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Doctoral Thesis

Enhancement of *Kluyveromyces marxianus* biotechnological potential

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ABSTRACT

Kluyveromyces marxianus is non-conventional food grade yeast capable of consuming a wide spectrum of substrates. This yeast also offers other great benefits including a high growth rate, thermotolerance and possesses hight activities of such biotechnologically important enzymes as β-galactosidase and inulinase. These advantages of *K. marxianus* make it promising for the production of bio-enriched chemicals from renewable substrates such as lignocellulose (xylose), polyfructans (inulin) and dairy whey (lactose). We developed an unstructured kinetic model and a biomass-linked stoichiometric model of central metabolism. The former can serve to develop and optimise processes for producing bioethanol from lactose- and inulin-containing resources, the latter in turn reveals the biotechnological potential of *K. marxianus* for industrial applications and metabolic engineering. The kinetic model of *K. marxianus* shows the highest ethanol yield (90.2 % of the theoretical yield) on whey permeate as substrate, while ethanol and biomass productivity were lower compared to semi-synthetic media with lactose or inulin due to nitrogen deficiency in whey permeate. In turn the stoichiometric model predicted that ethanol, acetate, L-lactate and ethyl acetate could be produced in near to theoretical yields and without the need for gene engineering, using substrates such as lactose, glucose and inulin. However model predicted that a high proportion of the theoretical yields of phenylethanol and succinate could not be achieved without metabolic engineering, for which the proposed model is a powerful tool.

Acetic acid is weak acid with pKa 4.76, formed as a by-product of cellular metabolism or as a result of hydrolysis of a complex substrate, is the main inhibitors that hamper a wide industrial usage of *K. marxianus*. Here, we investigate the effects of acetate in *K. marxianus* DSM 5422. Our results indicate that acetate inhibits growth in a pH-dependent manner and has pronounced effects if yeast is grown on lactose or galactose. When the culture is exposed to acetate, there is an extension of the lag phase, during which the cells adapt to higher concentrations of acetate, and when part of the population becomes resistant to acetate, growth resumes at a slower rate.

Bearing in mind the intraspecific diversity in *K. marxianus* and the differences in the ability to efficiently assimilate lactose between the different strains, we determined the total β-galactosidase activity in the four strains *K. marxianus* (DSM 5422, DSM 5418, DSM 4906 and NCYC 2791) as well as the distribution of activity between periplasmic and cytoplasmic β-galactosidases in this study. We found a correlation between the ability of the strain to grow on lactose medium and the distribution between the localization of β-galactosidase in

the strain in the precence of acetic acid. The specific growth rate of the strain decreases as the percentage of cytosolic β-galactosidase in total enzyme activity increases. The negative effect of elevated concentration of acetic acid is represented by decrease in the specific growth rate and reducing the maximum optical density of the culture. However, the degree of negative effect on the above mentioned growth parameters depends on both the carbon source in the medium and the strain that is used.

KOPSAVILKUMS

Kluyveromyces marxianus ir netradicionāls pārtikas raugs, kam ir raksturīgs plašs substrātu loks. Šim raugam ir arī citas izcilas priekšrocības, tostarp augsts augšanas ātrums, termotolerance un augsta tādu biotehnoloģiski svarīgu fermentu kā β-galaktozidāzes un inulināzes aktivitāte. Šīs *K. marxianus* priekšrocības padara to par labu kandidātu bioloģiski bagātinātu ķīmisko vielu ražošanai no tādiem atjaunojamiem substrātiem, kā lignoceluloze (ksiloze), polifruktāni (inulīns) un piena sūkalas (laktoze). Mēs izstrādājām nestrukturētu kinētisko modeli un ar biomasu saistītu centrālā metabolisma stehiometrisko modeli. Pirmais var tikt izmantots bioetanola ražošanas procesu izstrādei un optimizācijai no laktozi un inulīnu saturošiem resursiem, otrais savukārt atklāj *K. marxianus* biotehnoloģisko potenciālu industriālam pielietojumam un metabolisma inženierijai. *K. marxianus* kinētiskais modelis parāda augstāko etanola iznākumu (90,2 % no teorētiskā iznākuma) izmantojot sūkalu permeātu kā substrātu, savukārt uzrādot zemāku etanola un biomasas produktivitāti, salīdzinot ar pussintētiskām barotnēm ar laktozi vai inulīnu, slāpekļa limita sūkalu permeātā dēļ. Savukārt stehiometriskais modelis paredzēja, ka etanolu, acetātu, L-laktātu un etilacetātu var ražot ar gandrīz teorētisko iznākumu un bez gēnu inženierijas pielietošanas, izmantojot tādus substrātus kā laktozi, glikozi un inulīnu. Tomēr modelis paredzēja, ka feniletanola un sukcināta iznākumu kas būtu tuvs teorētiskam nevar sasniegt bez metabolisma inženierijas rīkiem, kam piedāvātais modelis ir spēcīgs instruments.

Etiķskābe ir vāja skābe ar pKa 4,76, kas veidojas kā blakusprodukts šūnu metabolismā vai sarežģīta substrāta hidrolīzes rezultātā, un ir galvenais inhibitors, kas limitē *K. marxianus* plašu rūpniecisku izmantošanu. Šī pētījuma ietvaros mēs parādījām acetāta ietekmi uz *K. marxianus* DSM 5422. Iegūtie rezultāti liecina, ka acetāts inhibē augšanu atkarībā no pH un tam ir izteikta ietekme, ja raugs tiek audzēts uz laktozes vai galaktozes. Kad kultūra tiek pakļauta acetāta iedarbībai, pagarinās lag fāze, kuras laikā šūnas pielāgojas augstākām acetāta koncentrācijām, un, kad daļa populācijas kļūst izturīga pret acetātu, augšana atsākas, bet ar zemāku augšanas ātrumu.

Ņēmot vērā *K. marxianus* sugas iekšējo daudzveidību un atšķirīgu starp dažādu celmu efektīvi asimilēt laktozi, šajā pētījumā tika noteikta kopējā β-galaktozidāzes aktivitāte četros celmos *K. marxianus* (DSM 5422, DSM 5418, DSM 4906 un NCYC 2791), kā arī β-galaktozidāzi aktivitātes sadalījums starp periplazmā un citoplazmā lokalizēto fermentu. Mēs konstatējām korelāciju starp celma spēju augt uz laktozes barotnes un β-galaktozidāzes

lokalizācijas sadalījumu celmā etiķskābes klātbūtnē. Celma specifiskais augšanas ātrums samazinās, palielinoties citosoliskās β-galaktozidāzes īpatsvaram kopējā fermenta aktivitātē. Paaugstinātas etiķskābes koncentrācijas negatīvā ietekme izpaužas kā specifiskās augšanas ātruma samazināšanās un kultūras maksimālā optiskā blīvuma samazināšanās. Tomēr negatīvās ietekmes pakāpe uz iepriekš minētajiem augšanas parametriem ir atkarīga gan no barotnē esošā oglekļa avota, gan no izmantotā celma.

TABLE OF CONTENT

ABBREVIATIONS

μ - specific growth rate, h-1

μ_{max} – maximum specific growth rate, h-1

α - growth-associated term

CCCP – carbonyl cyanide-m-chlorphenylhydrazone

DE – differential evolution

DW – dry weight

FBA – flux balance analysis

FVA – flux variability analysis

GOGAT - glutamine oxoglutarate aminotransferase

GPR – gene-protein-reaction

$K_{I,P}$ – product inhibition constant, g/l

$K_{I,S}$ – substrate inhibition constant, g/l

K_s – half-saturation constant, g/l

m_S – maintenance coefficient

NADH - nicotinamide adenine dinucleotide

NADPH - nicotinamide adenine dinucleotide phosphate

OD – optical density

ODE – ordinary differential equations

P – product, g/l

R^2 – coefficient of determination, %

RMSE – Root-Mean-Square-Error

S – substrate, g/l

WP – whey permeate

X – biomass g/l

X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

$Y_{X/S}$ – yield coefficient for biomass (X) on substrate (S)

β – non growth-associated term

INTRODUCTION

The non-traditional *K. marxianus* yeast has great biotechnological potential for numerous industrial applications. *K. marxianus* has a number of characteristics that are industrially useful, including a wide range of substrates, thermotolerance, limited fermentation in excess sugar and secretion of extracellular glycolytic enzymes. Due to its GRAS and QPS status, *K. marxianus* can be used in the food and pharmaceutical industries. *K. marxianus* can utilize such carbon sources as glucose, fructose, xylose, galactose and lactose, many of them are of special interest because they are wastes from forestry (xylose), from dairy industry (lactose) or inulin-containing substrates that do not compete with food crop. *K. marxianus* is a promising producer of a number of important food additives and chemicals as well as yeast biomass.

Despite significant industrial advantages these food yeasts have not yet been subjected to sufficient research, and quantitative studies of the technological processes. Such in-depth studies are particularly necessary because different growth parameters have been reported not only for different strains of *K. marxianus*, but also for the same strain in different scientific studies. Such metabolic diversity makes it difficult to generalize knowledge about these yeasts. It is known that the behaviour of microbial systems can be estimated from growth kinetic parameters, which represent appropriate mathematical models. These kinetic models may be indispensable for the design and successful operation of industrial bioprocesses and to obtain quantitative information on the function microbial cells. One of the objective of the present study is to develop a kinetic model based on the time-course measurements of substrate, product and biomass changes during the ethanol production by the yeast *K. marxianus* DSM 5422 at varied initial concentrations of lactose and inulin containing substrates.

Stoichiometric models and reconstructions greatly facilitate the analysis of the metabolic effects and limitations of microbial metabolism as well as the prediction of the phenotype of recombinant strains. Recent attempts at metabolic engineering of *K. marxianus* highlight the need to model the limitations of the metabolic potential of these yeasts. Another objective of this study is develop biomass-coupled model of central metabolism to be a basis for design of metabolic engineering and to assess in silico production of different valuable metabolic products from different substrates.

Another several disadvantages are reported that hamper a wider industrial usage of *K. marxianus*, for example, the relatively low ethanol tolerance and weak-acid intolerance has

also been reported. Acetic acid is weak acid with pKa 4.76, formed as a by-product of cellular metabolism or as a result of hydrolysis of a complex substrate, is the main inhibitors that hamper a wide industrial usage of *K. marxianus*. Until now, *K. marxianus* has been widely studied from a biotechnological point of view, and less attention has been directed to its basic physiology. Indeed, very little is known about its stress response and adaptation mechanisms induced by acetic acid in this yeast. Here we investigated the inhibitory effects of biotechnologically relevant concentrations of acetate on the industrially promising strain *K. marxianus* DSM 5422 in more detail.

Keeping in mind the metabolic and physiological differences between *K. marxianus* strains as well as conflicting data on β-galactosidase localisation in literature, in this study we evaluated the ability and efficiency of different strains to utilise different substrates in medium with an increased concentration of acetic acid at a low pH value.

The aim of the dissertation was to evaluate the possibility to enhance the biotechnological potential of *K. marxianus* using modelling techniques. As well as to evaluate the effect of acetic acid at low pH levels on growth and metabolism of *K. marxianus*.

The tasks of this study:

1 To develop an unstructured kinetic model for the batch production of bioethanol by *K. marxianus* from renewable sources of agricultural and food processing origin.

2 To estimate the parameters and to validate the kinetic model by data obtained from experiments.

3 To develop a mass and charge balanced stoichiometric model of central *K. marxianus* carbon metabolism including biomass production.

4 Using a stoichiometric model to reproduce the experimentally observed mix of industrially valuable products, as well to explain the formation of unwanted side products.

5 Compare the product values suggested by the stoichiometric model with the theoretical ones.

6 To investigate the inhibitory effects of biotechnologically relevant concentrations of acetate on the industrially promising yeast *K. marxianus*.

7 To determine the localization of β - galactosidase in four different *K. marxianus* strains. Evaluate the possible correlation between the acetate tolerance of the *K. marxianus* strains on lactose medium and distribution of the β-galactosidase activity between cytosol and periplasm.

1 LITERATURE REVIEW

1.1 Characteristics of K. marxianus

Yeast *Kluyveromyces marxianus* has been isolated from a wide range diary and non-diary environments. It was first described in 1888 by Danish mycologist Emil Christian Hansen and named *Saccharomyces marxianus* after zymologist Louis Marx of Marseille who first isolated it from grapes. Amongst ten strains of *S. marxianus* was a strain marked as *Zygosaccharomyces marxianus* and deposited at the Centraalbureau voor Schimmelcultures (CBS) in 1922, that was chosen as the type strain, presently known as CBS 712 strain (Lodder and Kreger-van Rij, 1952). In 1956 basing on differences in spore morphology, in the ability of metabolizing different sugars, and occurrence of hybridization between strains compared to *Saccharomyces* yeast motivated van der Walt to transfer the *S. marxianus* species to the genus *Kluyveromyces* (van der Walt, 1956). After several classification reorganizations in *Kluyveromyces* genus and application of genetic based analysis (multigene sequence) rather than phenotypic analyses, number of species in the *Kluyveromyces* genus decreased to six including *K. marxianus* (Kurtzman, 2003; Lachance, 2007).

Kluyveromyces marxianus is a homothallic hemiascomycetous yeast species together with its sister species *K. lactis* that are known for its ability to assimilate lactose, which is rare among the yeast (Lachance, 2011; Barnett et al., 2000). In addition to ability to metabolize lactose *K. marxianus* is able to grow on inulin containing substrates (Rouwenhorst et al., 1988). Also, this yeast species is well-known for its hight thermotolerance (up to 45 °C) (Steensma et al., 1988) and in consequence of the highest tricarboxylic acid cycle flux during batch growth on glucose, it has the highest specific growth rate among eukaryotes (Blank et al., 2005; Groeneveld et al., 2009). Similarly to *S. cerevisiae* numerous strains within *K. marxianus* species shows dimorphism. The morphology of several *K. marxianus* strains depends on cultivation conditions, it can be in unicellular yeast like form or in the pseudo-hyphal (O'Shea and Walsh PK, 2000). This aspect can be important for the biotechnological application especially for downstream processes (McIntyre et al., 2002).

Unlike to *S. cerevisiae*, facultatively fermentative *K. marxianus* yeast is classified as Crabtree-negative, meaning that no ethanol formation is detected even after excess glucose addition to aerobically cultivated cells (Verduyn et al., 1992). This results in the inability of *K. marxianus* to grow in a strictly anaerobic conditions and ethanol synthesis is directly related to oxygen limitation (Bellaver et al., 2004). Due to a capability of rapid conversion of

carbon sources to cell biomass this can be useful in yeast biomass production from industrial substrates and for heterologous protein production (Wolf et al., 2003).

1.2 Carbohydrate transport and metabolism in K. marxianus

In contrast with *S. cerevisiae's* narrow set of the strains that are used in biotechnology, *K. marxianus* is known for its wide variety of strains that have application in research and industry. *K. marxianus* yeast isolation from various habitats resulted with hight metabolic diversity and ability to metabolize wide spectrum of substrates (e.g., glucose, lactose, galactose, sucrose, xylose, inulin, raffinose and arabinose) (Fonseca et al., 2008). Sugar metabolism of *K. marxianus* notably differs from that of *S. cerevisiae*, with differences possibly related to sugar transport to the cell.

In spite of the fact that components of the glycolytic and Krebs cycle are conserved among the yeast, regulation of the sugar metabolism differs substantially (Flores et al., 2000). Even extrapolating from studies of such genetically close sister species as *K. marxianus* and *K. lactis* incorrect conclusions can be done (Blank et al., 2005). Until recent years information about transport of such sugars as glucose, galactose and lactose in *K. marxianus* was limited.

1.2.1 Glucose, fructose and galactose

Monosaccharide transport in *K. marxianus* occurs through HXT class of transporters which are part of major facilitator superfamily (MFS) (Varela et al., 2019a). Two types of structurally and functionally different sugar transport systems are described in *K. marxianus*. One system is low-affinity transporter which activity is not associated with H^+ movement. Another transport system type in *K. marxianus* is a proton-sugar symporter. These transporters exhibited different substrate specificities. Activity of low-affinity and hight-affinity transporters is regulated by the carbon source of cultivation medium. Low-affinity transporters are used for glucose transport to the cell in glucose-grown cell culture, whereas hight-affinity carriers are used in lactose-grown cells (Gasner, 1987; de Bruijne et al., 1988; Postma and Van den Broek, 1990). Depending on the cultivation conditions these both glucose transporters can be used for fructose entering to the cell. Fructose is preferential for low-affinity carrier in glucose-grown cells but it enters lactose-grown cells through a proton symport transporter (Gasner, 1987).

While there are some common features in galactose transport in *K. marxianus* and *K. lactis*, upatake in *K. marxianus* is more complicated. Galactose transport to the cell in *K.*

marxianus strains can occur using eight different galactose transporters. Three of four LAC12 (except LAC12-3 with unknown function) genes are identified, four HGT and one KHT gene showed its ability to transport galactose (Varela et al., 2019a). Using above mentioned low and hight affinity transport systems galactose is taken up to the cell only in lactose or galactose growing cells by proton-symport system. This system requires induction by lactose or galactose substrate (Gasner, 1987).

Glucose is a metabolically important monosaccharide that can be used by cells as an energy source and as a metabolic intermediate. This monosaccharide is catabolised through the process of glycolysis, cleaving the six-carbon molecule into two pyruvate molecules. Like glucose, galactose contains six carbon atoms and differs from glucose only in the stereochemistry of the single carbon atom in the C-4 position. Before it can enter glycolysis with the help of enzymes of the Leloir pathway, galactose must be converted into a glucose derivative (Caputto et al., 1949). The enzyme galactokinase phosphorylates galactose to form galactose-1-phosphate by a stereospecific reaction. However, as this enzyme only converts the alpha form of D-galactose, the initial step in the Leloir pathway is the conversion of beta-D-galactose to alpha-anomer by the enzyme mutarotase. Galactose-1-phosphate is then exchanged with the glucose group from UDP-glucose to form UDP-galactose and release glucose-1-phosphate. This reaction is catalysed by the third enzyme of the Leloir pathway, galactose-1-phosphate uridyltransferase. In turn, epimerase changes the stereochemistry of C-4 in UDP-galactose, forming UDP-glucose. The transfer reaction then releases the glucose in the form of glucose-1-phosphate. Eventually, after the conversion of glucose-1-phosphate to glucose-6-phosphate, followed by entry into glycolysis for energy production.

1.2.2 Lactose

Lactose is a reducing sugar, disaccharide formed from two hexoses: one molecule of D-glucose and one molecule D-galactose joined by β-1,4- glycosidic bond. Lactose hydrolysis in *K. marxianus* ensured by enzyme β-galactosidase (EC 3.2.1.23) which belongs to glycoside hydrolase enzymes family (Guidini et al., 2010). *K. marxianus* possess the highest β-galactosidase activity among yeasts when lactose is used as carbohydrate source (Belem and Lee, 1998). β-galactosidase localization in the cell is strain dependent, lactose hydrolysis can occur in the cytosol or extracellularly (Carvalho and Spencer, 1990). Therefore its activity is similar in poor and good lactose consuming *K. marxianus* strains and is not limiting factor for growing on this disaccharide (Varela et al., 2017).

Lactose assimilation is not a common trait for *K. marxianus,* but is strain dependent (Lane et al., 2011). Different strains can exist in three states: haploid, diploid and triploid. Ploidy have strong correlation with the environment niche of strain (Ortiz-Merino et al., 2018). Non-diary isolates are mainly haploids (A and C haplotypes) with non-functional lactose transporter allele *LAC12-A* and are Kluyver effect positive for lactose. They can respire, but cannot ferment the lactose and can be classified as poor lactose consumers. Meanwhile B haplotype strains with functional lactose transporter *LAC12-B* allele are diploids or triploids, and are associated with diary environment, can be classified as good lactose consumers. It was demonstrated that in spite of the fact that all *K. marxianus* have four copies of LAC12 gene in three different chromosomes, ability to effectively utilize lactose depends on the amino acid sequence of lac12p transporter. Polymorphism in LAC12 gene alleles translate into 13 amino acid variation determine functionality of lactose transporter (Ortiz-Merino et al., 2018; Varela et al., 2017). The lac12p is an integral membrane protein and belongs to major facilitator superfamily it works via a proton symport mechanism. It is inducible by substrate (Riley et al., 1987).

1.2.3 Inulin

K. marxianus is the only one species of *Kluyveromyces* genus able to assimilate inulin as carbon source (Barnett et al., 1983; Snyder and Phaff, 1962). Inulin is general term for all β(2→1) linear fructans with different degree of polymerisation (Roberfroid, 2005). Inulin containing non-food grade biomaterials (*Jerusalem artichoke*, chicory, dahlia, and yacon) have advantages as lower price, easer cultivation conditions over the starchy materials (Chi et al., 2011). This fructose polymer can be hydrolysed by invertase or inulinase but the first process is much slower than the second one. Localization of inulinase and invertase in yeast is dependent on the yeast strain and cultivation conditions. Invertase and inulinase enzymes have different structure, kinetic and physiological functions. Physiological role of invertase is intracellular hydrolysis of sucrose whereas inulinase insures fructan cleavage outside the cell wall (Rouwenhorst et al., 1990). Inulinase is non-specific β-fructofuranosidase that ensures hydrolysis of 2,1 and 2,6-linked β-D- fructofuranose residue in fructan with the release of β-D-fructose. It's production depends on the composition of the cultivation medium and the type of carbohydrate used. Optimal conditions for maximal enzyme activity were found in the pH range 3.5-5.0 and temperature diapason from 50 to 60 °C (Pessoa and Vitolo, 1999). For inulinase induction sucrose or inulin should be present in the culture medium. Depending on environment conditions the enzyme can be secreted to the

medium or remain associated to the yeast cell wall (Rouwenhorst et al., 1988; Barranco-Florido et al., 2001).

