Similarly, Be05 was clearly inhibited by the recombinant TLP but the fermentation rate was also higher compared to the extract. In case of the RP-HPLC fraction, the difference in inhibition strength could possibly be ascribed to dilution of the sample during the analysis (**Figure S3**). The observed distinction could furthermore be caused by the fact that the recovery during purification is never 100% or by the separation of the inhibitor from other non-toxic co-acting wheat flour components.

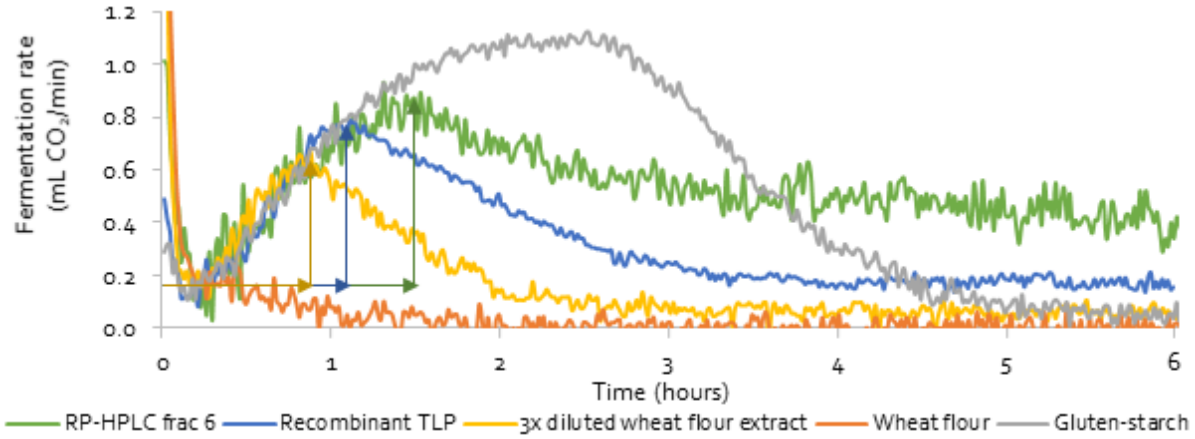

***Figure B**: Fermentation rate (mL CO_2/min) in function of time of the inhibition-sensitive Be05 in wheat flour dough (orange) and gluten-starch dough with a three times diluted wheat flour extract (yellow), with the recombinant TLP fraction (blue), with the inhibiting RP-HPLC fraction 6 (green) and with water (gray).*

In the present study, it was shown that barley, maize and rye also inhibited yeast fermentation, but the research only dealt with the identification of the inhibiting wheat component. According to literature, the mentioned cereals also contain thaumatin-like proteins of the fifth PR class, suggesting that their inhibition activity could also be ascribed to this kind of proteins. Although the TLP (AAM15877.1) in this study was already proven to be active against snow mould and barley malt thaumatin-like proteins had already been shown to have antifungal activity against some *S. cerevisiae* strains, it is to our knowledge the first time that the antifungal activity of this particular TLP (AAM15877.1) against specific *S. cerevisiae* strains during wheat flour dough fermentation has been described. These findings, together with knowledge about the mode of action of this inhibitor might be of relevance for industrial applications involving cereals and yeast-based fermentation processes. Genetic modification of yeast strains or the creation of new strains through breeding in a way that they become resistant to the action of the TLP (AAM15877.1) can be considered if inhibitor-sensitive yeast strains prove to have superior other properties. In the longer run modification of cereals to eliminate the unwanted inhibitor can also be an option.

Representativity of model systems for real life conditions

Going back again to the results of Chapter 2, the first thing to notice was the distinction in fermentation capacity of yeast strains between a liquid (non-wheat based) fermentation medium and wheat flour dough. Although the CO_2 production of all yeast strains in the liquid system seemed promising, this could not be extrapolated to their fermentation performance in wheat flour dough, making fast screenings in liquid media not representative for dough fermentations.

Although the overall fermentation capacity of the good fermenting yeast strains in the gluten-starch dough was lower than in wheat flour dough, this system seems valid as a model dough. However, if you want to screen yeast strains for their fermentation potential in wheat (or other cereal)-based fermentation processes, it is necessary to include flour extracts or to test *in situ*.

Impact of non-bakery *S. cerevisiae* strains on dough and bread quality

The fermentation capacity of yeast strains in wheat flour dough varied not only between fermentation inhibition-sensitive and insensitive strains but also amongst the group of insensitive strains. This suggested that dough and bread made with different *S. cerevisiae* strains might also demonstrate distinct characteristics. Indeed, the metabolites and volatile compounds

produced during fermentation are known to impact dough rheology and bread flavor. We examined to what extent the diverse fermentation profiles of different *S. cerevisiae* strains were reflected in dough and bread properties. In order to select a few strains for further analyses, all 45 yeast strains were first screened for their metabolite and aroma production. Since some yeast strains were inhibited during fermentation in wheat flour dough, this screening was done in gluten-starch dough, enabling all strains to perform to their best ability. Subsequently, two strains from each industrial category (bread, beer, bioethanol, sake & spirits and wine) were chosen, based on similar, lower or higher metabolite and aroma production compared to the control bakery strain Bro4. The selection contained two inhibition-sensitive yeast strains. Further analyses were performed in wheat flour dough, since we wanted to make the results as representative as possible for the bread making industry.

As expected, the first conclusion that could be drawn was that the inhibition-sensitive strains were clearly inferior to the other eight strains in wheat flour dough. Although these strains were not useful for bread making applications, taking them along the analyses provided us extra information about the impact of the inhibitor on the fermentation metabolism. Concerning their metabolite production, next to the fact that the absolute amounts produced by both yeasts were significantly lower compared to the other strains, most remarkable was that they did not produce succinic acid (in detectable amounts). Because succinic acid is a product of the respiration metabolism (TCA cycle) of yeast, this suggested that the TLP (AAM15877.1) also impacted the residual respiration activity, which could be linked to the retardation of yeast growth in the presence of the inhibitor. Indeed, TLP (AAM15877.1) is suggested to induce ROS in the inhibition-sensitive yeast strains, affecting the functionality of the TCA cycle. Another noteworthy observation in this context was the extremely low succinic acid production in gluten-starch dough, despite the significantly higher fermentation capacity of the yeasts in gluten-starch dough compared to wheat flour dough. The amounts of acetic acid were contrastingly high and significantly higher than the insensitive yeast strains in the model system. Although the mechanism behind this remains unclear, it can be suggested that the inhibition-sensitive yeast strains were more sensitive towards low oxygen concentrations in both dough systems compared to the other strains and regardless of the presence of the inhibitor. Indeed, the presence of oxygen is needed for the (residual) functionality of the TCA cycle and glyoxylate shunt, two pathways producing succinic acid during fermentation. As the glyoxylate shunt makes use of acetate for the production of succinic acid, this might explain the accompanying high acetic acid concentrations in gluten-starch dough.

Disregarding the poorly fermenting yeast strains, the metabolite production varied amongst the different yeast strains. Despite this variation, no clear differences on dough spread nor correlations between the metabolites and dough rheological properties could be established. Indeed, the continuous change in dough density through CO_2 production probably overruled the potential effects of metabolites on the rheology. Similarly, the diverse metabolite profiles did not result in the same variation in bread volume, crumb texture and structure. The use of one wine strain resulted in a final bread volume significantly higher than the control bakery strain, making this strain interesting when it comes to improvement of the loaf volume. However, no yeast strain differed significantly in all the bread characteristics from the control bakery strains, making them all potentially applicable in bread making. Indeed, using them would result in breads of comparable and good quality. Based on these results only, it was difficult to pick one or more strains as good replacers for industrially used bakery strains. The volatile compound profiles of the yeast strains were analyzed to see if they could provide more discrimination. The BioE03 strain seemed to be outstanding as it produced more pleasant and less undesired aroma compounds. Based on the results of the GC-MS only, it was however difficult to know if the noticed differences would effectively lead to noticeable variations in the final bread aroma. Therefore, performing sensory analyses in future trials is suggested.