Production of inulinase can be used in applied biotechnology for fructose syrup and fructooligosaccharides. Obtained sugar substrates can be converted to such bioproducts as ethanol, lipids, single-cell proteins, acids and other chemicals (Chi et al., 2011; Ma et al., 2015; Wang et al., 2014; Zhang et al. 2015b, Liu et al. 2013; Cui et al., 2011).

Inulinase activities available are still insufficient for industrial uses. This enzyme production can be enhanced using metabolic engineering techniques.

1.2.4 Other carbohydrates

Beside above mentioned carbon containing sources, unlike *S. cerevisiae*, which is poorly able to metabolise pentose sugars, *K. marxianus* is able to effectively assimilate pentoses xylose and arabinose via the aldose reductase pathway. First step in sugar metabolism is transport to the cell. In *K. marxianus* are certain transporters that are able to support growth in environment with xylose or arabinose. Four HGT – family proteins enable growth in low or hight pentose concentration medium and six HXT-like transporters with low affinity activity are active only at hight sugar concentration (Knoshaug et al., 2015; Donzella et al., 2021). Although *K. marxianus* can ferment xylose and arabinose, the fermentation capacity is strain dependent. This assimilation differences between *K. marxianus* strains are not caused by key metabolic enzymes structural polymorphism, but by transcription or translation regulation level. Fermentation performance depends on fermentation temperature (Hou et al., 2017).

Xylose is the most prevailing pentose from hydrolysis of hemicellulose and constitutes about 18-30% of lignocellulose hydrolysate sugars (Mosier et al., 2005). There are several limitations of using this pentose sugar as renewable resource for producing biofuels and chemicals. Firstly, only a limited number of microorganisms have the native xylose assimilating pathways (Kim et al., 2013). Secondly, xylose utilization can be repressed by glucose, so called carbon catabolite repression and results in diauxic growth (Young et al., 2014).

The isomerization of xylose to xylulose is two-step pathway employing NADPH - dependent xylose reductase (XR) reduces xylose to xylitol and NAD - dependent xylitol dehydrogenase (XDH) oxidizes xylitol to xylulose (Wang et al., 1980). Before entering to the pentose phosphate pathway (PPP) xylulose should be phosphorylated to D-xylulose-5-phosphate by xylulokinase (XK) (Bruinenberg and Van Dijken, 1983). The process of NADH

oxidation to NAD+ requires oxygen presence, under anaerobic conditions co-factor imbalance leads to accumulation of xylitol and ethanol concentration decreasing. Because of this wild-type strains of *K. marxianus* are not efficient xylose fermenters. Metabolic engineering methods can be used for improving ethanol production from xylose by *K. marxianus* wild strains.

L-arabinose is the second most abundant pentose in plant. Unlike xylose, where only three enzymes are needed to convert D-xylose to D-xylulose 5-phosphate, in the case of arabinose, five enzymes are needed (Seiboth and Metz, 2011). In L-arabinose conversion to xylitol three enzymes are involved. First step is L-arabinose reduction to L-arabitol catalysed by aldose reductase, next step is L-arabitol oxidation to L-xylulose by L-arabinitol 4-dehydrogenase, and final step is L-xylulose reduction to xylitol by L-xylulose reductase (Hahn-Hägerdal et al., 2007).

1.3 Stress tolerance

Microorganisms like yeast must be able to maintain homeostasis in the cell despite changes in the environment by adapting the processes occurring in the cell to survive in new growing conditions. Sudden changes in the environment can lead to the cell wall damage and plasma membrane disorders, induces protein denaturation, increases reactive oxygen species level and cause endoplasmic reticulum stress. These damages and changes can negatively affect metabolism and even lead to cellular apoptosis. In order to avoid the above-mentioned irreversible and lethal effects, the cell must be able to adapt rapidly to changes in the extracellular environment.

Yeasts are widely used in the field of industrial biotechnology (Nielsen and Keasling, 2016). During the fermentation process microorganisms can be exposed to such negative environmental stresses like hight temperature, oxidative stress, hight osmolarity, acid induced stress and others. Many of this stress conditions have been examined in the context of industrial application, such as alcoholic beverages and bread making since during these processes the cells are subject to such stresses as heating, chilling, desiccation and oxidation (Attfield, 1997). Changing environmental conditions away from their optimum value can cause the induction of many complex stress responses. These strategies are usually directed towards survival rather than growth and most often take the form of lag phase prolongation and biomass reduction (Ray, 1986; Russell, 1990).

1.3.1 Ethanol induced stress in yeasts

In the late stage of fermentation, yeast must resist the damage caused by increased ethanol concentration, and the cell membrane is the main target of ethanol attack. As the concentration of ethanol in the medium increases, cell viability decreases. Therefore, when the ethanol concentration exceeds the maximum allowable concentration, cell growth is inhibited and the yeast dies, eventually leading to a reduction in ethanol yield (Mo et al., 2019). Therefore, the ability of yeast to produce ethanol determines its ability to tolerate ethanol. Yeast resistance to ethanol is a complex phenotype and is regulated by multiple genes. In the case of *S. cerevisiae*, there are hundreds of genes involved in the response to ethanol, encompassing ethanol metabolism, glycolysis, plasma membrane composition, protein folding, cell wall biogenesis, lipid metabolism etc. Using lactose as a carbon source, Diniz and colleagues compared the transcriptomics of unstressed and short-term exposure to 6% (v/v) ethanol and found that central metabolic flux, including the TCA cycle, was impaired when exposed to ethanol, and biosynthesis of unsaturated fatty acids was also decreased (Diniz et al., 2012). In turn, Li and colleagues screened a library of random mutagenesis of the TATA-binding protein Spt15 of *K. marxianus* to produce a tolerant strain with maximum tolerance to 5% ethanol, whereas the original wild-type strain could tolerate only 2% (v/v) (Li et al., 2018). Ethanol alters the structure of cell membranes by increasing the interdigitations and reducing membrane thickness. Protein structure and function is also a target for ethanol.

1.3.2 Weak organic acid induced stress in yeasts

Due to the peculiarities of yeast metabolism, for many centuries they were used to produce fermented foods and alcoholic beverages. During fermentation processes with yeast, competing microorganisms release weak organic acids as lactic and acetic acids. These metabolic products can not only slow down the growth and proliferation of yeast, but also have a lethal effect. Also production of such metabolites as succinate, lactate and acetate, that are produced by yeasts itself, leads to a decrease in pH value of the environment. Probably it was the ability to survive in acidic environmental conditions that made the evolutionary pressure on yeast and as a result, the tolerance of yeast to a reduced pH of the environment is higher than for example in *E. coli*. Along with sulphite, weak organic acids as succinate, benzoic and acetic in combination with a reduced pH are widely used as preservatives in the food industry (Piper, 2011). Due to its high acid resistance, *S. cerevisiae* and *Zygosaccharomyces bailii* yeasts are among the most common spoilage yeasts in food and

beverage industries. As a result, the most studied yeast strains in the issues of adaptive mechanisms to survive in environment conditions with increased weak organic acid concentration are above mentioned.

In solutions, weak organic acid as sorbic, benzoic, lactic and acetic acids are present in a pH-dependent equilibrium between their undissociated and dissociated forms. Each of these acids has a pH at which half of the molecules lose the proton (pKa), a decrease in pH level form the pKa value leads to increasing of the level of undissociated form of acid. After addition of the weak organic acid to the cultivation medium, unlike dissociated acid form, the undissociated acid form freely enters the cell by passive diffusion. Dissociated weak organic acid form is transported to the cell by active transport system. After the non-dissociated form is released from the acidic environment into the more neutral pH in cytosol, the protonated form dissociates into a proton and an respective counter-anion. Accumulation of released protons can lead to a decrease of the intracellular pH value (Ullah et al., 2012), which, in turn, leads to a decrease in the metabolic activity of the cell (Orij et al., 2012 ; Pearce et al., 2001). Also, a decrease in intracellular pH has a negative effect on cell signalling (Dechant et al., 2010), interactions between proteins (Young et al., 2010) and cell division (Orij et al., 2012). Due to their charged form, anions also accumulate in the cell cytosol and depending of its nature, can cause increase of the cell turgor and reduce cell growth (Piper, 1999; Ullah et al., 2012). Because of the energy required to pump released protons and anions out of the cell via ATPases and the inhibition of glycolysis, the intracellular pool of ATP is depleted (Holyoak et al., 1996).

1.3.3 Mechanism of adaptation to acetic acids in yeasts

Understanding adaptation and tolerance mechanisms of yeasts to elevated concentrations of acetic acid in the environment have practical importance in the context of biotechnology and the food industry (Palma et al., 2018; Palma and Sa-Correia, 2019). Acetic acid is not only one of the major inhibitory chemicals present in the hydrolysate of lignocellulose that is used as a biotechnological substrate, but is also a normal metabolite produced during yeast growing process and preservative in foods and beverages industry (Cunha et al., 2019).

After exposing yeast cells to sublethal concentrations of acetic acid, an extension of the period of cell adaptation to the environment conditions and a reduction in the specific growth rates after culture adaptation are observed (Fernandes et al., 2005; Guerreiro et al., 2012). In turn, lethal concentrations of acetic acid can induce regulated cell death, through

either cell apoptosis or necrosis, depending on the sternness of the acetic acid-induced stress (Ludovico et al., 2001, 2003).

Acetic acid entry into the cell depends on both the extracellular pH value and the specific growth conditions. During cultivation of *S. cerevisiae* in glucose containing medium at a pH below that of acetic acid pKa (=4.76), the acetic acid is in an undissociated form that passively diffuses through the cell membrane (Casal et al., 1996). Aquaglyceroporin Fps1 is also involved in the transport of acetic acid to the yeast cell (Mollapour and Piper, 2007). If *S. cerevisiae* is cultivated without the presence of glucose in the growth medium, or if the pH of the environment is above the pKa value of acetic acid, the cell transports the acetic anion by the acetate carrier Ady2 (Casal et al., 1996; Paiva et al., 2004).

Unlike *S. cerevisiae*, during cultivation of *Z. bailii* in the presence of glucose and acetic acid, the entry of the undissociated form of acid into the cell causes no significant effect on the total flow of acetate into the cell was observed (Sousa et al., 1996). During cultivation of cells in medium where glucose or fructose is present as the main carbohydrate source, the presence and amount of glucose non-repressible transporters is regulated by the intracellular concentration of acetate (Sousa et al., 1998). However, if acetate is the main source of carbon in the growth medium, the transport of acetic acid takes place through a saturable transport system, which also can transport propionic and formic acids (Sousa et al., 1996).

It is thought that the main reason for the inhibition of *S. cereviaise* cell growth in the presence of acetic acid is the acidification of the cell cytosol (Stratford et al., 2013a). However, the inhibition of cell growth is most likely not due to a decrease in intracellular pH, but to the cell's ability to recover physiological intracellular pH levels (Ullah et al., 2012). The ability to maintain physiological levels of pH inside the cell is key to the survival and growth of *S. cerevisiae* in medium with elevated concentrations of acetic acid at low pH. To maintain a proton gradient across the cell membrane, the yeast cell depends on plasma and vacuolar H^+-ATPase activity. The activity of these two pumps ensures that the protons accumulated in the cytosol, as a result of acetic acid dissociation, are carried out into the extracellular medium and the vacuole lumen (Ullah et al., 2012; Carmelo et al., 1997). The recovery of pHi in combination with the inhibition of glycolysis is an energy-consuming process, which in turn leads to a reduction in the level of pH in the cell (Pampulha and Loureiro-Dias, 1990). It has been observed that ATP concentration level in the cell correlate with the concentration of acetic acid in the growth medium; the higher the acid concentration, the higher the ATP level in the cell. This strategy most likely helps the cell to conserve

energy to activate growth under more favourable conditions (Ullah et al., 2012). Spoilage yeast of *Z. bailii* can tolerate a significant drop in intracellular pH during the exponential phase and a subsequent recovery in intracellular pH levels during the stationary phase, which may underlie the exceptional acid tolerance of this yeast species (Dang et al., 2012).

Researches in genomics, transcriptomics and proteomics has led to a better understanding of the mechanisms of acetic acid tolerance to *S. cerevisiae* (Kawahata et al., 2006; Li and Yuan, 2010; Antunes et al., 2018; Longo et al., 2015; Almeida et al., 2009). These include changes in the composition and properties of the plasma membrane and cell wall, leading to a decrease in cell membrane permeability (Godinho et al., 2018; Lindahl et al., 2016). Such adaptation is important to reduce the permeability of the cell membrane, resulting in a reduced rate of diffusion of this weak acid from the outside environment of the cell into the intracellular space. This cellular response in turn counteracts the re-entry of the acidic form after active efflux of acetate from the inner cell environment, presumably catalysed by efflux pumps (Tpo2, Tpo3, Aqr1, Pdr18), and thus limits the related futile cycle (Godinho et al., 2018; Godinho et al., 2019; Ullah et al., 2013).

In yeast, tolerance to acetic acid is tightly controlled at the transcriptional level by several transcription factors, especially factors such as Haa1, which is noted in *Z. bailii* (Antunes et al., 2018), *S. cerevisiae* (Mira et al., 2011) and *Candida glabrata* (Bernardo et al., 2017). The transcription factor Hog1 is also required for yeast adaptation to acetic acid-induced stress (Mollapour and Piper, 2007). The role of this transcription factor in the resistance of *K. marxianus* to acetate stress was established by comparing the growth kinetics of wild type and delta mutant Hog1 cells exposed to increasing concentrations of acetate from 0.1 to 0.4 % (v/v). Acetic acid was more inhibitory for delta hog1 cells than the identical concentration for the wild type, at lower concentrations no significant difference was noted between these strains, showing the importance of Hog1 for the resistance of *K. marxianus* cells to acetic acid stress (Qian et al., 2011).

1.4 Applications of Kluyveromyces marxianus in biotechnology

Yeast is a microorganism with a remarkable diversity and properties, including many that have a strong impact on human lifestyle. For thousands of years, these organisms have played an essential role in traditional biotechnology. In the past century, yeast has become an important tool in modern biotechnology, acting as a host for the production of compounds with added value, including recombinant proteins, primary and secondary metabolites. Despite the fact that the number of described yeast species is increasing every year, the

number of yeast species that are used in industrial biotechnology still is limited. They mainly belong to five different yeast genera (de Winde, 2003). *S. cerevisiae* still takes main position in applied microbiology and is the widely used microorganism and in the biotechnological industry. But several limitations with *S. cerevisiae*, however, has led to a greater focus on the development potential of the so-called non-conventional yeasts, such as *Pichia*, *Zygosaccharomyces*, and *Kluyveromyces*.

Kluyveromyces marxianus GRAS (Generally Recognized as Safe) and QPS (Qualified Presumption of Safety) status makes possible to use this microorganism for heterologous protein production and for pharmaceutical and food applications (Leonel et al., 2021). *K. marxianus* is widely used in industry, mainly because it possesses some special properties of industrial interest that *K. lactis* lacks, such as the ability to produce the enzyme inulinase (Arrizon et al., 2011), and is also known for its rapid growth even at high temperatures (> 40 °C) (Rouwenhorst et al., 1988). Unlike *S. cerevisiae*, *K. marxianus* undergoes aerobic alcoholic fermentation, which is an advantageous phenotype for the industrial production of those compounds whose titre is associated with the formation of biomass, as ethanol production as a toxic or unintended by-product can be avoided under aerobic conditions (Wagner and Alper, 2016).

The high demand for biologically synthesised molecules for use in food and other products provides unique conditions for exploiting the potential of *K. marxianus* in food and industrial biotechnology. The main bottleneck in the development of this species has been restricted fundamental knowledge of its physiology and genetics, but this scenario is changing (Morrissey et al., 2015). Improvements are focused on optimising growth conditions and fermentation processes, as well as developing new strains using evolutionary or rational engineering approaches. To achieve improvements, it is necessary to study the physiology, metabolic mechanism and gene sequences in *K. marxianus* in more details. This yeast is considered as a favourable host for extracellular protein production due to its ability to grow on a variety of low-cost substrates as well as various polysaccharides (Fonseca et al., 2008). All of the aforementioned substrates are hydrolysed extracellularly due to the natural ability to release enzymes by yeast (Chi et al., 2011).

The extracellular enzyme inulinase has recently attracted great interest for hydrolysis of inulin for further production of bioethanol, fructose and fructooligosaccharides, which have wide application in food and pharmaceutical industries (Gao et al., 2009; Hoshida et al., 2018). Although this enzyme has been derived from various microorganisms, the yeast strains *A. niger* (Kango, 2008) and *K. marxianus* (Selvakumar and Pandey, 1999; Zhang et al., 2012)

are the most promising. *K. marxianus* inulinase remains stable at high temperature (45 °C) and at low pH (4.0) (Passador-Gurgel et al., 1996; Rouwenhorst et al., 1988). The optimal temperature for maximal enzyme secretion is the same as the optimal growth temperature for this microorganism (37 - 42 °C). Such a high temperature for optimal growth is also interesting for commercial production as it reduces the cost of cooling fermenters and reduces the risk of contamination. To optimise the production process, fermentation and culture medium variables such as temperature, pH, inoculum size, incubation time and type of microbial strain and substrate can be managed. An increase in secretory inulinase expression can also be achieved using genetic engineering methods. For example, by enhancing the efficiency of the inulinase encoding gene promoter (INU1) and signal sequence engineering (Zhou et al., 2018).

β-galactosidase is the most exploited enzyme in the food industry and is used for whey saccharification and in milk processing for lactose reduction (Singh et al., 2016). Various strains of *Kluyveromyces* have been found effective for industrial production of β-galactosidase (Hensing et al., 1994; Oliviera et al., 2011) from different industrial medias (Morrissey et al., 2015; Rech et al., 1999). Similarly, β-galactosidase from *K. lactis* and *K. marxianus* can be successfully used for lactulose oligosaccharide synthesis due to the transgalactosylic activity of this enzyme (Padilla et al., 2015). Successful attempts at genetic engineering to increase lactase activity have also been described in the scientific literature. Deletion of the MIG1 gene leads to an increase in β-galactosidase production as the presence of this gene in the medium with glucose inhibits lactase synthesis (Zhou et al., 2013). Similarly, high activity of this enzyme was also achieved after addressing to the INU1 promoter and the signal sequence (Bergcamp et al., 1993).

Also important in the context of *K. marxianus* exoenzyme secretion are enzymes such as pectinase and lipase. Pectinases (endopoligacturonases) are enzymes that hydrolyse pectin, which is the main structural element of plant cell walls. *K. marxianus* is widely used for pectolytic activity, producing polygalacturonases, pectinlyases, pectin esterases or pectan lyases, depending on temperature, pH and substrate availability. Depending on the genetic and environmental background, pectolytic yeast can produce different types of pectinases, *K. marxianus* mainly synthesizes endopoligalacturonase (de Mansoldo et al., 2019). Pectinases are used in the production of juices and wine due to their ability to break down plant cell walls (Alimardani-Theuil et al., 2011). *K. marxianus* is interesting because its production is constitutive and its synthesis is not enhanced by the presence of carbohydrates in the medium. The potential effect of carbon sources on PG activity depends on the physico-

chemical parameters of fermentation. Yeast pectinases could potentially be used in various industries such as fruit and vegetable processing, winemaking, coffee and tea fermentation, textile and paper industry (Alimardani-Theuil et al., 2011).

Lipases (triacylglycerolacyl hydrolases) catalyse both hydrolysis and synthesis of esters, usually responsible for the hydrolysis of acyl-glycerides required for lipid bioconversion (Vakhlu, 2006). Lipases of microbial origin are of greater industrial importance than those of animal or plant origin because they are more stable (Vakhlu, 2006) and have a wide range of catalytic activities (Siekstele et al., 2015). Lipases are mainly intracellular enzymes. Since *K. marxianus* lipases, in comparison with these enzymes of other microorganisms, are stable at acidic pH, they are of particular interest for commercial applications (Corzo and Revah, 1999; Sharma et al., 2002). Microbial lipases are very universal enzymes with a wide range of applications, in detergent production, food processing, paper production, pharmaceuticals, cosmetics, tanning, chemicals, biosensors and bioremediation (Sarmah et al., 2018).