Although all strongly fermenting yeast strains gave the impression to be as good as the bakery yeast, based on their comparable impact on bread quality parameters, some important characteristics of the yeast strains themselves were not examined in this study. Maltose is the major carbohydrate source for yeast strains in wheat flour. However, in this study 6% of glucose was added in dough formulations to make yeast strains independent of the sugar content of wheat flour, to reduce extra complexity of the system. As not all yeast strains are able to consume maltose, it is most certainly worth to dig into this in the light of the present findings. Furthermore, with commercialization of non-bakery strains for bread making purposes in mind, attention needs to be paid on yeast growth conditions, viability after drying and other potential drawbacks during yeast production.

Suggestions for future work

The recombinant expression of TLP (AAM15877.1) demonstrated the antifungal effect of the protein towards *S. cerevisiae*. However, additional experiments could provide more evidence and allow to build a stronger case. In this work, after recombinant expression of the TLP (AAM15877.1), its inhibition activity was shown by using the crude *E. coli* cell lysate. As a

control, to be sure that no other cell components act as fermentation or growth inhibitors, the cell lysate of *E. coli* without vector should be tested. Furthermore, purification of the rTLP and analysis with SDS-PAGE should be performed. Comparing the results of the purified rTLP (risograph, chromatography, SDS-PAGE) with the results of TLP (AAM15877.1) presented in this study should give irrefutable proof of the inhibitor's identity.

Similarly, the inhibitory activity of other cereals could be examined in more detail. In Chapter 3 barley, rye and maize were shown to possess antifungal activity against *S. cerevisiae* during dough fermentation. However, it was not proven that this inhibition was caused by the same or a similar TLP as identified in wheat flour. To do so, starting from crude flour extracts of the three mentioned cereals, activity-guided purification and identification of the inhibitor should be performed, in analogy to what has been done in this work. Full proof could be given by recombinant expression of the inhibitors found in these cereals.

The most important piece of research that should be pursued following this dissertation, is the analysis of the mode of action of the TLP (AAM15877.1). We already know that the inhibitor impacts growth and fermentation metabolism of the sensitive yeast strains. However, the search for the mode of action was not possible within the timeframe of this dissertation. Despite the hypothetical analogy to the antifungal protein osmotin, the mechanism behind the demonstrated inhibition still remains unclear. In a first step, the analogy with the mode of action of osmotin could be checked. As Ibeas and coworkers (2001) showed, the resistance of *S. cerevisiae* cells to osmotin depends on the allele or deletion of the SSD1 gene. Experiments following up growth of deletion mutants in the presence of the TLP (AAM15877.1) could answer the question whether or not the susceptibility of the yeast strains towards this inhibitor is determined by the SSD1 gene. Subsequently, the intracellular presence of ROS could be examined using fluorescent or chemiluminescent probes (Zhang et al., 2018). In case no analogy with the mode of action of osmotin is noticed, the growth of a whole collection of deletion mutants of *S. cerevisiae* in the presence of the TLP (AAM15877.1) is suggested to gain more insight in the processes the inhibitor is involved with. To gather information on the strength of the inhibition, the minimal inhibitory concentration (MIC) should be determined. As growth experiments in 96 well plates allow to analyze plenty of dilutions of the inhibitor at the same time, these kind of experiments are suggested for the MIC examination.

Because most of the characterization and identification experiments were performed with only a few yeast strains, it is suggested to perform the experiments done in chapter 3 and 4 also with a broader selection of inhibition-sensitive yeast strains.

As a follow-up to the results of Chapter 5, sensory analyses are suggested to give a holistic view on the impact of different non-bakery *S. cerevisiae* strains on bread quality. Analytical data, e.g. metabolite production and bread volume – as already determined – together with the consumer's perception of the product allow us to draw better conclusions concerning the applicability of the different yeast strains in bread making.

Conclusion

Although belonging to the same species, no two yeast strains are the same. In the search for alternative, non-bakery *S. cerevisiae* yeast strains for bread making, we encountered a hurdle that that was relevant to some of them. It appeared that not all yeast strains were able to ferment wheat flour dough due to the presence of a yeast specific inhibiting TLP (AAM15877.1) in wheat. The inhibition-sensitive yeast strains do possess the capacity to ferment in semi-solid state conditions. These findings can be of interest for yeast- and cereal-based industries. The improvement of existing strains by breeding, the creation of new strains through genetic modification, or genetic modification of wheat to eliminate the harmful component might provide some solutions to enable yeast strains with, for example a beneficial impact on dough and bread quality parameters or with a pleasant volatile compound profile, to be used in the bakery industry. However, in this research, quantification of the inhibitor in wheat flour was not performed. As similar studies in the past proved the importance of the concentration of an inhibitor for the impact it has on yeast cells, analysis of the concentration is certainly recommended. Obviously, to fully understand the inhibition activity and to facilitate the search for improvement of existing or the creation of new yeast strains, the mode of action of the inhibitor needs further research.

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Appendix

***Table S1**: Overview of the medians, means and standard deviations of the different fermentation metabolites (glycerol, acetic acid, succinic acid, ethanol and CO_2) produced by 45 S. cerevisiae strains. The yeasts are divided into five categories: bakery, beer, bioethanol, sake & spirits and wine. For each component, means with different letters are significantly different from each other (Tukey, p<0.05).*

	Median	Mean ± standard deviation
Glycerol (mmole/10 g dry dough)		
Overall	0.502	0.486 ± 0.105
Bakery	0.459	0.483 ± 0.0595 (ab)
Beer	0.449	0.427 ± 0.0965 (b)
Bioethanol	0.569	0.587 ± 0.0959 (a)
Sake & spirits	0.471	0.462 ± 0.108 (b)
Wine	0.535	0.550 ± 0.068 (a)
Acetic acid (mmole/10 g dry dough)		
Overall	0.182	0.182 ± 0.0609
Bakery	0.165	0.168 ± 0.0532 (a)
Beer	0.202	0.196 ± 0.0524 (a)
Bioethanol	0.185	0.162 ± 0.0614 (a)
Sake & spirits	0.158	0.165 ± 0.0539 (a)
Wine	0.183	0.199 ± 0.0672 (a)
Succinic acid (mmole/10 g dry dough)		
Overall	0.054	0.049 ± 0.024
Bakery	0.051	0.054 ± 0.013 (abc)
Beer	0.047	0.036 ± 0.028 (c)
Bioethanol	0.064	0.069 ± 0.017 (a)
Sake & spirits	0.050	0.044 ± 0.022 (bc)
Wine	0.058	0.060 ± 0.015 (ab)
Ethanol (mmole/10 g dry dough)		
Overall	4.77	4.56 ± 1.38
Bakery	4.98	5.66 ± 2.09 (a)
Beer	4.18	4.18 ± 1.51 (b)
Bioethanol	4.30	4.42 ± 0.90 (ab)
Sake & spirits	4.05	3.92 ± 1.14 (b)
Wine	5.11	5.20 ± 0.72 (a)
CO_2 (mmole/10 g dry dough)		
Overall	4.64	4.58 ± 1.18
Bakery	4.44	4.46 ± 0.86 (a)
Beer	4.37	4.31 ± 1.36 (a)
Bioethanol	4.88	5.00 ± 0.95 (a)
Sake & spirits	4.84	4.54 ± 1.40 (a)
Wine	4.82	4.86 ± 0.83 (a)

Table S2: *Volatile aroma compounds in bread produced by the 45 yeast strains together with their specific odor and odor threshold value in water (µg/kg).*