Among the microorganisms that produce single cell protein (SCPs), yeast is a good candidate because of its high protein content, small particle size, ease of processing and relatively low cost. *K. marxianus* is of great interest for the production of SCPs and for use as a feed organism in various countries. Monocultures as well as co-cultures with *S. cerevisiae*, *C. kefir* and *T. reesei* have shown great potential for biomass utilization for livestock and fish feed enrichment (Aggelopoulos et al., 2014; Overland et al., 2013; Kim et al., 1998).

Bioethanol production by fermentation at high temperature can reduce the cost of cooling and the permanent transition from fermentation to distillation, decrease the risk of contamination, perform simultaneous saccharification and fermentation, and can be used in tropic countries (Fonseca et al., 2008). From the aforementioned, the high-temperature optimum for the growth of *K. marxianus* is of particular interest for this process. Currently, in industrial bioethanol production mainly uses strains of *S. cerevisiae* due to its high production rate and better tolerance to high ethanol levels, but the suitable temperature for this yeast strain is comparatively low (Qui and Yiang, 2017). In terms of thermotolerance, *K. marxianus* isolates have particular promise for bioethanol production at temperatures above 40 °C (Madeira and Gombert, 2018). In addition, K. *marxianus* can utilise a wide range of cheap substrates for ethanol production (Hang et al., 2003; Martinez et al., 2017; Ozmihci and Kargi, 2007). All these advantages make *K. marxianus* a valid alternative to *S. cerevisiae* as an ethanol producer. It has recently been found that bioethanol production at 48 °C using *K. marxianus* strain NSYC 3396 from sugarcane reduces the final cost of bioethanol

production, decreases risk of contamination, cooling costs, use of antibiotic, use of sulphuric acid for cell recycling, water and energy consumption during distillation (Madeira and Gombert, 2018). Due to its ability to utilise xylose at high temperature, *K. marxianus* can be favourable for second generation bioethanol production using lignocellulosic biomass as substrate, as the temperature for saccharification of this substrate is usually higher than the normal fermentation temperature (Madeira and Gombert, 2018). Currently, *K. marxianus* tolerates only a maximum ethanol concentration of 6% (v/v). Low ethanol tolerance results in low ethanol yields and is the main bottleneck hampering its practical industrial application to date. Like *S. cerevisiae*, ethanol tolerance of *K. marxianus* is also a complex process involving many genes and different physiological pathways and it is difficult to improve by a rational engineering approach, as the molecular basis of its resistance to ethanol has not yet been fully understood. Mo and colleagues as a result of laboratory adapted evolution under 6% (v/v) ethanol exposure after 100 days obtained a strain with improved tolerance to ethanol from 7% to 10% (v/v) (Mo et al., 2019). Phenotyping and genetic analyses revealed that protein tolerance-related pathways, antioxidation, osmotic pressure, membrane lipid biosynthesis, cell wall biosynthesis and the secretory pathway were enhanced to prepare the cell for the upcoming ethanol stress. Data from RNA-sequencing showed that the obtained strain can also develop resistance to osmotic, oxidative and thermal stress, which was confirmed by a cell viability test. Finally, the improved resistance of *K. marxianus* lead to increased ethanol production under multiple stress conditions.

S. cerevisiae var *boulardii* has for some time been described as a probiotic (More and Suidsinski, 2015), but in recent years there has been growing interest in the probiotic potential of other non-traditional yeasts. Due to its ability to modify cellular immunity, adhesion and gut microbiota, as well as its antioxidative, anti-inflammatory and hypocholesterolemic properties, *K. marxianus* is a promising candidate for use as a probiotic (Cho et al., 2018; Xie et al., 2015). Among various yeasts, several strains of *Kluyveromyces* are promising candidates for the synthesis of a significant number of aromatic compounds. *K. marxianus* has a high potential to produce various aromatic compounds such as 2-phenylethanol, alcohols, furanones, fruit esters, ketones, carboxylic acids and aromatic carbons (Guneser et al., 2016; Morrissey et al., 2015).

2MATERIALS AND METHODS

2.1 K. marxianus strains

Three yeast *Kluyveromyces marxianus* strains (DSM 5422, DSM 4906 and DSM 5418) were obtained from the Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures. *Kluyveromyces marxianus* strain NCYC 2791 was obtained from the National Collection of Yeast Cultures. All *K. marxianus* strains were maintained on YPD agar. YPD contained (per litre of distilled water) 10 g of yeast extract (Biolife), 20 g of peptone (Biolife), 20 g of glucose (Sigma), and 20 g of agar (Biolife).

2.2 Cultivation

2.2.1 Cultivations in flasks and 96-well plates

For all experiments, preculture was grown from a single colony, maintained on YPD plate. For 96-well plate and flasks experiments, preculture and main culture were grown on a liquid semisynthetic medium: carbon source (glucose, galactose, fructose, xylose, inulin and lactose) 20.0 g/L, yeast extract 5.0 g/L, $MgSO_4$ 0.7 g/L, KH_2PO_4 1.0 g/L, K_2HPO_4 0.1 g/L, $(NH_4)_2SO_4$ 5.0 g/L (Sigma).

For 96-well plate experiments (TECAN infinite 200 M Pro), fresh overnight culture inoculated from a single colony was used. The plates were incubated at 30 °C for 24 h, shaken with amplitude 3.5 mm, and optical density readings were taken every 10 min followed by a minute-long pause in shaking. Cells from preculture were washed with sterile distilled water and resuspended in a cultivation medium with initial $OD_{600} = 0.15$.

Cultures in flasks were cultivated at 30°C with an agitation speed of 180 rpm in orbital Shaker – Incubator ES-20 (bioSan).

2.2.2 Cultivations in bioreactors

For bioreactor experiments, second preculture was grown. All precultures were grown in semisynthetic media or with carbon source corresponding to one used in experiments or on whey permeate (Smiltene milk factory) as stated in Results.

The main cultivations were carried out in a 6-fold bioreactor system *Statorius Biostat Qplus* with a working volume 0.4 litre or in a bioreactor *Infors* 2HT with a working volume 1.0 litre. Stirring speed, airflow rate, temperature, pH control and inoculum volume as stated in Results.

2.2.3 Determination of acetate effect on growth rate

A 96-well plate reader (Tecan infinite 200 M Pro) was used for *K. marxianus* DSM 5422 growth rate measurements at different pH and substrates with four biological replicates. Medium pH was maintained by acetic or citric buffer solutions (sodium acetate, acetic acid, sodium citrate, and citric acid buffer solutions with final acetate or citrate molarity of 40 mM). From the microcultivation data, we calculated growth rates and doubling times as described by Toussaint et al. (2006).

2.2.4 Adaptation to acetate

In order to assess acetate tolerance plating on YPD and solid semisynthetic medium supplemented with 40 mM acetic acid, pH 4.3 was used. Adapted cells were counted as the percentage of colonies that formed on semisynthetic media against colonies on YPD. For each biological replicate, three independent dilutions and plating on each medium were made. Serial dilutions ranging from 1×10^7 to 1×10^2 cells/mL were made, thus allowing to discriminate survival from 0.001 to 100 %.

2.3 Analytical methods

2.3.1 Yeast growth monitoring

The yeast growth was monitored spectrophotometrically by the OD_{600}. To determine culture dry weight, exponentially growing cells were washed three times with distilled water and dried in 104 °C until a constant weight was achieved. A conversion coefficient 0.33 g DW/OD unit was obtained.

2.3.2 Carbohydrate and metabolite measurements

Lactose, glucose, fructose, xylose, galactose, ethanol, acetate and glycerol contents were measured simultaneously using an Agilent 1100 HPLC system with a Shodex Asahipak SH1011 column. Metabolites were quantitated with a refractive index detector (RI detector RID G1362A). The flow of the mobile phase (0.01 N H_2SO_4) was 0.6 ml min^{-1} ; the sample injection volume was 5 µL.

To estimate the inulin concentration a sample was first incubated (10 µl for 1 ml of sample at 60 ºC for 3 h) with a commercial preparation of inulinases Fructozyme L (Novozymes A/S, Denmark). After inulin hydrolysis by Fructozyme L glucose and fructose were determined enzymatically using the K-SUFRG assay kit (Megazyme, Ireland).

2.3.3 β-galactosidase activity measurement

β-galactosidase activity was measured by modified Domingues et al. 1997 method. For β-galactosidase activity measurements in native and permeabilized cells 50 µL/mL 10 mM X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used. The 5,5'-dibromo-4,4'-dichloro-indigo – 2 produced was determined by absorbance at 650 nm. To ensure optimal conditions for β-galactosidase activity 1 mM $MgCl_2$ and 1 mM $MnCl_2$ were added.

2.3.4 pO_2 measurements

Exponential or post-diauxic phase *K. marxianus* cultures were grown in shake flasks, growth monitored by optical density measurements. After reaching desired growth phase, cells were washed twice and starved in water. Starvation was carried out in shake flasks, at the same conditions as cultures were grown. After 4-h starvation, they were resuspended at 15 ml in Falcon tubes at OD_{600} = 10 (cell concentration was 3.3 gDW/L) in acetate buffers of various molar concentrations at pH 4.5, containing 20 g/L lactose. Cell suspensions were saturated with oxygen, carbon source added, and changes in pO2 were measured with Hamilton Oxy Ferm 120 oxygen sensors for a time course of 20 min.

2.4 Cell treatment

2.4.1 Cell disruption

Cell disruption was performed by 2-times stirring (1min 30 times per second) and cooling cycles with 0.3 ml ⌀ 0.45 glass beads (Sigma) in phosphate buffer with protease inhibitors 20 µl/ml (Sigma). The cell extract was prepared by centrifugation (30 min 13000 rpm at 4°C).

2.4.2 Yeast cells permeabilization

Cells were washed twice with 0.1 M potassium phosphate buffer (pH 7.0) at 8000 rpm +4°C 10 min. To cell suspension (OD_{600} = 0.5) was added 20 µg/mL protease inhibitor (Complete Protease inhibitor cocktail, Roche CO-RO). Permeabilization was carried out by incubation with 10 µg/mL 200 µM proton gradient uncoupler CCCP (carbonyl cyanide-m-chlorophenylhydrazone) (Sigma) or treated with 10 µL/mL 2 mM digitonin (Sigma). Method modified Bacci et al., 1996.

2.5 Modelling techniques

2.5.1 Kinetic model formulation

Equation 1 describes the kinetics of biomass formation featuring the Monod-type substrate limitation in combination with the Haldane substrate inhibition model (Andrews, 1968; Guisasola et al., 2006) and the Jerusalimsky term for the non-competitive product inhibition (Moser, 1988). It is also known as the Haldane-Boulton model (Arellano-Plaza et al., 2007). The system of Ordinary Differential Equations (ODE) summarized below (eq. 2, 3, 4) represents a general mathematical model capable of describing the batch kinetics of ethanol fermentation by *K. marxianus* from lactose- and inulin- containing substrates as mentioned above:

$$\mu = \frac{\mu_{\max} \cdot S}{K_S + S + \frac{S^2}{K_{I,S}}} \cdot \left(\frac{K_{I,P}}{K_{I,P} + P} \right). \quad (1)$$

$$\frac{dX}{dt} = \mu \cdot X, \quad (2)$$

$$\frac{dS}{dt} = -1 \left(\frac{\mu}{Y_{\frac{X}{S}}} \right) \cdot X + m_S \cdot X, \quad (3)$$

$$\frac{dP}{dt} = \frac{\alpha \cdot dX}{dt} + \beta \cdot X. \quad (4)$$

Here *S*, *X*, *P* are substrate, biomass and product concentrations ($g \cdot L^{-1}$), respectively, μ denotes the specific growth rate (h^{-1}), μmax denotes the maximum specific growth rate (h^{-1}), K_S is the substrate limitation constant ($g \cdot L^{-1}$), $K_{I,S}$ is the substrate inhibition constant ($g \cdot L^{-1}$), $K_{I,P}$ is the product inhibition constant ($g \cdot L^{-1}$). $Y_{X/S}$ is the yield coefficient for cells on substrate ($g\ X \cdot g\ S^{-1}$), m_S denotes the maintenance coefficient for cells ($gS \cdot gX^{-1} \cdot h^{-1}$), α and β are growth- and non-growth-associated terms, respectively. Therefore, the impact of both inhibition constants (K_{IS}, K_{IP}) appears not only in the expression of the specific growth rate (eq. 1), but also is carried over the whole ODE system (eqns. 2, 3, 4) containing this essential process variable. Thus, equation 2 represents the generalized population growth model where the rate constant μ values are determined by equation 1. As well as in equations 3 and 4 describing the rates of substrate consumption (Zafar et al., 2005; Moser, 1988) and product formation respectively (Luedeking and Piret, 2000). The whole system of ODE describing the

batch kinetics of fermentation by *K. marxianus* was solved using the Real-valued Variable-coefficient ODE solver, with the fixed-leading-coefficient implementation.

Computational methods

Simulations were done using *Python* on a laptop computer with Intel i5 processor and 6GB of RAM. Differential equations used in the models were integrated using *SciPy integrate.odeint* function from *SciPy* (Jones et al, 2001). The experimental data were stored and maintained in *pandas*. *DataFrame* (McKinney, 2010). The processing and visualization of the simulation results was done using the *Matplotlib* (Hunter, 2007) and *Statgraphics Centurion* (Manugistics,Inc, USA). To standardize the range of variables the feature scaling was employed (eq. 5) before calculation the sum of square errors between the values of the observed and predicted concentrations.

$$x' = \frac{x - \min(x)}{\max(x) - \min(x)} \qquad (5)$$

The model parameters were estimated by the Differential Evolution (DE) algorithm from *SciPy* library by minimizing the sum of square errors calculated between the measured and model prediction for biomass, substrate and ethanol. The population size for the Differential Evolution (Storn and Price, 1997) algorithm was set to 100, which is sufficient taking into account the number of the model parameters. At the end of the parameter estimation the L-BFGS-B method is used to polish the best population member. The leave-one-out cross validation (LOOCV) (Arlot and Celisse, 2010) procedure as employed to validate the kinetic models and the linear plots of the actual data against those predicted by models used to assess the goodness-of-fit for them according to adjusted R^2 values.

2.5.2 Stochiometric modelling methodology and software

Two major strands of stoichiometric modelling are the constraint-based flux balance analysis (FBA) (Orth et al., 2010; Varma and Palsson, 1994) and elementary modes analysis (Schuster and Dandekar, 2000). A constraint-based model of central metabolism including biomass production of *K. marxianus* was created adapting and combining the high quality genome-scale metabolic reconstructions protocol (Schellenberger et al., 2011) and structural modelling approach for development of medium-scale reconstruction and models (Kalnenieks et al., 2014).

Our medium-scale *K. marxianus* central carbon metabolism model is based on the general mass balance equation:

$$dX/dt = r_{met} - \mu X_{met}.$$

With respect to intermediate metabolite accumulation, a cell's metabolism is in pseudosteady state and can be described by the following equation (Stephanopoulos et al., 1998):

$$0 = r_{met} - \mu X_{met}.$$

We also assume the following:

• the specifc growth rate (μ, h^{-1}) during the exponential growth phase is constant,

• the cells are at pseudosteady state: substrate uptake, metabolite and product fluxes are constant when μ is constant.

For constraint-based and structural analysis, the *ScrumPy* modelling package (Poolman, 2006) was used. Flux balance analysis (FBA) was carried out by setting a constant rate of substrate uptake to 10 mM g^{-1} DW h^{-1}, and searching for the maximum yield of one of the following products: ethanol, acetate, lactate, glycerol, ethylacetate, succinate, glutamate, phenylethanol or phenylalanine. Solutions were further examined using flux variability analysis (FVA) (Mahadevan and Schilling, 2003) to determine the ranges of internal fluxes that are consistent with the maximum if there were multiple equivalent FBA solutions. Inconsistencies in the model formulation were additionally detected through null space analysis (Fell et al., 2010) combined with determination of inconsistent enzyme subsets (Pfeiffer et al., 1999) using *ScrumPy*. The essentiality of genes and reactions was analysed using FBA to check whether biomass production was feasible after deleting the relevant reaction(s) from the model. The gene essentiality test took into account the gene–protein-reaction (GPR) associations (Thiele and Palsson, 2010) that were determined for the model (next subsection). FVA was also used to calculate the potential range in product production taking into account minimal and maximal oxygen respiration levels at a fixed substrate uptake value.

Reactions

The *K. lactis* genome-scale reconstruction (Dias et al., 2014) was used as a starting point given the high degree of similarity between its metabolic networks and that of *K. marxianus*. The amino acid sequences of *K. lactis* genes from the NIH genetic sequence database GenBank (Benson et al., 2013) were compared against fungal species using NCBI BLAST (Johnson et al., 2008). The corresponding *K. marxianus* genes were also checked for presence in the Uniprot database (Magrane and Consortium, 2011). For each reaction, its

Enzyme Commission number (E.C. number) and reaction directionality were checked and validated. The IntEnz (Fleischmann et al., 2004) (available at http://www.ebi.ac.uk/intenz/) database was the main reference source for mass and charge balance validation. To represent the *K. marxianus* biomass growth reaction, we used the *S. cerevisiae* biomass composition as described by Gombert et al. (Gombert et al., 2001).

Metabolites

Metabolite names, their neutral and charged formulas and InChI (International Chemical Identifer) strings (Fleischmann et al., 2004) were taken from the CheBi database (Degtyarenko et al., 2008) (available at http:// www.ebi.ac.uk/chebi/), and the yeast-specific Metacyc (Caspi et al., 2012). The PubChem database (Wang et al., 2012) (available at http://pubchem. ncbi.nlm.nih.gov/) was used to get additional information about metabolites (Fleischmann et al., 2004).

2.6 Statistical methods

All experiments, except 96-well experiments, were carried out in biological triplicates. Ninety-six-well plate experiments were carried out in four biological repetitions. Data shown in figures is average from biological replicates with error bars depicting standard deviation. Concentration of undissociated acetic acid (HA) was deduced from the Handersson Hasselbach equation:

$$pH = pKa + \log_{10}\left(\frac{[A^-]}{[HA]}\right)$$

3 RESULTS

3.1 Kinetic modelling of ethanol fermentation by yeast Kluyveromyces marxianus from lactose- and inulin- containing substrates

KINETIC MODELING OF ETHANOL FERMENTATION BY YEAST *KLUYVEROMYCES MARXIANUS* FROM LACTOSE- AND INULIN- CONTAINING SUBSTRATES

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Abstract. An unstructured kinetic model was developed in this study for the batch production of bioethanol by the yeast *Kluyveromyces marxianus* DSM 5422 from the renewable sources of agricultural and food processing origin, such as whey permeate or inulin, which include the terms of both substrate and product inhibitions. Experimental data collected from multiple fermentations in bioreactors with three different initial concentrations for each substrate were used to estimate the parameters and to validate the proposed model. The growth of *K.marxianus* can be expressed by the Haldane-type extended Monod model in combination with the Jerusalimsky term for the non-competitive product inhibition and the Luedeking–Piret equation was adequate to describe the growth-associated formation of ethanol as the target product. The parameters in the models were estimated by minimizing mean-squared errors between the predictions of the models and the experimental data using the differential evolution (DE) algorithm and the L-BFGS-B nonlinear optimization code. In all cases, the model simulation matched well with the fermentation data being confirmed by the high R-squared values (0.984, 0.992 and 0.965 for WP, lactose and inulin, respectively). The kinetic models proposed here can be employed for the development and optimization of the bioethanol production processes from renewable resources.

Keywords: bioethanol, kinetic model, whey permeate, inulin, *Klyuveromyces marxianus*.

Introduction

In recent years the yeast *Kluyveromyces marxianus* has attracted an increasing attention due to versatile biotechnological applications. It can be used as an efficient producer for valuable microbial products including a number of enzymes, flavor and fragrance compounds as well as bioethanol, particularly from renewable resources [1-3].

The ability to utilize a variety of carbon sources, an enhanced thermotolerance, a rapid growth and a strong Crabtree-negative character of cells are the advantageous traits, which promote the use of *K. marxianus* for industrial bioprocesses [1; 3]. Although, these non-conventional food-grade yeasts have been subjected to still insufficient investigation efforts and quantitative studies of technologically important processes are rarely reported [4; 5]. Such in-depth studies are particularly needed because significantly different growth parameters have been reported not only for different strains of *K. marxianus* but also for the same strain when investigated in different laboratories [2; 6]. Such a metabolic diversity makes it difficult to generalize the knowledge about these yeasts and therefore encourages researchers to focus at least initially, on the reduced number of strains chosen from key culture collections [2].

A substantial phenotypic variation in the growth parameters can be observed in the production of bioethanol from lactose- or inulin-containing substrates, which are provided by the operation of β-galactosidase or endo- and exo-inulinases in *K. marxianus* cells [2; 3; 7-9]. Within the foregoing, particularly the proposed need [2] to study a limited number of strains, a certain attention should be given to the *K.marxianus* DSM 5422. This strain has been proposed as an efficient producer of ethyl acetate and appears relatively well-studied in this context [10, 11]. Although, it has been also used for the production of bioethanol from the renewable lactose-containing substrate such as cheese whey [12-14]. There are several reports on the ethanol production from another technologically promising substrate such as inulin and inulin - containing raw materials by a variety of *K. marxianus* strains [15-21] although DSM 5422 remains unrepresented among them.