Volatile compound	Odor	Odor threshold in water (µg/kg)
Alcohols		
1-Octen-3-ol	Mushroom	1 [1]
2,6-Dimethyl-4-heptanol	Mushroom	1.3 [2]
Isoamyl alcohol	Whisky, malt	250 [1]
3-Methylthio-1-propanol	Sulfurous, meaty	500 [3]
1-Nonanol	Floral, rose	50 [2]
Phenethyl alcohol	Floral, honey	1100 [1]
1-Hexanol	Green grass, floral	2500 [2]
Aldehydes		
Dodecanal	Floral, soapy, citrus	2 [2]
Hexanal	Green, grassy	4.5 [1]
Nonanal	Citrus, soapy	1 [1]
2E-Nonenal	Fatty, cucumber	0.08 [1]
2-Heptenal	Green, fatty	13 [2]
Phenylacetaldehyde	Honey, sweet	4 [1]
2,5-Dimethylbenzaldehyde	Cherry, almond	not available
2E,4E-Decadienal	Fatty	0.07 [4]
Benzaldehyde	Almond, caramel	350 [1]
Esters		
Phenethyl acetate	Sweet, honey, floral	233 [5]
Pentyl octanoate	Iris, sweet, fruity, fatty	not available
Ketones		
2-Octanone	Floral, green, cheesy	50 [1]
3E-Octen-2-one	Earthy	not available
Acetophenone	Sweet, pungent	65 [2]
Acids		
Hexanoic acid	Cheesy, fatty, sour	3000 [2]
Benzoic acid	Balsamic	85000 [2]
Isobutyric acid	Buttery, sour, rancid	8100 [2]
Others		
D-limonene	Citrus	10 [6]
2-Acetylthiazole	Nutty, roasted	4 [2]
2,6-Dimethylpyrazine	Cocoa, roasted, nutty	200-9000 [2]
Dimethyl trisulfide	Sulfurous, onion	2.5 [5]
3-Ethyl-2,5-dimethylpyrazine	Potato, cocoa, roasted	not available