Therefore, a comparative analysis for the above two substrates could give a fuller insight into the potential of *K. marxianus* DSM 5422 for production of bioethanol from the renewable, inexpensive and abundant raw materials [8; 22]. It is well known that the behavior of microbial systems can be evaluated by the growth kinetic parameters, which constitute appropriate mathematical models [23-25]. Even relatively simple kinetic models could be indispensable for the design and successful operation of industrial bioprocesses and for obtaining quantitative information about the function of microbial cells [23]. Thus, relevant parameters of Monod kinetics such as the maximum specific growth rate (μ_{max}), the saturation constant (K_s) and the yield of biomass ($Y_{X/S}$) can be considered as

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passport data for a particular organism [26]. Several kinetic models have been developed, which describe the ethanol production by different *K. marxianus* strains on lactose – containing substrates [27-30], including the strain DSM 5422 [12]. However, these models appear as differently structured, may contain quite distinctive parameters, as well as often disregard the possible effects of product and/or substrate inhibition [12; 27] and remain restricted by too narrow range of substrate concentrations. The kinetics of ethanol production by *K. marxianus* on the inulin – containing substrates has been only partly described [15; 16], and the full kinetic model is still not developed, which would allow to predict the concentration profiles of substrate, product and biomass during alcoholic fermentation.

The objective of the present study is to develop a kinetic model based on the time-course measurements of substrate, product and biomass changes during the ethanol production by the yeast *K. marxianus* DSM 5422 at varied initial concentrations of lactose- and inulin – containing substrates.

Materials and methods

Organism and cultivation conditions

The yeast *Kluyveromyces marxianus* DSM 5422 was obtained from the Leibniz-Institute DSMZ (German Collection of Microorganisms and Cell Cultures), and maintained on YPD agar. YPD contained (per liter of distilled water) 10 g of yeast extract (Biolife), 20 g of peptone (Biolife), 20 $g \cdot l^{-1}$ of glucose (Sigma) and 20 $g \cdot l^{-1}$ of agar (Biolife).

For the preculture 5 mL of liquid semi-synthetic medium with lactose as carbon source 50 $g \cdot l^{-1}$, yeast extract 5 $g \cdot l^{-1}$ (Biolife), $MgSO_4$ 0.7 $g \cdot l^{-1}$ (Sigma), KH_2PO_4 1 $g \cdot l^{-1}$ (Sigma), K_2HPO_4 0.1 $g \cdot l^{-1}$ (Sigma), $(NH_4)_2SO_4$ 5 $g \cdot l^{-1}$ (Sigma) in 25 mL test tube was inoculated with a single colony from YPD agar plate. Preculture were cultivated at 30 ºC with an agitation speed of 180 rpm in orbital Shaker – Incubator ES-20 (bioSan).

For cultivation in bioreactors, the first preculture was grown in liquid semi-synthetic medium with lactose as carbon source. For the second preculture, 1 l of defined medium in a 2 l flask with cotton stopper was inoculated with 0.05 % (v/v) of the first preculture. Cultivations were carried out at 30°C with agitation speed of 180 rpm.

For bioreactor experiments the second preculture and main culture were grown on whey permeate (WP) (lactose concentrations 120 $g \cdot l^{-1}$, 135 $g \cdot l^{-1}$ and 150 $g \cdot l^{-1}$) obtained from Smiltene milk factory or liquid semi-synthetic medium containing: lactose (Sigma) (120$g \cdot l^{-1}$, 135$g \cdot l^{-1}$ and 150$g \cdot l^{-1}$) or inulin (Dion-Bioline) (80 $g \cdot l^{-1}$, 150 $g \cdot l^{-1}$ and 200 $g \cdot l^{-1}$) as a carbon source, yeast extract 5 $g \cdot l^{-1}$, $MgSO_4$ 0.7 $g \cdot l^{-1}$, KH_2PO_4 1 $g \cdot l^{-1}$, K_2HPO_4 0.1 $g \cdot l^{-1}$, $(NH_4)_2SO_4$ 5 $g \cdot l^{-1}$. Cultures in shake flasks were cultivated at 30 ºC with an agitation speed of 180 rpm.

The main cultivations were carried out in a bioreactor BIOSTAT Q PLUS (Sartorius Stedim Biotech GMbH, Goettingen, Germany) with a working volume 0.4 L, at 30 ºC and 40 ºC, the stirring speed 400 rpm and airflow rate (0.2, 0.8 and 1.4 1 $l \cdot l^{-1} \cdot min^{-1}$) were used. The fermentation medium pH 5.0 was controlled by adding 10 % KOH.

Analytical methods

The yeast growth was monitored spectrophotometrically at the OD_{600}, according to the calibration curve: Biomass dry weight ($g \cdot l^{-1}$) = $0.33 \cdot OD_{600}$. To determine the biomass dry weight, exponentially growing cells were washed three times with distilled water and dried at 104°C until a constant weight.

To estimate the inulin concentration a sample was first incubated (10 µl for 1 ml of sample at 60 ºC for 3 h) with a commercial preparation of inulinases Fructozyme L (Novozymes A/S, Denmark). After inulin hydrolysis by Fructozyme L glucose and fructose were determined enzymatically using the K-SUFRG assay kit (Megazyme, Ireland).

The ethanol and lactose concentrations were determined by HPLC (Agilent 1100 Series), using column Aminex HPX-87H (length 300 mm, i.d. 7.8 mm) with a refractive index detector. The column temperature was 45°C, mobile phase 0.005 $mol \cdot l^{-1}$ H_2SO_4, flow rate 0.6 $ml \cdot min^{-1}$ and sample volume 20 µl.

All analytical measurements were performed at least in duplicate.

Model formulation

Equation 1 describes the kinetics of biomass formation featuring the Monod-type substrate limitation in combination with the Haldane substrate inhibition model [31; 32] and the Jerusalimsky term for the non-competitive product inhibition [33]. It is also known as the Haldane-Boulton model [34].

The system of Ordinary Differential Equations (ODE) summarized below (eq. 2, 3, 4) represents a general mathematical model capable of describing the batch kinetics of ethanol fermentation by *K. marxianus* from lactose- and inulin- containing substrates as mentioned above:

$$\mu = \frac{\mu_{\max} \cdot S}{K_S + S + \frac{S^2}{K_{I,S}}} \cdot \left(\frac{K_{I,P}}{K_{I,P} + P} \right). \tag{1}$$

$$\frac{dX}{dt} = \mu \cdot X , \tag{2}$$

$$\frac{dS}{dt} = -1 \left(\frac{\mu}{Y_{\frac{X}{S}}} \right) \cdot X + m_S \cdot X , \tag{3}$$

$$\frac{dP}{dt} = \frac{\alpha \cdot dX}{dt} + \beta \cdot X . \tag{4}$$

Here *S*, *X*, *P* are product, biomass and product concentrations ($g \cdot l^{-1}$), respectively, μ denotes the specific growth rate (h^{-1}), μ_{max} denotes the maximum specific growth rate (h^{-1}), K_S is the substrate limitation constant ($g \cdot l^{-1}$), $K_{I,S}$ is the substrate inhibition constant ($g \cdot l^{-1}$), $K_{I,P}$ is the product inhibition constant ($g \cdot l^{-1}$). Y_{XS} is the yield coefficient for cells on substrate ($gX \cdot gS^{-1}$), m_S denotes the maintenance coefficient for cells ($gS \cdot gX^{-1} \cdot h^{-1}$), α and β are growth- and non-growth-associated terms, respectively.

Therefore, the impact of both inhibition constants (K_{IS}, K_{IP}) appears not only in the expression of the specific growth rate (eq. 1), but also is carried over the whole ODE system (eqns. 2, 3, 4) containing this essential process variable. Thus, equation 2 represents the generalized population growth model where the rate constant μ values are determined by equation 1. As well as in equations 3 and 4 describing the rates of substrate consumption [27; 33] and product formation [35], respectively. The whole system of ODE describing the batch kinetics of fermentation by *K. marxianus* was solved using the Real-valued Variable-coefficient ODE solver, with the fixed-leading-coefficient implementation.

Computational methods

Simulations were done using *Python* on a laptop computer with Intel *i*5 processor and 6GB of RAM. Differential equations used in the models were integrated using *SciPy integrate.odeint* function from *SciPy* [36]. The experimental data were stored and maintained in *pandas. DataFrame* [37]. The processing and visualization of the simulation results was done using the *Matplotlib* [38] and *Statgraphics Centurion* (Manugistics,Inc, USA) .

To standardize the range of variables the feature scaling was employed (eq. 5) before calculation the sum of square errors between the values of the observed and predicted concentrations.

$$x' = \frac{x - \min(x)}{\max(x) - \min(x)} \tag{5}$$

The model parameters were estimated by the Differential Evolution (DE) algorithm [39] from *Sci Py* library by minimizing the sum of square errors calculated between the measured and model prediction for biomass, substrate and ethanol. The population size for the Differential Evolution algorithm was set to 100, which is sufficient taking into account the number of the model parameters.

At the end of the parameter estimation the L-BFGS-B method is used to polish the best population member.

The leave-one-out cross validation (LOOCV) procedure [40] was employed to validate the kinetic models and the linear plots of the actual data against those predicted by models used to assess the goodness-of-fit for them according to adjusted R^2 values.

Results and discussion

A set of conventional batch fermentation experiments was carried out at varied concentrations of distinctive substrates (Table 1) in order to perform the parameter estimation for the proposed model (equations 1-4) using the obtained data of biomass, substrate and ethanol concentration changes. During these procedures the ordinary differential equations (ODE) were integrated numerically by means of the Differential Evolution (DE) algorithm [39] as described above.

Table 1

Parameters and indices of the goodness -of-fit statistics of kinetic models describing the ethanol fermentation of whey permeate, lactose and inulin by the yeast *Kluyveromyces marxianus* DSM 5422

Parameter / Statistical Index	Description	Unit	Medium A whey permeate as the lactose (120-150 g·l^{-1}) source	Medium B lactose (120-150 g·l^{-1})	Medium C inulin (80-200 g·l^{-1})
μ_{max}	Maximum specific growth rate of biomass	h^{-1}	0.7500	0.6567	0.7500
$Y_{X/S}$	Yield coefficient for biomass (X) on substrate (S)	GX·gS^{-1}	0.0394	0.0673	0.0785
ms	Maintenance coefficient	GS·gX^{-1}·h^{-1}	0.4287	0.0620	0.0000
K_S	Half-saturation constant	g·l^{-1}	0.1000	26.4858	2.0833
$K_{I,S}$	Substrate inhibition constant	g·l^{-1}	13.9648	589.2454	584.7218
$K_{I,P}$	Product inhibition constant	g·l^{-1}	21.2391	13.9478	7.4423
α	growth-associated term [a]	-	5.0000	5.0000	4.1232
β	non growth-associated term [a]	-	0.4185	0.0686	0.0033
RSME	Root-Mean-Square-Error of the model	-	0.0398	0.0201	0.0397
R^2	R-square (coefficient of determination) of the model	%	98.40	99.21	96.34
R^2	Leave-one-out-cross-validated (LOOCV) R-square	%	97.76	98.77	93.95

a - term of the Luedeking-Piret equation [35]

Table 1 also demonstrates that the different substrates for the ethanol fermentation by *Kluyveromyces marxianus* do not affect the structure of the model, which follows from the identical parameter sets being eligible for the system of relevant ODE. Although, the numerical values of the parameters are noticeably affected. Thus, a substantially reduced *Ks* value corresponding to whey permeate (WP) fermentations at a whole range of concentrations indicates a high affinity of *K. marxianus* with respect of this substrate [26; 33]. At the same time the WP exhibits a much more pronounced non-competitive substrate inhibition when compared (Table 1) with the other two carbon

sources (lactose, inulin), presumably due to its complex composition containing a wide range of osmolytes [41]. Such WP specificities could also cause the apparent increase in the maintenance coefficient (*ms*) value reflecting the impact of metabolic costs for osmotic adjustment and, as a consequence, a relatively reduced yield coefficient ($Y_{x/s}$) for biomass on substrate (Table 1). It is essential that despite noticeable differences in parameter values the maximum growth rates (μ_{max}; equation 1) remain at rather high level and are almost unaffected for all three substrates under study. This is well in line with the notions on *K.marxianus* as the fastest growing eukaryote on Earth [4]. Besides, in all cases the formation of ethanol can be described (equation 4) according the Luedeking-Piret kinetics [35] as the almost solely growth-associated process where the specific rate of product formation is proportional to the specific growth rate of the yeast *K. marxianus*. This is indicated by significantly higher values for the growth associated (α) parameters when compared (Table 1) with those non-growth-associated (β) terms [35]. However, for the WP fermentation also partially mixed-growth-associated ethanol formation could occur as indicated by a slightly elevated β value (Table 1).

The matching quality of the proposed kinetic models was evaluated by the linear plots (Fig.1) of the actual experimental data against those predicted by the models. The highly significant R-square values (coefficient of determination) indicate that the model adequately describes the actual changes of biomass, substrate and product concentrations during ethanol fermentation of whey permeate, lactose or inulin by the yeast *K. marxianus* since only a relatively small proportion (0.79-3.66 %) of the total variance remains unexplained (Table 1). This is also confirmed by the relatively low RMSE (Root-Mean-Square-Error) values of the model (Table 1).

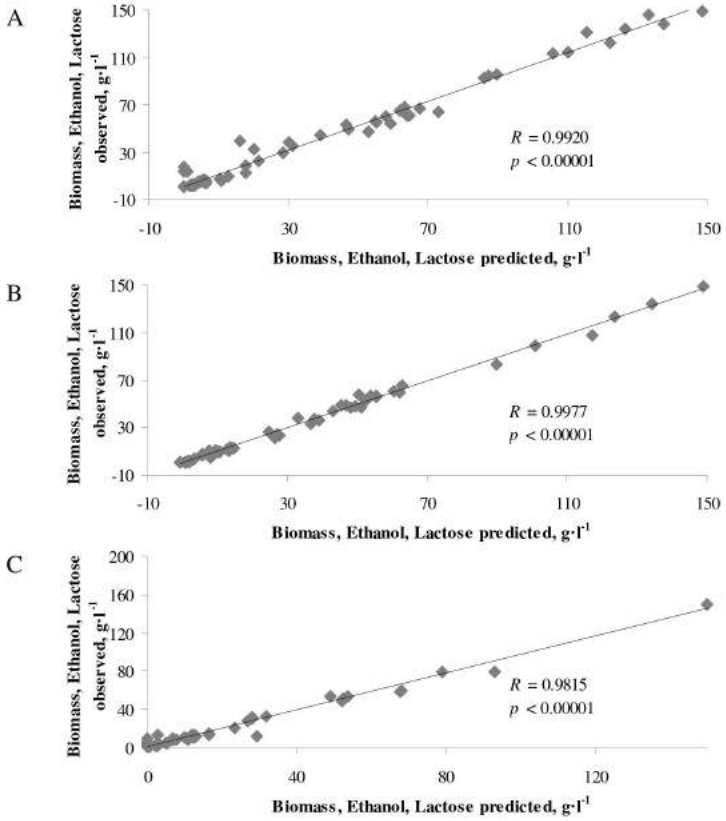

Fig. 1. **Linear plots of the actual concentrations of biomass, substrate /lactose or inulin/ and ethanol against those predicted by unstructured kinetic models (equations 1-4) for the yeast *Kluyveromyces marxianus* DSM 5422.** The observed versus predicted plots (A,B,C) for the estimates obtained during the ethanolic fermentation of Whey Permeate (WP), lactose or inulin, respectively, as specified in Table 1

In addition, the validation of the model using the leave-one- out cross-validation [40] procedure (LOOCV) resulted in slightly reduced R-square values (Table 1), which still remain within the limits of high ($p < 0.00001$) statistical significance.

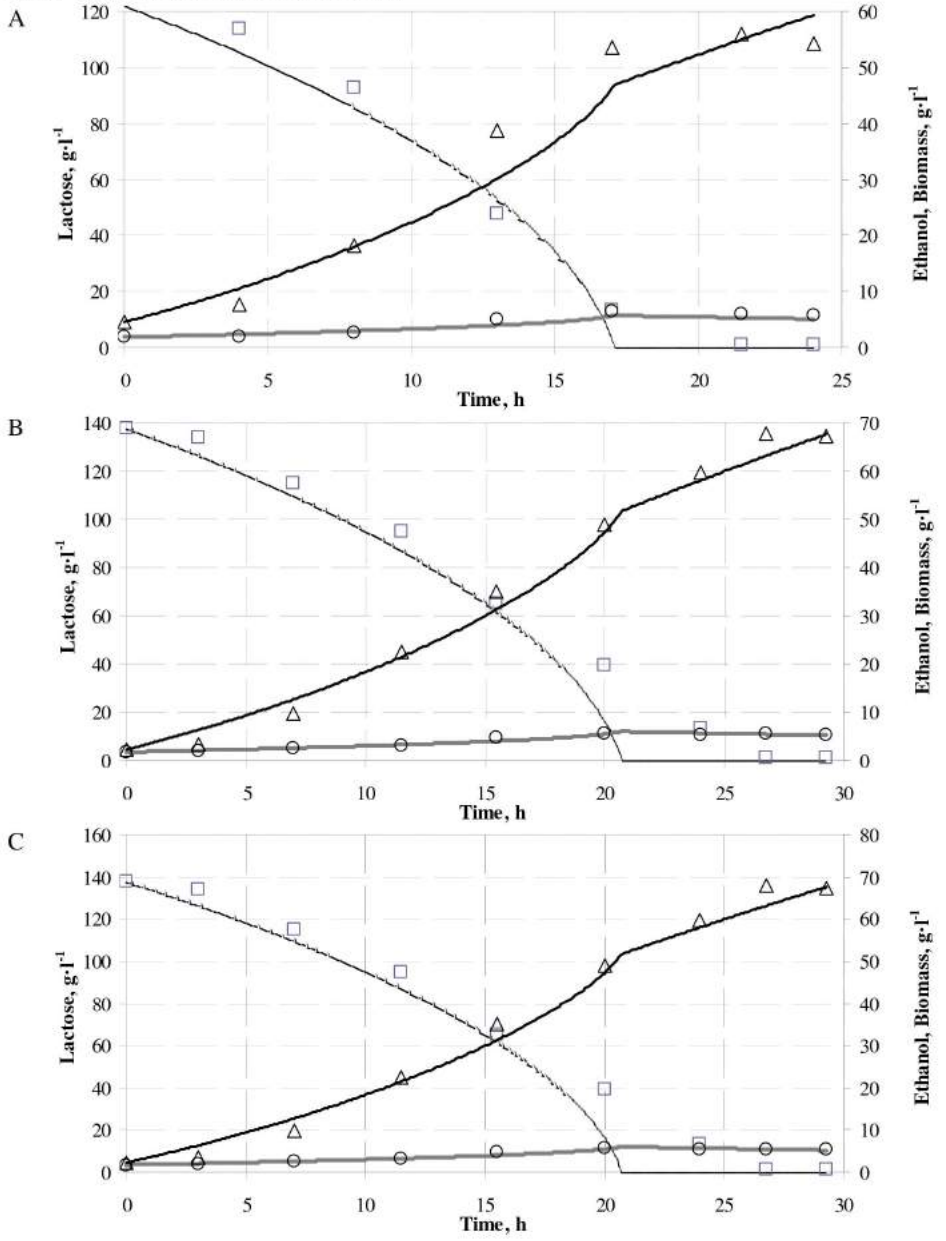

Fig. 2. **Experimental (symbols) and predicted (lines) profiles for biomass (Δ), ethanol (○) and lactose (□) concentrations in batch fermentations of the yeast *Kluyveromyces marxianus* DSM 5422 in the Whey Permeate (WP) medium containing lactose 120 g·l^{-1} (A), 135 g·l^{-1} (B) and 150 g·l^{-1} (C)**

The batch kinetics of biomass and ethanol production was studied at different initial substrate concentrations (S_0) of distinctive substrates (Table 1). Figure 2 shows the time course profiles of batch

fermentations of whey permeate to ethanol by *K. marxianus* at different initial lactose concentrations (S_0 = 120, 135 and 150 g·l^{-1}).

According to the model, the most of initial lactose can be metabolized by the yeast within 23 h or even earlier (fig. 2A) and the ethanol concentration and cell mass achieved 72.85 g·l^{-1}and 6.37 g·l^{-1}, respectively, at a maximum lactose concentration osf 150 g·l^{-1} (Fig. 2C). Besides, both the ethanol concentration and the cell mass increase in proportion to growing initial concentrations of lactose in the fermentation medium (Table 1, Fig.2 A-C). In cases with two other fermentation media, containing pure lactose or inulin as the carbon sources, the cell growth, substrate consumption and ethanol production profiles (data not shown) appeared as rather similar. In these fermentations the growing initial substrate concentrations also are followed by the proportionally increased ethanol and biomass concentrations. Although, there are substantial differences in both ethanol and biomass concentrations that can be achieved when using fermentation media with such carbon sources. Thus, the media containing pure lactose or inulin especially promote the formation of biomass, which concentration, for example, for inulin, can be achieved up to 16.41 g·l^{-1} (at S_0 = 200 g·l^{-1}). In turn, for pure lactose the biomass concentration may be lower, reaching 10.74 g·l^{-1} (at S_0 = 150 g·l^{-1}), however, significantly above that observed in the whey permeate medium. In turn, the concentration of ethanol, which can be achieved using these substrates, appears lower when compared to the whey permeate - containing medium. Although, even in these cases, at high initial substrate concentrations (S_0 = 150 g·l^{-1} or above) the ethanol concentration may exceed 60 g·l^{-1}.

Several technologically relevant parameters, obtained at the same initial concentration of the carbon source, are represented in Table 2, thus enabling an assessment of different substrate impacts on ethanol production and biomass formation by *K. marxianus* DSM 5422. It is obvious that the fermentation medium containing pure lactose or inulin as the carbon source provides a much higher biomass productivity (Q_x) and yield ($Y_{x/s}$) per unit of the substrate consumed. This could be explained by differences in the substrate composition, for instance, by the presence of osmolytes in the whey permeate as mentioned above [41] and perhaps even more by a possible nitrogen deficiency in this source unlike the pure lactose- and inulin-containing media containing the yeast extract supplement [42]. At the same time the differences in the volumetric ethanol productivity (Q_p) and the specific rate of product formation (*qp*) for the whey permeate medium are relatively less pronounced when compared with the other two media (Table 2). Of particular note is the fact that the whey permeate-containing medium turns out to be the most appropriate to achieve the highest yield of ethanol per unit of substrate consumed ($Y_{p/s}$ = 0.460 gg^{-1}), which accounts for 90.2 % of theoretical, which is substantially higher than 79.2 % and 64.5 % for the pure lactose- and inulin-containing medium, respectively (Table 2). This fact in conjunction with the highest achievable ethanol concentration mentioned above indicates that the whey permeate - containing medium and the yeast *K. marxianus* DSM 5422 could find technological applications to produce ethanol from this renewable source.

Table 2

Summary of parameters for ethanol fermentation of whey permeate, lactose and inulin by the yeast *Kluyveromyces marxianus* DSM 5422

Parameter	Carbon source		
	Whey permeate (medium A)	Lactose (medium B)	Inulin (medium C)
Substrate consumption $S_0 - SI$, g·l^{-1}	148.74	148.71	149.99
Ethanol volumetric productivity *Qp*, g·(lh)$^{-1}$	2.506	4.056	3.167
Ethanol yield *Yp/s*, g·g^{-1}	0.460	0.404	0.329
Biomass volumetric productivity *Qx*, g ·(L·h)$^{-1}$	0.179	0.742	0.764
Biomass yield *Yx/s*, g·g^{-1}	0.0293	0.0654	0.0785
Specific rate of ethanol formation *Qp*, g· (g·h)$^{-1}$	0.394	0.434	0.296
Specific rate of substrate consumption *qs*, g ·(g·h)$^{-1}$	1.443	1.214	0.909

Conclusions

A simple unsegregated and unstructured kinetic model has been developed for the batch production of bioethanol by the yeast *Kluyveromyces marxianus* DSM 5422 from the renewable sources of agricultural and food processing origin, such as whey permeate or inulin, which includes the terms of both substrate and product inhibition. *Kluyveromyces marxianus* shows the highest yield of ethanol (90.2 % of theoretical) on whey permeate as substrate, while ethanol and biomass productivity was lower as compared with semi-synthetic lactose or inulin medium due to nitrogen deficiency in whey permeate. It can be concluded that whey permeate is a suitable raw material for bioethanol fermentation by *Kluyveromyces marxianus*. There is reverse correlation between the biomass yield and ethanol yield on all three substrates. In all cases, the model simulation matched well with the whey permeate, inulin and lactose fermentation data of biomass growth, ethanol production and substrate consumption being confirmed by the high R^2 values.

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3.2 Model-based biotechnological potential analysis of Kluyveromyces marxianus central metabolism

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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY - ORIGINAL PAPER

Model-based biotechnological potential analysis of *Kluyveromyces marxianus* central metabolism

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Abstract The non-conventional yeast *Kluyveromyces marxianus* is an emerging industrial producer for many biotechnological processes. Here, we show the application of a biomass-linked stoichiometric model of central metabolism that is experimentally validated, and mass and charge balanced for assessing the carbon conversion efficiency of wild type and modified *K. marxianus*. Pairs of substrates (lactose, glucose, inulin, xylose) and products (ethanol, acetate, lactate, glycerol, ethyl acetate, succinate, glutamate, phenylethanol and phenylalanine) are examined by various modelling and optimisation methods. Our model reveals the organism's potential for industrial application and metabolic engineering. Modelling results imply that the aeration regime can be used as a tool to optimise product yield and flux distribution in *K. marxianus*. Also rebalancing NADH and NADPH utilisation can be used to improve the efficiency of substrate conversion. Xylose is identified as a biotechnologically promising substrate for *K. marxianus*.

Keywords *Kluyveromyces marxianus* · Modelling · Central metabolism · Metabolic engineering · Essentiality analysis

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Introduction

Kluyveromyces marxianus is an ascomycoteus yeast with enormous biotechnological potential for multiple industrial applications. There are a number of characteristics of *K. marxianus* that are industrially useful, including fast growth, broad substrate spectrum, thermotolerance, limited fermentation at sugar excess, and secretion of extracellular glycolytic enzymes. In addition, *K. marxianus* enjoys GRAS (Generally Regarded as Safe) status and therefore is useful in food- or pharma-related applications [30, 46].

Kluyveromyces marxianus can grow on glucose, fructose, xylose, galactose, lactose and inulin as the sole carbon sources [24]. Many of these carbon sources are of particular interest since they are waste products of forestry (xylose) or dairy (lactose) industries. Xylose is a pentose and the main sugar of plant hemicellulose; its content in hard wood wastes can be up to 30% [50]. *K. marxianus* has been engineered for xylitol production from xylose [42]. Cheese whey is a lactose-rich byproduct of the dairy industry produced in an approximate 10–1 (v/w) ratio to cheese. Currently, whey is considered as a potential substrate for future microbial fermentations [25, 65]. Inulin is one of the widely available plant polysaccharides common in many taxonomic groups (*Asteraceae* family, wheat, onion, banana, etc.). Some of those (e.g. Jerusalem artischoke, chicory) accumulate inulin in their underground tubers in vast amounts [11, 18]. These plants might serve as "niche" substrates for fermentations by yeasts including *K. marxianus*, [11] if not deprecated on account of competition with food use.

Kluyveromyces marxianus is a prospective producer for a range of important food additives and chemicals: phenylethanol, phenylalanine [60], hexanoic acid [10], xylitol [107, 108] and ethylacetate [52]. Due to its protein

excretion, *K. marxianus* is suitable for extracellular protein production (galactosidase, inulinase, etc.) [26, 98].

Stoichiometric models and reconstructions significantly facilitate analysis of metabolic effects and limitations of microorganism metabolism, as well as predicting the phenotype of recombinant strains [39, 40]. Modelling attempts on *K. marxianus* to date have been concentrated on particular problems: e.g. kinetic models of ethanol batch fermentation [77], and of growth on cheese whey [51]. The first attempt at a genome-scale metabolic reconstruction [47] is patented in unreadable form and cannot be used for metabolic flux calculations. A genome-scale metabolic model for the related species *K. lactis* has been published [16]. Analysing medium-scale stoichiometric models of central metabolism, where the most significant metabolic fluxes are, has been successful for biotechnological applications. Examples include assessment and selection of productive routes in *Escherichia coli* [88–90] and *Zymomonas mobilis* [68]. Medium-scale modelling also proved to be a successful strategy for describing the uncharacterised central metabolism of the non-conventional yeast *Pichia pastoris* on the basis of limited wet experimentation [87]. Recent extensive attempts at *K. marxianus* metabolic engineering [33, 42, 43, 105, 109] underline the immediate need for modelling of limits of its metabolic potential.

The aim of this study was to assess the biotechnological potential of *K. marxianus* by a constraint-based stoichiometric [79] modelling approach. A biomass-coupled model of central metabolism was developed to be a basis for design of metabolic engineering and to assess in silico the production of ethanol, acetate, lactate, glycerol, ethylacetate, succinate, glutamate phenylethanol, phenylalanine. As well as being useful products in their own right, they are also representatives of other products that could be derived from the same precursor metabolites.

Materials and methods

Modelling methodology and software

Two major strands of stoichiometric modelling are the constraint-based flux balance analysis (FBA) [63, 94] and elementary modes analysis [80]. A constraint-based model of central metabolism including biomass production of *K. marxianus* was created adapting and combining the high-quality genome-scale metabolic reconstructions protocol [79] and structural modelling approach for development of medium-scale reconstruction and models [39].

Our medium-scale *K. marxianus* central carbon metabolism model is based on the general mass balance equation:

$$dX/dt = r_{met} - \mu X_{met}.$$

With respect to intermediate metabolite accumulation, a cell's metabolism is in pseudosteady state and can be described by the following equation [85]:

$$0 = r_{met} - \mu X_{met}.$$

We also assume the following:

- the specific growth rate (μ, h^{-1}) during the exponential growth phase is constant,
- the cells are at pseudosteady state: substrate uptake, metabolite and product fluxes are constant when μ is constant.

For constraint-based and structural analysis, the ScrumPy modelling package [71] was used. Flux balance analysis (FBA) was carried out by setting a constant rate of substrate uptake to 10 mM g^{-1} DW h^{-1}, and searching for the maximum yield of one of the following products: ethanol, acetate, lactate, glycerol, ethylacetate, succinate, glutamate, phenylethanol or phenylalanine. Solutions were further examined using flux variability analysis (FVA) [55] to determine the ranges of internal fluxes that are consistent with the maximum if there were multiple equivalent FBA solutions. Inconsistencies in the model formulation were additionally detected through null space analysis [21] combined with determination of inconsistent enzyme subsets [69] using ScrumPy. The essentiality of genes and reactions was analysed using FBA to check whether biomass production was feasible after deleting the relevant reaction(s) from the model. The gene essentiality test took into account the gene–protein-reaction (GPR) associations [86] that were determined for the model (next subsection). FVA was also used to calculate the potential range in product production taking into account minimal and maximal oxygen respiration levels at a fixed substrate uptake value.

Reactions

The *K. lactis* genome-scale reconstruction [16] was used as a starting point given the high degree of similarity between its metabolic networks and that of *K. marxianus*. The amino acid sequences of *K. lactis* genes from the NIH genetic sequence database GenBank [3] were compared against fungal species using NCBI BLAST [38]. The corresponding *K. marxianus* genes were also checked for presence in the Uniprot database [54]. For each reaction, its Enzyme Commission number (E.C. number) and reaction directionality were checked and validated. The IntEnz [22] (available at http://www.ebi.ac.uk/intenz/) database was the main reference source for mass and charge balance validation. To represent the *K. marxianus* biomass growth reaction, we used the *S. cerevisiae* biomass composition as described by Gombert et al. [31].

Metabolites

Metabolite names, their neutral and charged formulas and InChI (International Chemical Identifier) strings [22] were taken from the CheBi database [13] (available at http://www.ebi.ac.uk/chebi/), and the yeast-specific Metacyc [7]. The PubChem database [96] (available at http://pubchem.ncbi.nlm.nih.gov/) was used to get additional information about metabolites [22].

Kluyveromyces marxianus strains and cultivation conditions

The results of original experiments carried out by us to provide data for model development are marked in Table 1 as "this study". *K. marxianus* strain DSM 5422 was cultivated in semi-synthetic medium containing (g/l) $KHPO_4$ (1.0), $CaCl_2$ (0.1), $MgSO_4 \cdot 7H_2O$ (0.5), NaCl (0.5), $(NH_4)_2SO_4$ (5.0) KH_2PO_4 (0.1) yeast extract (*Biolife*) (0.5). Different carbon sources (lactose or inulin) were added at concentrations of 5 or 10% w/v. All fermentations were carried out in 1 litre *Infors* 2HT or 0.4 litre *Sartorius Biostat Qplus* 6-fold system fermenters at 35 °C and 400 rpm.

Metabolite and biomass analyses

Extracellular lactose, ethanol, acetate and glycerol contents were measured simultaneously using an *Agilent 1100 HPLC* system with a *Shodex Asahipak SH1011* column. Metabolites were quantitated with a refractive index detector (RI detector *RID G1362A*). The flow of the mobile phase (0.01 N H_2SO_4) was 0.6 ml min^{-1}; the sample injection volume was 5 μL.

Biomass growth was estimated by absorbance measurements at 600 nm (OD600). The conversion coefficient of *K. marxianus* DSM 5422 strain OD600 to culture dry weight was determined gravimetrically: OD600 1.0 was equivalent to 0.3 g $dw.L^{-1}$.

Results and Discussion

Model construction and properties

The model is shown diagrammatically in Fig. 1 and is supplied in SBML [34] (Online Resource 1) format and in the form of a COBRA [79] MS Excel input file (Online Resource 2). Our *K. marxianus* metabolism model contains 113 reactions and 101 metabolites organised in 3 compartments: extracellular, cytoplasm and mitochondria. There are 72 cytosolic reactions (central metabolism pathways), 28 transmembrane transport reactions, 11 mitochondrial reactions, one extracellular and one biomass reaction (24 components).

Specific assumptions for our *K. marxianus* FBA model included the following:

- ammonium sulphate was the sole nitrogen and sulphur source and was available in excess;
- extracellular product accumulation had no effect on intracellular reactions;
- inorganic phosphate was available in excess;
- NADH and NADPH were assumed not to freely exchange between mitochondria and cytoplasm. Instead, redox equivalents could be translocated across the mitochondrial membrane by specific transport systems (shuttles). A malate–aspartate shuttle [17] and a 2-oxo glutarate–citrate carrier [8] were included to model NAD- and NADP-dependent redox exchange between cytosol and mitochondria.

To allow for succinate exchange across the mitochondrial membrane, a succinate–malate carrier was introduced [1, 64]. An electron transport chain was included in the model as a lumped reaction with the *P/O* ratio set to 1.2 [32].

AcetylCoA transport across the inner mitochondrial membrane occurs via a carnitine shuttle that is related to fatty acid metabolism [102]. Since the main fluxes for many biotechnologically important products stem directly from short chain carbon metabolites, we decided not to include a representation of fatty acid metabolism and described AcetylCoA transport across the mitochondrial membrane as a simple transport reaction (model reaction ACCOA_DIFF).

Kluyveromyces marxianus is an example of Crabtree negative yeast. Its physiology is believed to be closely related to its sister species *K. lactis* [95]. It is reported that ethanol production in *K. lactis* coincides with decreased oxygen supply [41]. It is assumed that flux regulation around pyruvate bypass is the reason for Crabtree negative yeasts to choose between fermentation or oxidative growth. The cytoplasmic pyruvate bypass in *K. lactis* consists of pyruvate decarboxylase, NADP-dependent acetaldehyde dehydrogenase and acetyl-CoA synthetase. The first step of the pyruvate bypass in *K. lactis* is strongly upregulated during fermentative growth thus increasing the cytoplasmic production of AcetylCoA [41]. In the case of disturbed functioning of the mitochondrial pyruvate dehydrogenase complex, oxygen limitation or blockage of respiration chain, this bypass can supply enough cytoplasmic acetyl-CoA to support growth [103, 104].

Acetyl-CoA production in *K. marxianus* mitochondria occurs via the pyruvate dehydrogenase complex (model

Table 1 Model validation data. Substrate uptake, biomass growth and product formation fluxes (mM g DW^{-1} h^{-1}) were calculated from exponential phase of batch fermentations

Lactose as substrate	Substrate consumption	Biomass μmax h^{-1}	Ethanol	Glycerol	Acetate	Ethylacetate	Aeration Vol/vol × min
This study, lactose	4.4 ± 0.4	0.39 ± 0.06	19 ± 2	0.72 ± 0.2	0.24 ± 0.05	ND	0.2
This study, lactose	5.0 ± 0.4	0.30 ± 0.05	9.0 ± 0.5	0.57 ± 0.16	0.17 ± 0.017	ND	1
Martynova et al. [57]	13.04	0.31	30.04	0.86	0.19	ND	1
Sansonetti et al. [77]	3.6	0.07	12	0.34	ND	ND	Self anaerobic
Longhi et al. [51]	2.3	0.48	2.73	ND	ND	ND	3
Longhi et al.[51]	2.1	0.40	7.2	ND	ND	ND	3
Löser et al. [53]	2.4	3.6	1.0	ND	3.0	0.97	1.32
Inulin, glucose or sucrose as substrates	Summary sugar/glucose consumption	Biomass μmax h^{-1}	Ethanol	Glycerol	Acetate	Phenylethanol	Aeration Vol/vol × min
This study, glucose,	15.20	0.48	26.69	1.33	0.013	ND	0.25
This study, glucose,	13.71	0.54	24.25	0.46	0.008	ND	2.5
This study, inulin	5.8 ± 0.5	0.25 ± 0.2	10 ± 1.0	0.16 ± 0.02	0.04 ± 0.002	ND	1.5
Kim et al. [43], glucose	0.47	0.02	ND	ND	ND	0.7	ND
Santharam et al. [78], inulin	2.60	0.2	ND	ND	ND	ND	Shake flasks
Gao et al. [27], inulin	42.6	0.26	72.4	ND	ND	ND	Self anaerobic
Yuan et al. [101], inulin	16	0.14	18	ND	ND	ND	Shake flasks
Garavaglia et al. [28], grape must	2.3	0.40	1.1	ND	ND	0.068	1 and 2
Etschmann et al. [20], sucrose	4.8	0.26	10	ND	ND	0.33	Shake flasks
Wittmann et al. [100], glucose	5.0	0.081	1.7	0.34	ND	0.14	1

Xylose as substrate	Substrate consumption	Biomass μmax h^{-1}	Ethanol	Acetate	Xylitol		Aeration Vol/vol × min
This study,	1.06	0.11	0.18	0.04	ND	ND	2.5
This study,	1.38	0.089	0.085	0.00007	ND	ND	0.25
Signori et al. [82]	0.55	0.014	0	0.4	ND	ND	1
Delgenes et al. [14]	0.43	0.007	0.28	ND	ND	ND	1
Kim et al. [42]	0.84	0.023	ND	ND	0.66	ND	Shake flasks
Nitiyon et al. [62]	5.78	0.09	3.02	ND	0.04	ND	Shake flasks, +30
Nitiyon et al. [62]	7.54	0.10	2.55	ND	2.44	ND	Shake flasks +37
Sharma et al. [81]	1.20	0.13	ND	ND	ND	ND	Shake flasks
Margaritis and Bajpai [56]	1.83	0.08	1.54	ND	ND	ND	Shake flasks
Behera et al. [2]	0.69	0.12	ND	ND	ND	ND	Shake flasks

Simultaneous uptake of xylose and glucose	Glucose/xylose consumption	Biomass μmax h^{-1}	Ethanol	Acetate	Glycerol	Xylitol	Aeration Vol/vol × min
Zhang et al. [105]	4.94/6.67	0.17	4.83	ND	ND	4.02	anaerobic

Data were extracted from other author publications or obtained from our fermentations (denoted as "this study"). Means and standard deviation are calculated from 3 technical replicates where applicable

reaction ed5). In the model, cytoplasmic AcetylCoA synthesis was catalysed by acetyl-CoA synthetase (model reaction ACS). To model anaerobic or semi-anaerobic fermentations, an AcetylCoA (reaction ACCOA_DIFF) transport reaction from cytoplasm to mitochondria was included.