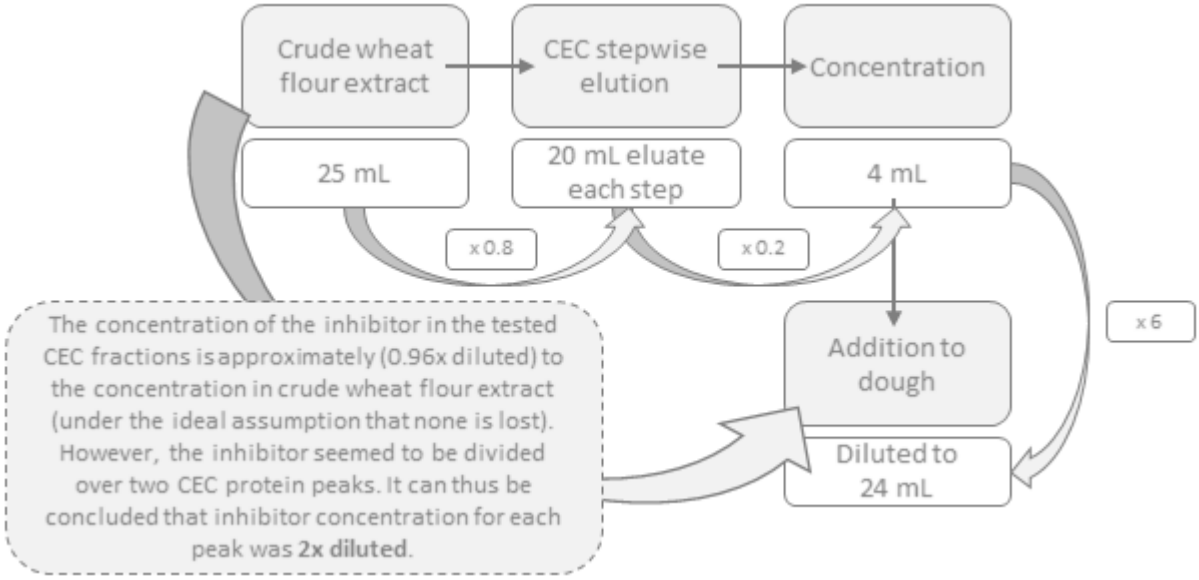

***Figure S1**: Dilution scheme CEC stepwise elution*

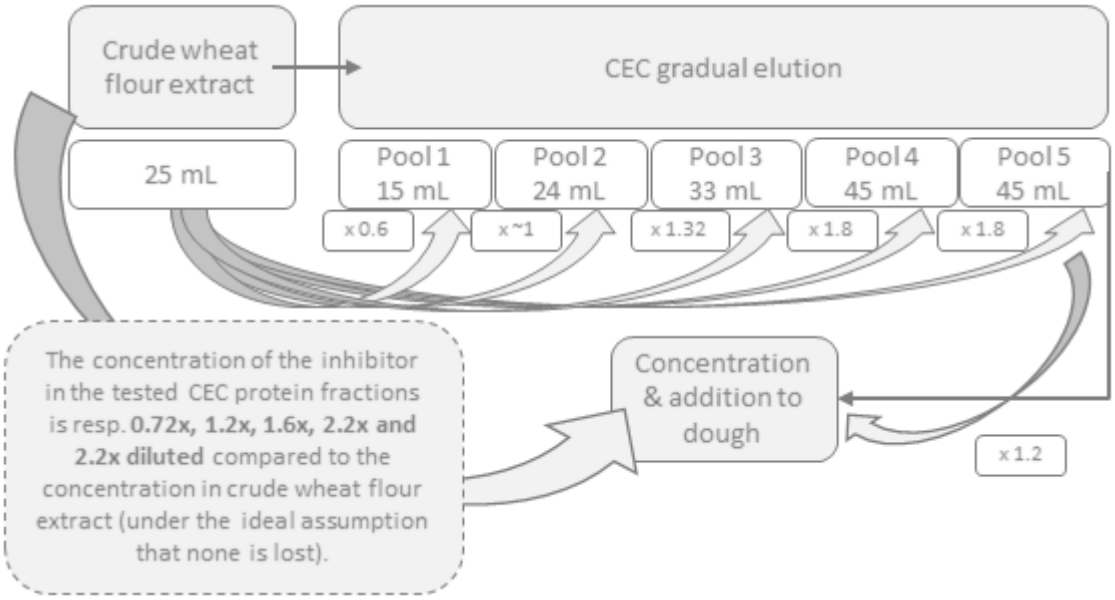

***Figure S2**: Dilution scheme CEC gradual elution*

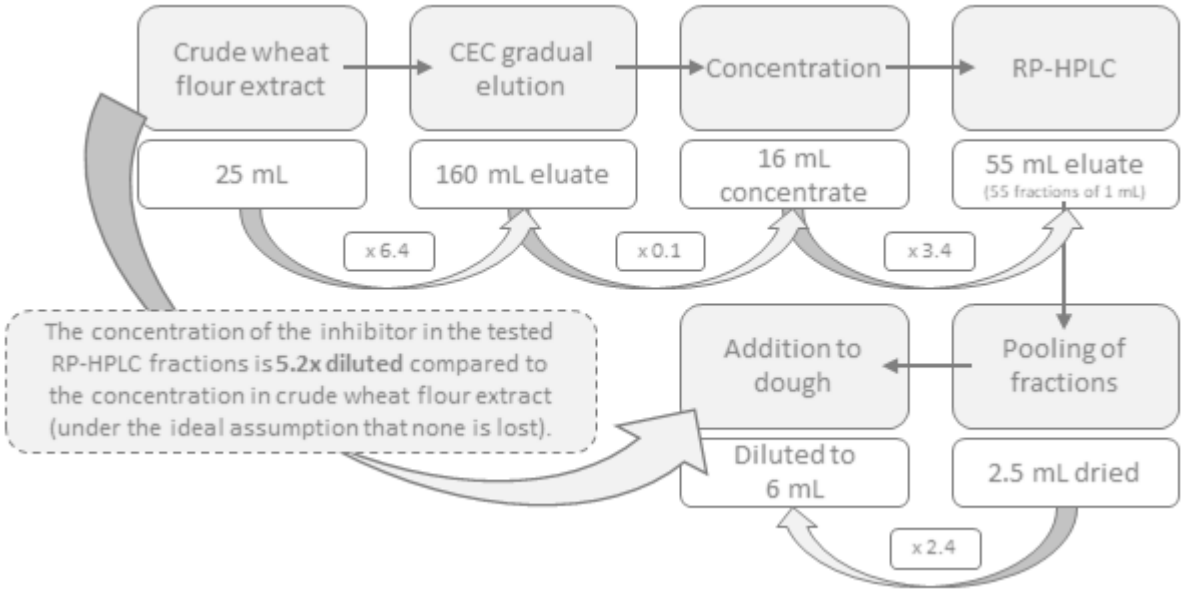

***Figure S3**: Dilution scheme RP-HPLC*

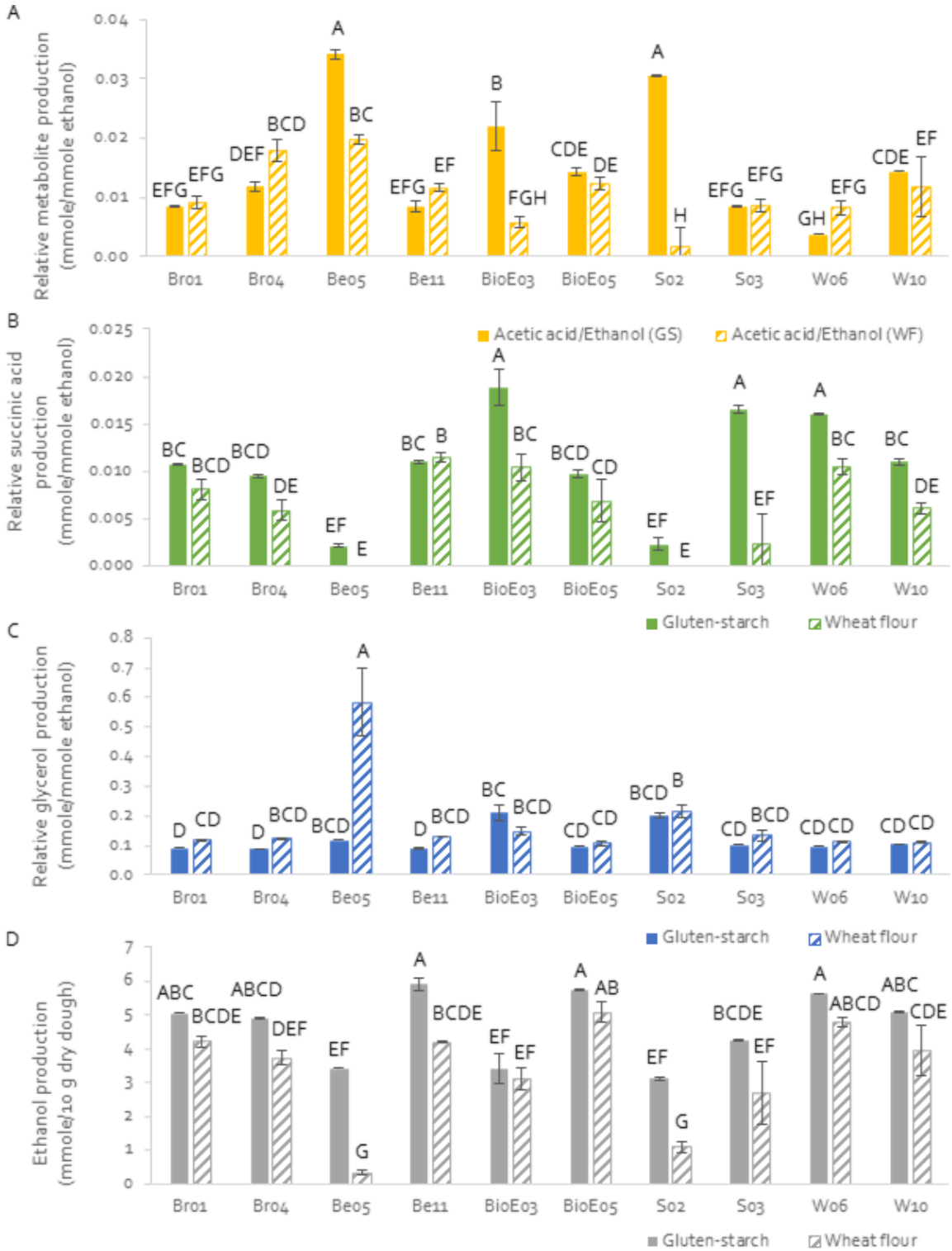

***Figure S4**: Comparison metabolite production in gluten-starch dough (fully colored columns) and in wheat flour dough (striped columns) of (A) acetic acid (mmole/mmole ethanol, yellow), (B) succinic acid (mmole/mmole ethanol, green), (C) glycerol (mmole/mmole ethanol, blue) and (D) ethanol (mmole/10 g dry dough, gray). Vertical error bars represent the standard deviations of two technical (gluten-starch) or three biological (wheat flour) replicates. Columns indicated with different capital letters within each graph differ significantly from each other (Tukey, p<0.05).*