Model validation

Data sources for validation/calibration

The main carbon fluxes for model validation in *K. marxianus* were as follows: substrate uptake, CO_2, ethanol,

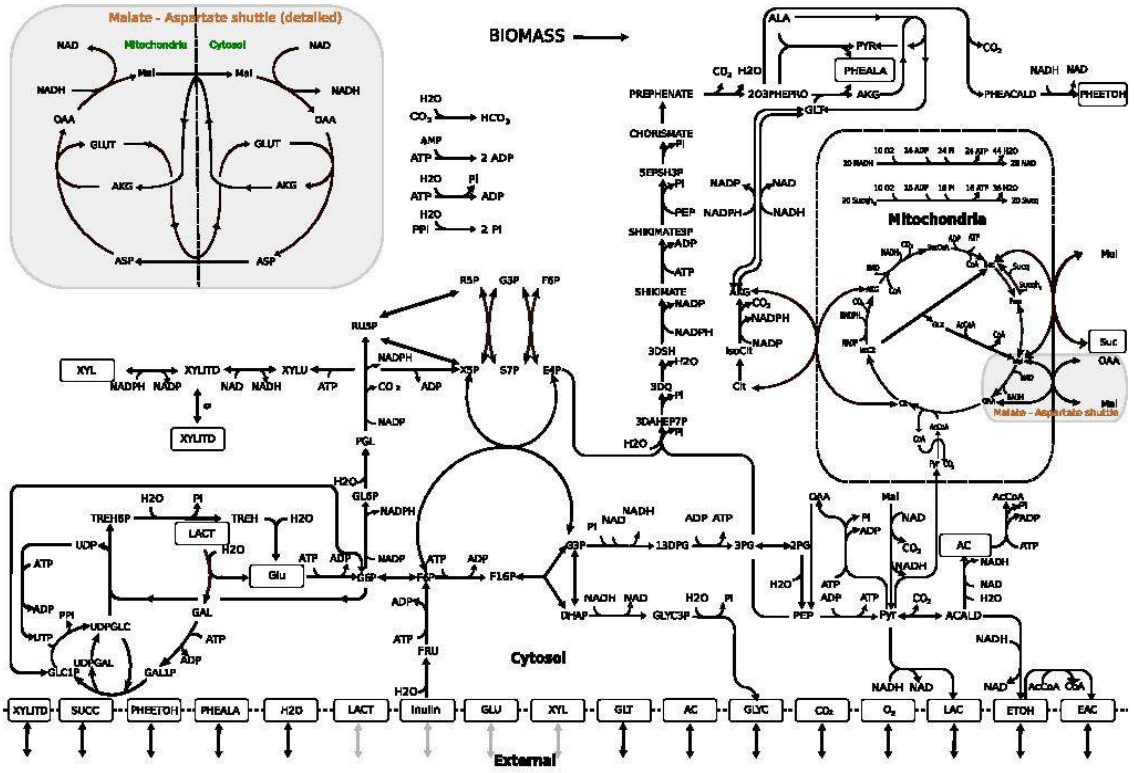

Fig. 1 The scheme of *Kluyveromyces marxianus* central carbon metabolism model

glycerol, acetate and biomass production. For a *K. marxianus* batch cultivation with limited oxygen supply these fluxes can account for up to 100% of total carbon [77]. Therefore, this set of fluxes is sufficient to validate this medium-scale model. A similar set of fluxes has been successfully applied to validate the medium-scale carbon metabolism model of *Pichia pastoris* [87].

Here, the model outputs were compared with previously published and original experimental data. Metabolite and biomass data from the exponential growth phase were extracted from numerous published studies involving *K. marxianus* batch cultivations on various substrates (Table 1).

Model validation on lactose as substrate

Lactose is the main carbohydrate in cheese whey. In *K. marxianus*, lactose is split by the enzyme β-galactosidase into glucose and galactose, then each of these monosaccharides enters glycolysis at different levels: glucose is converted to glucose-6P, but galactose is converted to glucose-1 phosphate by the Leloir pathway. Strains of *K. marxianus* differ with respect to the first steps of lactose metabolism—some strains have intracellular and some extracellular β-galactosidase [6]. We modelled *K. marxianus* lactose uptake with transport reaction lactD (lactose permease) and breakdown by reaction GALSID (β-galactosidase). The model was able to achieve a steady-state solution for all the experimentally measured flux distributions (Table 1).

In addition to the published studies, we performed aerobic fermentations (semi-synthetic broth with 7 or 10% lactose, aeration 0.2 or 1 vol vol^{-1} min^{-1} of fermentation volume). The measured fluxes of the extracellular metabolites are presented in Table 1 as "this study"; ethanol was the major product with glycerol and acetate as the main byproducts.

Depending on the oxygen supply, *K. marxianus* lactose fermentation is biomass (aerobic) or ethanol (anaerobic) orientated. Sansonetti et al. [77] demonstrated results for *K. marxianus* DSM 5422 strain lactose fermentation in "self anaerobic" mode reaching 3.33 units of ethanol per unit of lactose. In this case, biomass growth was slow ($\mu = 0.07$ h^{-1}) and glycerol was produced as the main

byproduct. On the other hand, rapid biomass production by *K. marxianus* strain CBS 6556 from lactose has been described under fully aerobic mode with comparatively low ethanol flux [51].

Interestingly, none of above-mentioned cases reported acetate accumulation, which seems to be related either to slow cytoplasmic consumption of AcetylCoA (as in the case of low μ), or sufficient AcetylCoA supply by mitochondria (in the aerobic case). Longhi et al. [51] reported possible accumulation of acetate during fermentation, albeit they did not report exact concentrations.

Model validation on glucose, sucrose and inulin as substrates

Glucose, fructose and their derived glucose and fructose oligo- and polysaccharides form an important group of substrates for industrial applications. Sugarcane or sugar beet molasses, starch, sucrose and inulin are typical examples [66].

Kluyveromyces marxianus is able to hydrolyze inulin directly due to its extracellular inulinase activity [75]. We performed fermentations with strain DSM 5422 in semi-synthetic broth with inulin as a sole carbon source; results are depicted in Table 1.

For strain DSM 5422, extracellular inulinase activity by far exceeded the uptake of released monosaccharides (data not shown). An ample amount of free fructose in the media due to extracellular inulinase activity was also demonstrated by other authors [101]. In the model we assumed that only fructose is produced after inulin hydrolysis; glucose is released in negligible amount and has no effect on fructose uptake. Similarly, when simulating the data of Etchmann et al. [20], we assumed that sucrose is split outside the cell and invertase activity exceeds the rate of monosaccharide uptake [75]. Subsequent simultaneous consumption of glucose and fructose happens when sucrose is hydrolyzed by invertase [23]. Fructose uptake (model reaction inulin_t) followed by fructose kinase (model reaction onoHLK) was considered as a starting point for inulin consumption. All results from inulin, glucose and sucrose fermentations described in Table 1 were replicated by the model.

Model validation on xylose as substrate

Kluyveromyces marxianus is able to ferment xylose. As for many yeasts and fungi, in *K. marxianus* xylose is taken up and converted to xylulose-5 phosphate (pentose phosphate pathway intermediate) via three sequential reactions: xylose reductase (reaction XYL1), xylitol dehydrogenase (reaction XDH) and xylulose kinase (reaction pengluc3). Moreover, xylose reductase in *K. marxianus* is exclusively NADPH dependent [106]. Xylose reductase reaction in our model was represented as exclusively NADPH dependent.

There are many reports of xylose fermentation by *K. marxianus*. We chose three example fermentations [14, 56, 82] to extract data for model validation. All three xylose fermentations yielded slow biomass growth with μ varying from 0.007 to 0.08 h^{-1}. Interestingly enough, the experimental μ values correlated with oxygen supply: increased oxygen supply led to increased μ [82].

Reaction and gene essentiality

In this study, we linked gene (or reaction) essentiality to the inability to form biomass (maximal biomass flux = 0) on deletion of all reactions catalysed by that gene product. Reaction deletion was performed by setting a zero flux for the reaction in model. Mostly, there were one-to-one relations between genes and reactions, but in some cases there were (1) redundant genes when each of alternative genes encoded enzyme (OR relationship), (2) two or more genes encoded polypeptides that form functional enzyme (AND relationship) and (3) one gene encoded more than one reaction. According to the analysis results (Online Resource 3), the model contained 38 essential reactions (Fig. 2). The 26 reactions (23%) essential for all analysed substrates belonged to central carbon metabolism. Due to the small model size (113 reactions), there were not many redundant or parallel pathways included. Large-scale experimental deletion studies with *S. cerevisiae* report 17% [99] and 19% [29] essential proteins for viability in rich medium which is close to our medium-scale model-based prediction. For comparison, 2–66% of reactions are essential across different eukaryotes [9].

Model optimisation

The model was optimised by FBA for production of ethanol, acetate, lactate, glycerol, ethylacetate, succinate, glutamate phenylethanol and phenylalanine at fixed substrate uptake rate (glucose/inulin, lactose and xylose) at 10 mM g^{-1} DW h^{-1} and μ at 0.4 h^{-1} as a compromise between different substrate consumption and growth rates. The substrate uptake flux was set high to make flux distribution and yield calculations more practical. The maximal percentage of substrate carbon atoms converted into product (Fig. 3a) always was below the maximal theoretical yield (Fig. 3b).

Kluyveromyces marxianus metabolism is sensitive to the oxygen consumption rate. To assess the opportunities of metabolic control by variable oxygen supply, optimisation

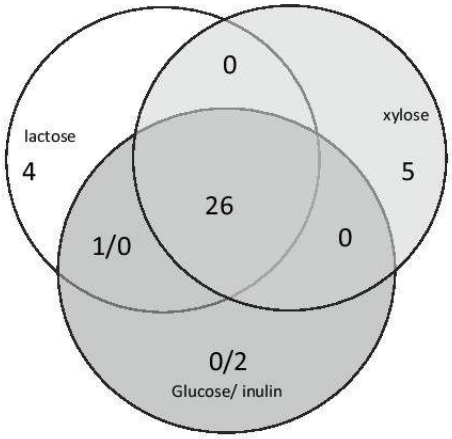

Fig. 2 Model reaction essentiality for biomass production depending on substrate. Analyses revealed 26 essential reactions for all substrates, 5 reactions exclusively essential for xylose, and 4 for lactose. Glucose and inulin are shown as one substrate. Fructose kinase and inulinase were essential reactions for inulin consumption, while hexokinase was essential for both lactose and glucose consumption

(FBA) and variability analysis (FVA) were performed for two extreme respiration cases—low (necessary for biomass production) (Fig. 3c) and high (Fig. 3d) oxygen consumption rates at fixed $\mu = 0.4\ h^{-1}$ in the following steps:

1. maximal and minimal oxygen consumption was determined minimised/maximised by FVA at $\mu = 0.4\ h^{-1}$ for each substrate;
2. 90 and 100% of maximum oxygen consumption rate (determined in step 1) were set as lower and upper oxygen consumption rate bounds FBA analysis at high oxygen consumption;
3. minimal and three minimal oxygen consumption rates (determined in step 1) were set as lower and upper oxygen consumption rate bounds FBA analysis at low oxygen consumption;
4. maximal product rate at low (Fig. 3c)/high (Fig. 3d) oxygen consumption was determined by FBA.

In the case of no constraints on oxygen consumption, high values of carbon flux to product (Fig. 3a) were predicted for lactate, glutamate and phenylalanine. Ethanol, acetate and ethyl acetate yields were identical and close to their theoretical maxima (Fig. 3b). The lowest fractions of carbon flux to product were in the cases of succinate, phenylethanol and glycerol. All other cases attained at least 75% of their theoretical yields. Succinate was the only product that had higher yields on xylose as substrate compared to other substrates (Fig. 3a). At minimal oxygen consumption (Fig. 3c) for most products the carbon flux to products was lower in the case of xylose as substrate. With lactose, inulin and glucose as substrates, ethanol and lactate

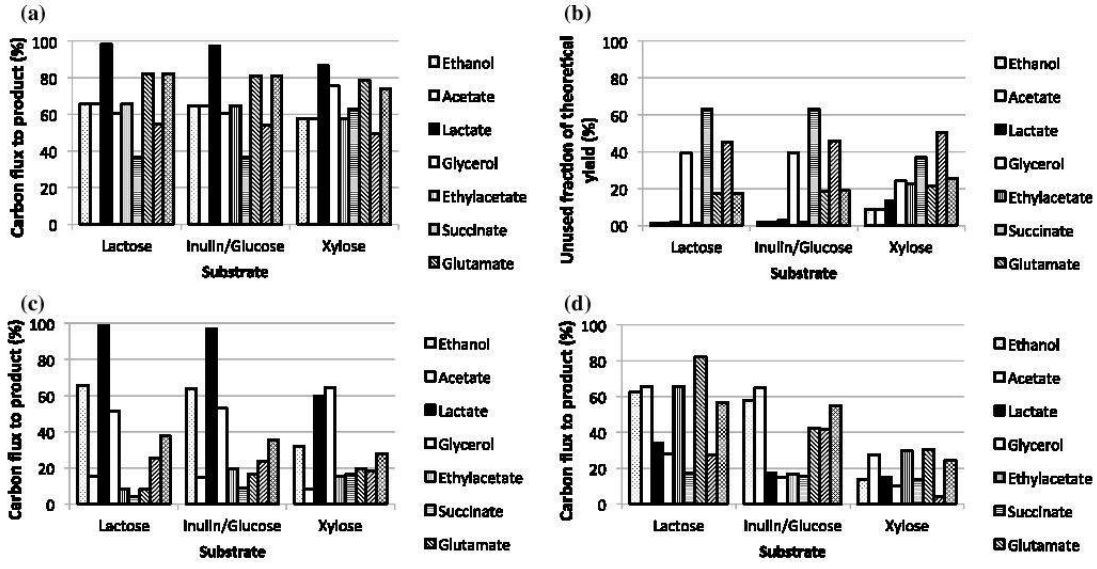

Fig. 3 **a** Maximal percentage of substrate carbon atoms converted into product at biomass growth $\mu = 0.4\ h^{-1}$. **b** Difference between theoretical yield and maximal carbon flux to product at biomass growth $\mu = 0.4\ h^{-1}$. **c** Maximal percentage of substrate carbon atoms converted into product at low oxygen consumption and biomass growth fixed at $\mu = 0.4\ h^{-1}$. **d** Maximal percentage of substrate carbon atoms converted into product at high oxygen consumption and biomass growth fixed at $\mu = 0.4\ h^{-1}$

is used. Microbial (bacterial, yeast or fungi) fermentation is one of the options for L-lactic acid isomer synthesis in industrial amounts [35]. *Kluyveromyces* sp. has been proposed as a prospective lactic acid producer due to its fast production rates and GRAS status [15]. Yeasts do not have lactate dehydrogenase; therefore, for lactate production recombinant strains harbouring LDH of eukaryotic origin (mammals, moulds) are used.

The theoretical molar yield of L-lactate from mole of glucose was 2, from lactose 4, but from xylose 1.6. Our model predicted L-lactate formation with the following molar ratios: 3.9 from lactose, 1.9 from glucose and 1.6 from xylose.

Introduction of heterologous lactate dehydrogenase alone does not lead to maximal L-lactate production in vivo. Carbon flow towards lactate or ethanol was divided at the level of pyruvate by pyruvate decarboxylase (reaction in the model PDC) or the pyruvate dehydrogenase complex in mitochondria. If the pyruvate gets decarboxylated, direct lactate production from pyruvate was not possible, instead carbon was routed to acetaldehyde and ethanol or acetate formation. Flux variability analyses revealed that this was the case—when simulating a decrease in lactate flux, an equimolar increase in CO_2 and ethanol fluxes was observed.

Kluyveromyces sp., unlike *Saccharomyces*, contain just one PDC gene; therefore, preparation of *pdc* functional knockouts is comparatively easy. Lactate dehydrogenase overexpression in a *K. lactis pdc* strain has proven to be an efficient strategy yielding a lactic acid: consumed glucose ratio up to 0.5 [72]. Lactate production close to the theoretical maximum was achieved when both pyruvate consuming branches (pyruvate decarboxylase and dehydrogenase) were inactivated. A molar lactate/glucose ratio close to 2 in *K. lactis pdc pdh* knockouts was obtained by Bianchi and colleagues [4]. Alternatively, additional heterologous expression of lactate dehydrogenase by increasing gene copy numbers can be a strategy to increase lactate production [67].

Glycerol production

Glycerol is a typical byproduct of yeast ethanol fermentation that forms in response to the need to balance cytoplasmic NADH oxidation. Glycerol formation as an NADH sink becomes crucial when NADH oxidation via the electron transport chain is not possible (limited oxygen supply). Although glycerol synthesis by microbial producers per se *h*as no applications in biotechnology, we included this metabolite in our analyses since this is one of the major carbon and redox sinks in the *K. marxianus* metabolism.

Theoretical maximal glycerol production from different substrates in molar ratios was as follows: from lactose 4, from glucose 2, from xylose 1.66. Our medium-scale *K. marxianus* metabolic model predicted maximum molar yields from lactose 2.4 from glucose 1.2 and from xylose 1.2. The model predicted the need for a certain respiratory activity (up to 0.5 units of O_2 per unit of substrate) for glycerol production to reach a maximum, and hence CO_2 was the only byproduct in the case of optimal glycerol production.

We and other researchers have observed similar effects in vivo in inulin and lactose fermentations with *K. marxianus*—higher aeration leads to smaller ethanol and glycerol flux and vice versa [82]. Severe fermentation dependence on oxygen supply has also been demonstrated in the physiology of *K. marxianus'* sister species *K. lactis* [58].

Ethyl acetate production

Ethyl acetate is a volatile, slightly polar molecule, used as an organic solvent. Nowadays, it has many applications in cosmetics (nail polish remover), electronics (cleaning circuit boards, etc.), and has a potential future application as an environmentally friendly acyl acceptor in biodiesel production instead of methanol. Currently, ethyl acetate is produced from petrochemical sources, but it can be produced through biotechnological synthesis by many yeasts. Currently, *K. marxianus* is regarded as the most productive ethyl acetate producer [52].

For ethyl acetate, the theoretical molar product/substrate yield, when considering pyruvate decarboxylation, was 2 for lactose, 1 for glucose and 1 for xylose. Our model predicted the maximum ethyl acetate-to-substrate ratio from lactose to be 1.97, 0.72 from xylose, and 0.97 for inulin or glucose. FVA results revealed strong effects of aeration on ethyl acetate formation. Most ethyl acetate was produced at increased aeration. However, the most effective ethyl acetate formation was not during growth with maximal respiration (Fig. 3c). Additionally, FVA revealed a notable increase in glycerol production during oxygen limitation, which indicated the necessity of cytoplasmic NADH reoxidation to support acetate production. In the case of respiration, cytoplasmic NADH could be reoxidised through the electron transport chain and mitochondrial shuttle activity.

Careful fine-tuning of oxygen consumption might be a strategy for maximum ethyl acetate production. A similar strategy was applied when limiting *K. marxianus* access to metal ions [91, 92]. Metal ion (Fe, Cu, Zn) limitation was found to affect ethyl acetate production. Amongst them, Fe limitation had the most effect. *K. marxianus* culture starving for Fe produced ethyl acetate at close to 50% of theoretical maximum. Fe limitation lowered the activities of Fe-dependent mitochondrial aconitase and succinate dehydrogenase; this subsequently led to accumulation of acetylCoA, which was used to increase ethyl acetate production [92].

Succinate production

Succinate is one of the 12 most recognised sugar-derived chemical precursors. There is biotechnological potential for succinate due to its wide application spectrum, since it can serve as a precursor for tetrahydrofuran, butanediol, succinonitrile etc. Cheap microbial production of succinate has huge market potential [97]. There are already several examples of microbial succinate production at industrial scale (Reverdia, Myriant, BioAmber, BASFPurac, etc.). At least one of the processes is yeast based (Reverdia, *S. cerevisiae*) [12]. Although succinate yields close to the theoretical stoichiometric maximum are reached by bacterial cells, yeast offer several advantages over bacteria: they are not obligately anaerobic; they are robust, acid and osmotically tolerant, and non-pathogenic organisms [73].

The theoretical maximal molar ratio for succinate production from lactose was 3, from glucose 1.5 and xylose 1.25. Our *K. marxianus* carbon metabolism model predicted the maximum succinate production ratio from glucose to be 0.55, from lactose 1.1 but from xylose 0.78. From here, it seems, that xylose might be the most potent substrate for succinate production; however, there are not many in vivo results on succinate production by *Kluyveromyces* sp. from xylose. Interestingly, a xylose/ethanol mixture is suggested as a prospective substrate for glyoxylate production along with succinate (isocitrate lyase reaction) in *S. cerevisiae* and *K. lactis* isocitrate lyase overexpressed strains [45].

Succinate can be produced via the tricarboxylic acid cycle or the glyoxylate shunt. It is not a redox neutral product with respect to carbohydrate substrates—theoretically, 2 NAD + are consumed per each molecule of succinate. Reduced cofactors can be oxidised in the electron transport chain or by production of glycerol or ethanol—NAD regenerating pathways. The model predicted accumulation of at least one byproduct when optimised for succinate production. FVA results demonstrated that, depending on oxygen supply, many byproducts were formed. Interestingly, the model predicted glycerol formation in the case of poor aeration, independent of substrate. The compensatory NADH reoxidation through increased glycerol production in *S. cerevisiae* strains, optimised for succinic acid production, was demonstrated in vivo [73].

Based on our medium-scale model, phenylalanine can also be formed as a byproduct in rather large amounts (0.3–0.6 units of phenylalanine per unit of lactose) if oxygen is supplied in surplus (3.7 units of oxygen per unit of lactose). In this case, production of a relatively large amount of phenylalanine is possible, since our medium-scale model is not nitrogen (ammonia) restricted (see model assumptions). In real applications, however, nitrogen bioavailability might prevent such high levels of phenylalanine production being reached.

Deletion of the genes for succinate dehydrogenase subunits is a popular strategy for yeast-based succinate production [73]. Succinate accumulation in the case of KlSDH1 (succinate dehydrogenase subunit) deletion was observed in the case of *Kluyveromyces lactis* [76]. Our model predicted that inactivation of the aspartate malate shuttle in combination with increased oxygen consumption (up to 1.3 per unit of glucose) would give maximum succinate yield, while inactivation of succinate dehydrogenase together with inactivated glyoxylate shunt would be preferable in the case of fermentation of xylose.

Glutamate production

In our central metabolism model, the *K. marxianus* biomass reaction consisted of 24 metabolites, excluding the amino acids, although phenylalanine and glutamate are included in the model as desired products. The amino acid content of yeast biomass is of particular industrial interest, since some of them (like glutamic acid) are responsible for developing of umami taste [37]. Random mutations are the typical method for generating yeast strain with increased glutamic acid content [61]. Here, we provide model-based theoretical analysis of possible scenarios for increasing glutamic acid yield from substrate in *K. marxianus.*

The theoretical maximal glutamate production from different substrates in molar ratios would be as follows: from lactose 2.4, from glucose 1.2, from xylose 1. Our medium-scale *K. marxianus* metabolic model predicted maximum molar yields from lactose 1.97, from glucose 0.97, and from xylose 0.8. Glutamate in *K. lactis* and, most probably also in *K. marxianus,* can be produced by either of two reactions: NADP-dependent glutamate dehydrogenase (EC 1.4.1.4 reaction GLUDE_nadp) or by GOGAT (EC 1.4.1.13, reaction Glude_NAD) [74]. Our model predicted the larger carbon flux to be routed through NADPH-dependent glutamate dehydrogenase. To recover enough cytoplasmic oxoglutarate an NADP—oxoglutarate—citrate shuttle was used (see Fig. 1). At the same time, a high respiration rate was needed to reach maximum glutamate production if glucose or lactose was used as substrates (approx. 1.5 O_2/glucose). Interestingly, a higher fractional of molar yield of glutamate was achieved by xylose fermentation (80% from theoretical) and less oxygen needed to be supplied per substrate moiety (1.2). In addition, the main glutamate synthesis flux in *K. marxianus* consuming xylose was predominantly, through the GOGAT reaction, unlike when lactose or glucose was consumed (see above).

In *K. marxianus* glutamate synthesis is tightly product-regulated by feedback inhibition. As in *S. cerevisiae,* glutamate synthesis via NADPH-dependent glutamate dehydrogenase is subject to nitrogen catabolite repression [59].

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3.3 Effects of acetate on Kluyveromyces marxianus DSM 5422 growth and metabolism

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Effects of acetate on *Kluyveromyces marxianus* DSM 5422 growth and metabolism

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Abstract Metabolically active cells produce a wide array of metabolites that can inhibit their growth. Acetate is a widely known preservative, and it is also produced by yeast cells during their growth. *Kluyveromyces marxianus* DSM 5422 is a promising yeast strain that could be employed in biotechnological processes, but the knowledge of its stress physiology is scarce. Here, we investigate the effects of acetate on growth and changes in cell population structure during adaptation to elevated concentrations of acetate in *K. marxianus* DSM 5422. Our results indicate that acetate inhibits growth in a pH-dependent manner and has pronounced effects if yeast is grown on lactose or galactose. When challenged with acetate, culture extends lag phase, during which cells adapt to elevated acetate concentrations, and growth reoccurs, albeit at a slower rate, when majority of the population is acetate resistant. Acetate resistance is maintained only if acetate is present in the media or if the culture has reached end of active growth phase. This study shows possible caveats in lactose fermentation with *K. marxianus* and gives a further perspective in non-conventional yeast applications in biotechnology.

Keywords *Kluyveromyces marxianus* · Acetate stress · Population heterogenity · Non-conventional yeasts · Lactose

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Introduction

Bioconversion of renewable resources is the only sustainable method for producing bioethanol, microbial biomass, and secondary metabolites. Bioethanol production from renewable resources is a topic gaining increasing attention, since the need for energy is persistent whilst fossil resource deposits are gradually being depleted. Furthermore, substrates containing compounds such as lactose or inulin are readily available in large amounts.

Although *Saccharomyces cerevisiae* is the most common eukaryotic organism used in bioconversion, many non-conventional yeasts, such as *Kluyveromyces marxianus*, are capable of consuming a wider spectrum of substrates. *K. marxianus* possesses high activities of β-galactosidase (Goncalves and Castillo 1982), inulinase, invertase (Rouwenhorst et al. 1990), and xylose reductase (Wilkins et al. 2008). Furthermore, it is thermo-tolerant with a maximum growth temperature as high as 52 °C (Banat et al. 1992) and it has a high specific growth rate (Fonseca et al. 2008). This yeast has been assigned GRAS (generally regarded as safe) and QPS (qualified presumption of safety) status. *K. marxianus* is also suitable for producing simple metabolites, such as ethyl acetate (Löser et al. 2015), ethanol, and succinate, as well as for producing biomass and protein (Lane and Morrissey 2010). Taken together, these characteristics and the lack of pathogenic strains make this yeast an attractive microbe for chemical precursor, food production, and even medical industries.

However, several disadvantages are reported that hamper a wider industrial usage of *K. marxianus*. For example, the relatively low ethanol tolerance of *K. marxianus* strain IGC 2671 compared with that of *S. cerevisiae* was described by Rosa and Sá-Correia (1992) and weak-acid intolerance has also been reported (Rugthaworn et al. 2014). Weak-organic acid-

induced physiological stress and inhibition of microbial growth have been widely explored in the context of food spoilage since acetates, benzoates, and sorbates have been used as food preservatives for a very long time. However, the presence of weak-acids is undesirable in many biotechnological applications, such as in the fermentation of cellulosic and lignocellulosic hydrolysates (Rugthaworn et al. 2014; Palmqvist and Hahn-Hagerdal 2000). Weak-organic acids typically inhibit cell growth and metabolism, thus making bioconversion processes less efficient. The main mechanism of weak-acid toxicity is probably based on intracellular acidification and anion accumulation. When the medium pH is lower than pKa of the given acid, the formation of undissociated acid molecules is facilitated more than dissociation into acid anions and protons. Uncharged acid molecules can cross the plasma membrane more easily than ionic forms of the same acids. As the pH of cytosol is higher than pKa values of most carboxylic acids, molecules that have entered cells dissociate into ions. These ions in turn become trapped in these cells due to their electric charge. This dissociation of acid molecules shifts the concentration gradient of undissociated acid, facilitating further uptake of even more acid. The resulting cytosol acidification and increase of intracellular acid anion concentration presumably lead to the inhibition of various metabolic processes (Arneborg et al. 2000; Mira et al. 2010). pKa of acetic acid and its salts is 4.76. Such pH values can occur in biotechnological processes putting yeast cells under risk of weak-acid stress induced by excessive acetate uptake.

Any accumulation of acetate and other organic acids can also result from other metabolic processes. In yeasts, acetic acid is synthetized from acetaldehyde in the pyruvate dehydrogenase (PDH) bypass pathway. Crabtree negative organisms (including *K. marxianus*) produce acetate as a result of overflow metabolism (Bellaver et al. 2004; Fonseca et al. 2007). As yeasts share common central carbon metabolism principles, we used our current knowledge of phylogenetically close yeasts, *Kluyveromyces lactis* and *S. cerevisiae*, to construct the putative metabolic network of *K. marxianus*. A brief overview of predicted central carbon metabolism in *K. marxianus* is shown in Fig. 1. Due to an increased carbon flow through the PDH bypass during oxygen limitation, an unwanted accumulation of acetate is expected, i.e., during ethanol fermentation or at high biomass concentrations, where it is hard to supply and distribute enough oxygen for all cells throughout the bioreactor (Koynov et al. 2007). In line with this, during poorly aerated fermentations with *K. marxianus*, an accumulation of acetate coinciding with a reduction in growth rate and ethanol production has been reported by various authors. Acetate accumulation of 10 (Sansonetti et al. 2013), 24 (Löser et al. 2015) up till 100 mM (Signori et al. 2014) was observed. So far, *K. marxianus* has been widely researched in terms of biotechnology and less attention has been focused on its basic physiology. Indeed, very little is known about its stress response and adaptation mechanisms induced by acetic acid in this yeast. Here, we investigated the inhibitory effects of biotechnologically relevant acetate concentrations on *K. marxianus* industrially perspective strain DSM 5422 in more detail.

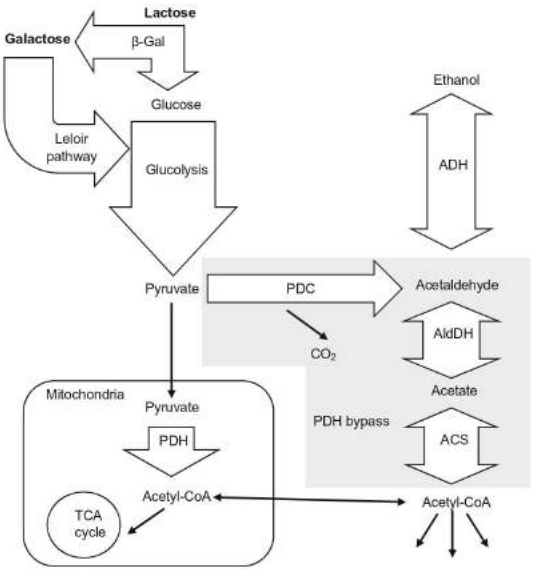


Fig. 1 Predicted scheme of acetate and lactose central metabolism in *K. marxianus*. *B-Gal* β-galactosidase, *ADH* alcohol dehydrogenase, *ACS* acetyl coenzyme A synthase, *AldDH* acetaldehyde dehydrogenase, *PDC* pyruvate decarboxylase, *PDH* pyruvate dehydrogenase. Compiled from Remize et al. (2000); Bianchi et al. (2001) and Sellick et al. (2008)

Materials and methods

Organisms and cultivation conditions

Yeast *Kluyveromyces marxianus* DSM 5422 was obtained from the Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures, and maintained on YPD agar. YPD contained (per liter of distilled water) 10 g of yeast extract (Biolife), 20 g of peptone (Biolife), 20 g/L of glucose (Sigma), and 20 g/L of agar (Biolife).

For 96-well plate and bioreactor experiments, preculture and main culture were grown on a liquid semisynthetic medium: carbon source (lactose, glucose, galactose, and fructose) 50 g/L, yeast extract 5 g/L, $MgSO_4$ 0.7 g/L, KH_2PO_4 1 g/L, K_2HPO_4 0.1 g/L, $(NH_4)_2SO_4$ 5 g/L. Cultivation and inoculation scheme is shown in Fig. 2.

For 96-well plate experiments, fresh overnight culture inoculated from a single colony was used. For bioreactor experiments, overnight culture was upscaled to 1 L flask culture. Cultures in flasks were cultivated at 30 °C with an agitation

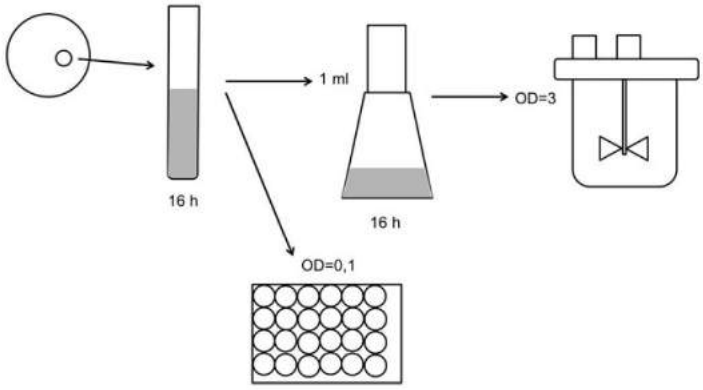


Fig. 2 Workflow for cultivation experiments. For all experiments, preculture was grown from a single colony, maintained on YPD plate. For bioreactor experiments, second preculture was grown. All precultures were grown in semisynthetic media with carbon source corresponding to one used in experiments

speed of 180 rpm, cultivation liquid not exceeding 20 % of total volume of vessel.

For acetate stress, acetate concentration of 40 mM was chosen as it corresponds to the literature data of concentration that accumulates during *K. marxianus* fermentations.

Determination of acetate effect on growth rate

A 96-well plate reader (Tecan infinite 200 M Pro) was used for *K. marxianus* DSM 5422 growth rate measurements at different pH and substrates with four biological replicates. Medium pH was maintained by acetic or citric buffers (sodium acetate, acetic acid, sodium citrate, and citric acid buffers with final acetate or citrate molarity of 40 mM). The plates were incubated at 30 °C for 24 h, shaken with amplitude 3.5 mm, and optical density readings were taken every 10 min followed by a minute-long pause in shaking. From the microcultivation data, we calculated growth rates and doubling times as described by Toussaint et al. (2006).

Bioreactor experiments

All experiments related to metabolite analysis and population adaptation were carried out in bioreactors. Precultures were grown as described in "Organisms and cultivation conditions." Before inoculation in fermentation vessels, cells were washed twice to avoid media carryover. The main cultivation was carried out in a 0.5-L Sartorius Stedium bioreactors with working volume 0.4 L at 30 °C, stirring speed 600 rpm, and airflow rate 1 L/L/min. Oxygen saturation was monitored and never dropped below 20 %. The fermentation medium pH (4.0, 4.5, and 5.0) was controlled by adding 5 % KOH and 1 M H_2SO_4 when needed. Yeast cells grew in lactose semisynthetic medium; 5 M sodium acetate was added to concentration of 40 mM at the start of cultivation when needed. Culture growth was monitored by absorbance measurements at 600 nm with Biochrom Libra S22 spectrophotometer. Metabolites were assessed by HPLC.

Adaptation to acetate

In order to assess acetate tolerance plating on YPD and solid semisynthetic medium supplemented with 40 mM acetic acid, pH 4.3 was used. Adapted cells were counted as the percentage of colonies that formed on semisynthetic media against colonies on YPD. For each biological replicate, three independent dilutions and plating on each medium were made. Serial dilutions ranging from 1×10^7 to 1×10^2 cells/mL were made, thus allowing to discriminate survival from 0.001 to 100 %.

pO_2 measurements

Exponential or post-diauxic phase *K. marxianus* cultures were grown in shake flasks, growth monitored by optical density measurements. After reaching desired growth phase, cells were washed twice and starved in water. Starvation was carried out in shake flasks, at the same conditions as cultures were grown. After 4-h starvation, they were resuspended at 15 ml in Falcon tubes at OD_{600} = 10 (cell concentration was 3.3 gDW/L) in acetate buffers of various molar concentrations at pH 4.5, containing 20 g/L lactose. Cell suspensions were saturated with oxygen, carbon source added, and changes in pO_2 were measured with Hamilton Oxy Ferm 120 oxygen sensors for a time course of 20 min.

Analytical methods

The yeast growth was monitored spectrophotometrically by the OD_{600}. To determine culture dry weight, exponentially growing cells were washed three times with distilled water and dried in 104 °C until a constant weight was achieved. A conversion coefficient 0.33 g DW/OD unit was obtained.

The lactose, ethanol, and acetate concentrations were determined by HPLC (Agilent 1100 Series), using column Aminex HPX-87H (length 300 mm, i.d. 7.8 mm) with a refractive index detector. Column temperature was 45 °C, mobile phase 0.005 mol/L H_2SO_4, flow rate 0.6 mL/min, and sample volume 20 μL.

Statistical methods

All experiments, except 96-well experiments, were carried out in biological triplicates. Ninety-six-well plate experiments were carried out in four biological repetitions. Data shown in figures is average from biological replicates with error bars depicting standard deviation.

Concentration of undissociated acetic acid (HA) was deduced from the Handersson Hasselbach equation:

$$\mathrm{pH} = \mathrm{pKa} + \log_{10}\left(\frac{[A^-]}{[\mathrm{HA}]}\right)$$

Results

Toxicity of acetate is pH and substrate dependent

Bearing in mind the ability of *K. marxianus* to utilize numerous different carbon sources and the role of pH in weak-acid entry into cells, the effect of 40 mM acetate on growth of DSM 5422 was assessed over a pH range of 4–6 and with different carbon sources. This experiment was done in 96-well plate in a semisynthetic medium supplemented with lactose, glucose, fructose, galactose, and inulin as the substrates and 40 mM acetate or citrate buffers (Fig. 3a). During routine fermentations without additional buffering, pH often drops to 4.5 and below, thus putting yeast cells under similar conditions as for Fig. 3a. A pronounced increase in doubling time was observed as the pH of the growth medium decreased and the calculated concentration of undissociated acetate increased. When the pH of media was below 4, no growth of *K. marxianus* was observed in the acetate-buffered medium with lactose or galactose as carbon source. On glucose, the yeast was affected by the presence of acetic acid. But acetic acid did not affect the growth of the yeast on inulin and fructose. An equimolar citrate buffer was used as a control, and no changes in doubling times were observed over the given range of pH (Fig. 3b). This allows to discriminate between pH-specific and acetate-specific responses. A reduced biomass yield was observed with all substrates in the case of acetate compared with citrate (see Fig. S1 and Fig. S2).

Growth in acetate-enriched medium is preceded by an extended lag phase

In order to further investigate the effects of elevated undissociated acetate concentration on DSM 5422, lactose consumption, ethanol production, and concentration of acetate were monitored during cultivation in bioreactors in the presence of 40 mM sodium acetate at controlled pH 4.0, 4.5, and 5.0. The obtained results are presented in Fig. 4. In all control fermentations without added acetate, growth, substrate consumption, and metabolite production dynamics were similar, regardless of medium pH, thus affirming that low pH alone is not responsible for the changes in growth rate seen in Fig. 3a.

In comparison with control fermentations, simultaneous delays in growth, lactose and acetate consumption, and ethanol formation were observed when 40 mM acetate was added to the medium. The delays in growth and metabolism were longer at lower pH (when more acetate is undissociated and able to freely enter yeast cells). Even though the lag phase lasted as long as 20 h and the final amount of biomass was slightly reduced in comparison with the control fermentations, all substrate was consumed eventually as acetate levels decreased. The start of rapid lactose consumption also coincided with a drop of acetate levels.

Changes in cell population structure during adaptation to acetate stress

Next, we examined the structure of yeast cell population regarding acetate tolerance. We performed bioreactor cultivation with controlled pH 4.5 in the presence of 40 mM acetate. We assessed culture growth and acetate tolerance expressed as percent of cells able to grow on semisynthetic agar plates enriched with acetate, pH 4.3 when compared to CFU on YPD plates (Fig. 5, filled symbols), further called primary fermentation.

After start of rapid growth (on the 26th hour), we harvested half of the culture from primary fermenters, washed with distilled water, and re-inoculated into fresh bioreactors with same cultivation conditions (Fig. 5 empty symbols), further called secondary fermentation.

During cultivation at pH 4.5 and 40 mM sodium acetate, an extremely long lag phase was observed in primary cultivations (Fig. 5) as in previous experiments (Fig. 4a).

At the beginning of the lag phase, less than 0.1 % of the cells were able to form colonies on agar plates supplemented with acetate. Approaching the end of the lag phase, the portion of acetate-tolerant cells had reached 78 % while biomass had not increased noticeably yet. Meanwhile, total viable cell count per OD_{600} unit (determined as CFU by plating on YPD) did not change over the course of primary cultivation (Fig. S3).

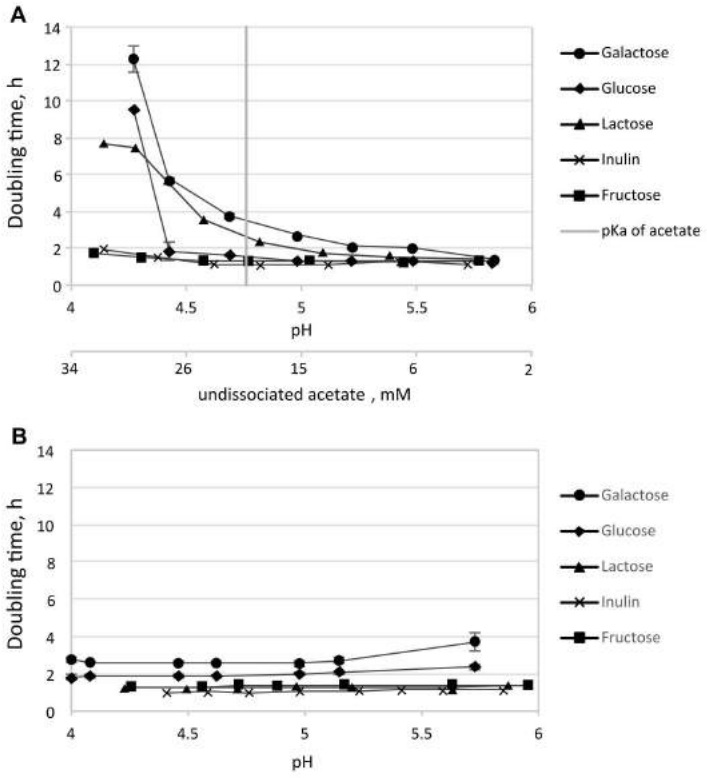


Fig. 3 Doubling times of DSM 5422 cultures over a range of medium pH values when grown on different carbon sources in 96-well plates. **a** Medium pH maintained with 40 mM acetate buffer. **b** Medium pH maintained with 40 mM citrate buffer. Data presented is average from four biological replicates, with *error bars* representing standard deviations

Cultures that were taken during an exponential phase of primary cultivation and inoculated in a fresh medium with added acetate under the same cultivation conditions had obviously adapted to acetate as they showed no lag phase and started growing immediately (Fig. 5).

We also looked for acetate tolerance in cultures that had not adapted to grow in the presence of acetate. Cultures progressing into post-diauxie showed similar acetate-tolerant cell content to cultures adapting to elevated acetate concentration through an extended lag phase (Fig. 6).

Combined effects of acetate concentration and growth phase on respiration rate

Finally, we investigated the influence of undissociated acetate on the respiration rate of DSM 5422 cells from different growth phases after a 4-h starvation period (Fig. 7).

Cells during the exponential phase showed a very sharp reduction in the respiration rate in response to an increasing concentration of undissociated acetate. The same was true for negative control (no lactose added) cells which apparently used acetate from the buffer as a carbon source. As the concentration of undissociated acetate increased, it also inhibited the use of acetate itself as a respiratory substrate. Starved post-diauxic cells were respirating at a lower rate by default, but they were also less responsive to acetate and maintained higher respiration rates than cultures from an exponential phase at higher undissociated acetate concentrations.

Discussion

Toxicity of acetate is pH and substrate dependent

Our results showed that an increased concentration of acetate slowed down yeast growth depending on medium pH and carbon source. The effect of added acetate alone was well distinguished from the effects of pH alone. A citrate buffer caused no weak-acid-related effects. pKa values for each of the three carboxyl groups of citric acid are 3.13, 4.76, and 6.40. This means that pH of the growth medium should approach 3.5 or even lower to yield significant amounts of undissociated citrate that could enter cells easily. As pH determines the amount of acid that is undissociated and is able to permeate lipid bilayers, these results suggest that unidissociated acetate is the agent that acts on yeast cells causing growth defects. Thomas et al. (2002) showed that not only the concentration of undissociated acetic acid but also

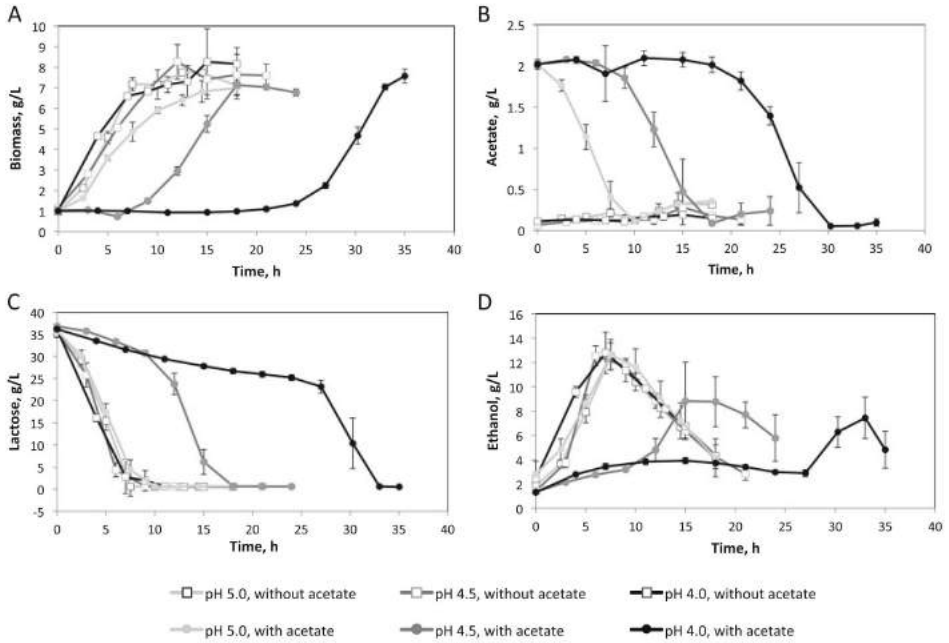


Fig. 4 Effects of 40 mM sodium acetate on growth, substrate consumption, and metabolite accumulation of DSM 5422 at different pH conditions when grown in well-aerated fermenters (1 v/(v*min)). **a** Growth curve (expressed as g/L of dry weight). **b** Acetate in medium. **c** Lactose in medium. **d** Ethanol in medium. Data presented is average from three biological replicates, with *error bars* representing standard deviations

the total concentration of acetic acid determined the extent of growth inhibition when cultivating *S. cerevisiae* in the presence of elevated concentration of acetate. In our case, the concentration of undissociated acetate seems sufficient to explain the reduced growth rates at biotechnologically relevant acetate concentrations. Different cellular mechanisms regarding acetic acid might work in *K. marxianus* and *S. cerevisiae* at different pH values.

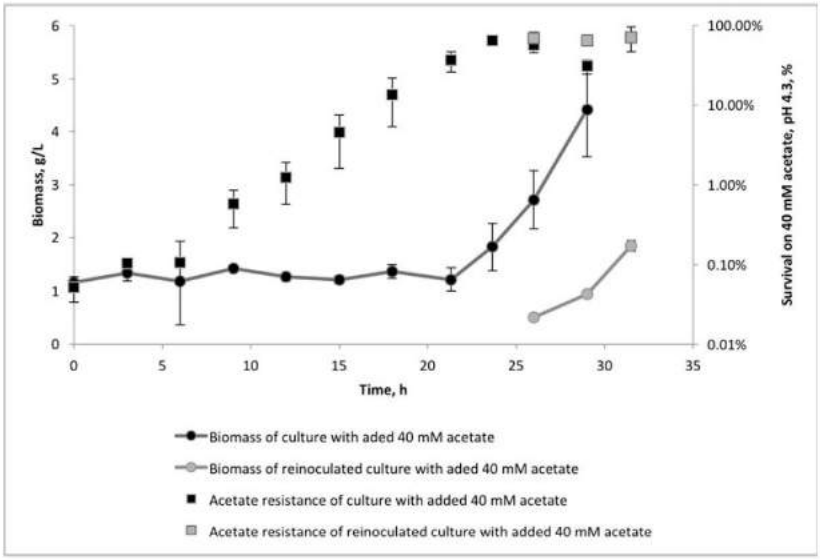


Fig. 5 Adaptation to elevated concentration of acetate during growth. DSM 5422 was cultivated at pH 4.5 and 40 mM acetate (yielding 25 mM non-dissociated acetate) growth curves and dynamics of acetate-tolerant cell proportion shown with *black symbols* and after population reached 90 % tolerance re-inoculated in an identical medium—*gray symbols*. *Circles* show growth curves (expressed as g/L of dry weight). *Rectangles* show proportion of acetate-tolerant cells in the respective culture. Data presented is average from three biological replicates, with *error bars* representing standard deviations

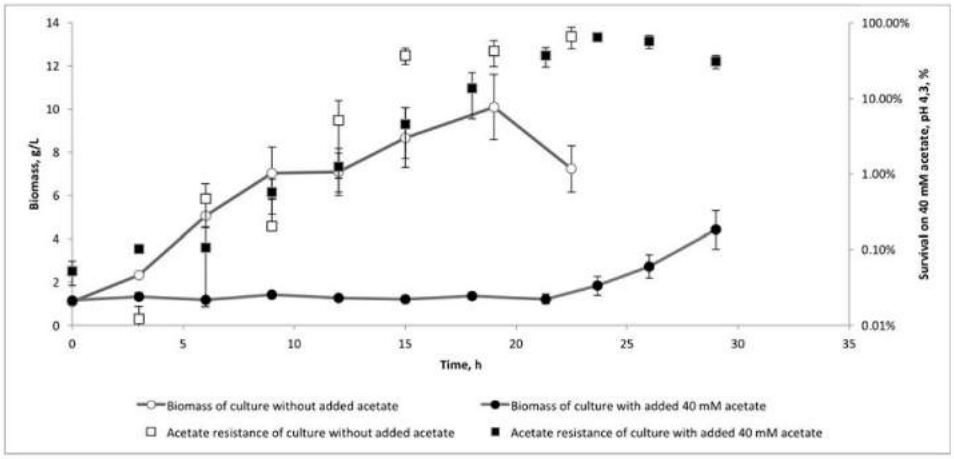
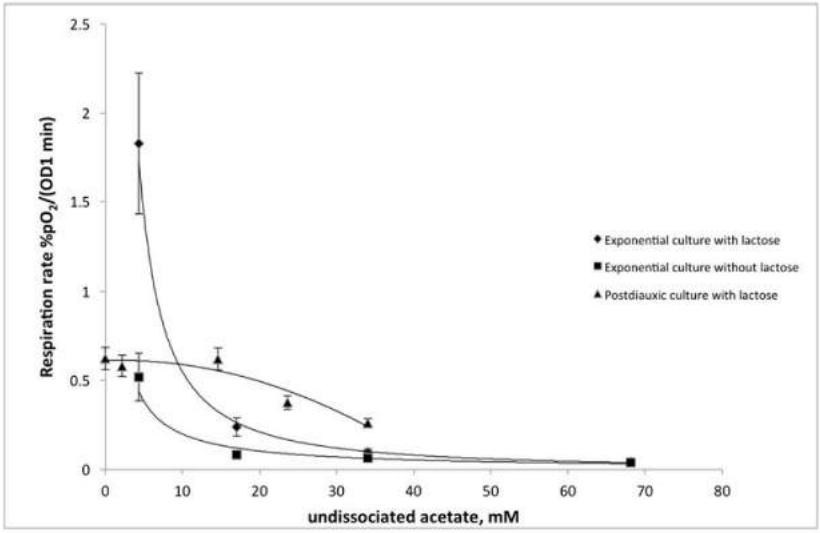


Fig. 6 Growth curves and dynamics of acetate-tolerant cell portion in DSM 5422 cultures grown in the same conditions at pH 4.5 with (*filled symbols*) or without 40 mM sodium acetate (*empty symbols*). *Circles* show growth curves (expressed as g/L of dry weight). *Rectangles* show proportion of acetate-tolerant cells in the respective culture. Data presented is average from three biological replicates, with *error bars* representing standard deviations

Interestingly, the effect of acetate on doubling time of DSM 5422 cultures was dependent on the carbon source that was used (Fig. 3a). A combination of low pH and 40 mM acetate increased doubling times dramatically only when yeast cultures were grown on lactose or galactose. The effect was not so well pronounced in the case of glucose. This makes us look for common traits and pathways in the metabolism of lactose and galactose because these common elements could be affected by the accumulation of acetate and acidification of cytosol. Two key steps are important for the metabolism of any substrate: uptake into cells and further biochemical processing until entry into central carbon metabolism.

Fructose, glucose, and galactose enter *K. marxianus* cells both via proton symport and alternative mechanisms not related to proton symport (Gasnier 1987; De Bruijne et al. 1988; Carvalho-Silva and Spencer-Martins 1990). However, the only known mechanism of lactose uptake is proton symport (De Bruijne et al. 1988). Besides, no extracellular lactose hydrolysis in DSM 5422 strain has been detected (Carvalho-Silva and Spencer-Martins 1990), which means that DSM 5422

Fig. 7 Rate of respiration in different types of DSM 5422 cells in relation to the concentration of non-dissociated acetate in the medium



growth on lactose can be limited by inhibiting lactose transport. At low pH when acetate can freely enter cells, the maintenance of a proton gradient between extracellular environment and cytosol is expensive in terms of ATP due to the acidification of cytosol (Piper et al. 2001). A decreased proton gradient could slow down lactose uptake via proton symport. An insufficient lactose uptake rate together with a high energy expenditure could in turn limit the growth rate. However, it is not clear whether lactose uptake is limited due to the effects on the proton gradient and lactose uptake mechanism itself or due to a reduced lactose consumption in subsequent catabolic reactions (Fig. 1).

Possession of extracellular inulinase allows *K. marxianus* to hydrolyse inulin to fructose monomers (Rouwenhorst et al. 1988). As seen in Fig. 3a, function of inulinase is not inhibited by acetate over the tested pH range. Furthermore, if glucose and galactose uptake is provided by facilitated diffusion (Carvalho-Silva and Spencer-Martins 1990) and proton symport (De Bruijne et al. 1988), it is possible that, during acetate stress, the capacity of facilitated diffusion transporters cannot supply enough sugar to satisfy the energy needs of the cell.

When growing on lactose or galactose, yeast cells employ the five enzymes of the Leloir pathway to convert galactose to glucose-6-phosphate which can be used in glycolysis (Sellick et al. 2008; Fonseca et al. 2013) (Fig. 1). Meanwhile, fructose is processed by hexokinase to yield fructose-6-phosphate that enters glycolysis. It seems most probable that the negative effect of acetate on culture growth rate comes from its direct or indirect intervention in the Leloir pathway. However, little is known about properties of the enzymes involved in this pathway in *K. marxianus*, so none of these speculations about the physiological and molecular consequences of acetate stress in lactose- and galactose-grown DSM 5422 cultures can be confirmed or dismissed yet.

Growth in acetate-enriched medium is energetically expensive

When comparing results from cultivations in fermenters and 96-well plates, we can see that biomass yield is affected in both situations, but more prominently in 96-well plates. It should be considered that aeration during cultivation in 96-well microtiter plate is poorly controlled and might be insufficient for growth of a particular microorganism (Hermann et al. 2003). This can explain differences in yeast biomass yields under acetate stress between 96-plate and fermenter cultivations. Examining Fig. 4c, d, we can see that cells are directing carbon toward ethanol and not biomass formation during the lag phase in acetate-treated fermenters.

Yeast culture growth inhibition by increased media acidity and subsequent decreased cytoplasmic pH is well characterized in *S. cerevisiae*. The functional effects of acid stress include decrease in specific growth rate and inhibition of mitochondria proliferation (Orij et al. 2012) and inhibition of substrate uptake (Ding et al. 2013). Cytoplasmic and vacuolar ATP-ases which export cytoplasmic protons on the expense of ATP are reported to be induced during acid stress, especially under pH 3.5 in the presence of succinic or acetic acid (Carmelo et al. 1997). We think that prolonged lag phase in the case of *K. marxianus* cultivation at pH 4 and 4.5 where cell growth is reduced and ethanol accumulates (Fig. 4a, c, d) can be explained by similar mechanisms as in *S. cerevisiae* (increased cytoplasmic ATP consumption by ATP-ases, that in turn hampers rapid growth and mitochondria function).

Changes in cell population structure during adaptation to acetate stress

Stratford et al. (2013) demonstrated that in the case of the notorious spoilage yeast *Zygosaccharomyces bailii*, resistance to high concentration of acetic acid and other weak-acid preservatives is limited to a small fraction of the initial cell population. Similarly, our results show that *K. marxianus* DSM 5422 populations are heterogeneous regarding tolerance to acetate. When acetate-supplemented medium is inoculated with DSM 5422 culture, an extended lag phase is typically observed (Figs. 4a, 5, 6). During this lag phase, the proportion of acetate-tolerant cells in the culture increases (Figs. 5, 6). Since the biomass concentration remains constant, it seems most probable that non-tolerant cells acquire resistance to elevated concentration of acetate instead of non-tolerant cells dying and tolerant ones growing. This is supported by data from S3. The different durations of the lag phase in Fig. 4a indicate that higher concentrations of undissociated acetate require longer time for adaptation. The key to adaptation of yeast cultures is changes within the cells rather than modification of the growth medium. This is illustrated by the fact that acetate-adapted DSM 5422 cultures can start growth without delay after inoculation in fresh medium containing acetate (Fig. 5). The same pattern of an extended adaptation period and immediate growth after reinoculation in identical medium has been described for other weak-acids in *S. cerevisiae* (Viegas et al. 1998; Cabral et al. 2001; Teixeira and Sa '-Correia 2002) and *Z. bailii* (Stratford et al. 2013). Findings of van Heerden et al. (2014) suggest that stochastic metabolic variation at cellular level could determine which cells eventually start growing on acetate and which cells do not.

Cells in cultures that were cultivated in a medium without added acetate also exhibited increased tolerance to acetate as the cultures were approaching diauxie (Fig. 6). It appears that slowly growing or stationary phase cells acquire tolerance to acetate as they become resistant to many other environmental stress factors, such as dessication for example (Klosinska et al. 2011). Schuller et al. (2004) showed that sorbate-activated genes and general stress response pathway genes share

common regulators in *S. cerevisiae*. If the same would be true for *K. marxianus* and other weak-acids, like acetic acid, this would at least partially explain why tolerance to acetate is acquired along with general stress tolerance in post-diauxic cultures.

Combined effects of acetate concentration and growth phase on respiration rate

Not only did post-diauxic phase cultures contain higher proportion of acetate-tolerant cells that could grow on acetate-enriched, low pH plates, they were also less sensitive to acetate when respiration rate was assayed (Fig. 7). This falls in line with the previously mentioned notion that tolerance to acetate is acquired along with a general stress resistance as the growth rate decreases and cells approach the stationary phase. The observed low respiration rate in post-diauxic cultures, when concentration of undissociated acetate was close to zero, can be explained with generally slower growth and metabolism as nutrients are becoming scarce after diauxie. The high sensitivity of exponential cultures can similarly be explained by the fact that rapidly growing cells are more sensitive toward different stress factors than stationary cells (Klosinska et al. 2011).

To summarize our findings, in this study, we showed that the toxicity of acetate is dependent on the carbon source and the culture growth phase. Increased tolerance to elevated concentrations of acetate can be acquired through adaptation during an extended lag phase. The mechanism of adaptation, however, remains unclear. We also identified sugar-proton symport and the Leloir pathway as the most probable acetate-inhibited processes that limit growth of *K. marxianus* DSM 5422. Further research is needed to discover the exact processes behind growth inhibition by acetate. In comparison with other yeasts (Thomas et al. 2002; Stratford et al. 2013), *K. marxianus* DSM 5422 is rather acetate-intolerant. Understanding these mechanisms of inhibition and adaptation should be useful for the design of detoxification strategies or new yeast strains that will combine the superior biotechnological capabilities of *K. marxianus* with high tolerance against acetic acid and similar inhibitors that are present in substrates or arise as byproducts of yeast metabolism.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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3.4 Acetic acid stress hampers Kluyveromyces marxianus growth on lactose

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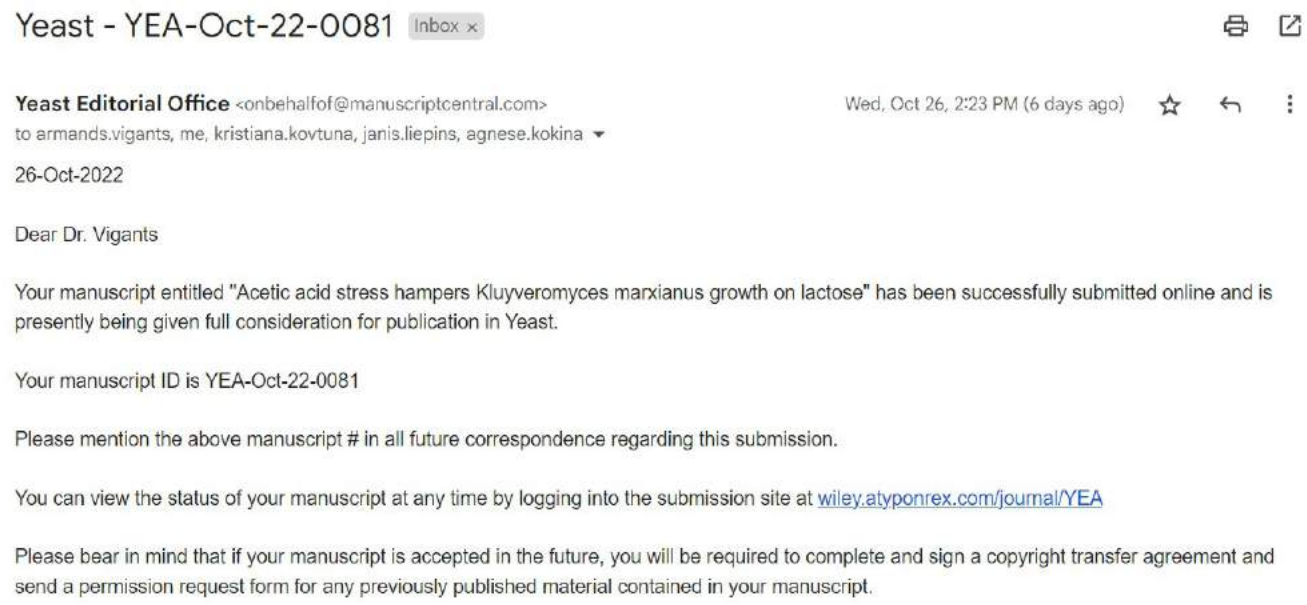

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Acetic acid stress hampers *Kluyveromyces marxianus* growth on lactose

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Abstract. *Kluyveromyces marxianus* is a non-conventional yeast that can efficiently metabolize lactose. Acetate is a weak organic acid and one of the fermentation by-products with pKa 4.76. Due to physiological and metabolic diversity within *K. marxianus* strains the yeast tolerance to acetate can differ. This study shows acetate tolerance in four different *K. marxianus* strains on three different carbon sources – glucose, lactose and equimolar mixture of glucose and galactose. The negative effect of 40 mM acetate on growth of four different *K. marxianus* strains was assessed over pH range of 4-6 at acetate and citrate buffers. The localization of β-galactosidase in *K.marxianus* strains DSM 5422, DSM 4906, DSM 5418 and NCYC 2791 was determined. The direct linear correlation between percentage of cytosolic β-galactosidase in strains and strength of inhibition of acetic acid on *K. marxianus* cell growth in lactose medium at 4.5 pH value was found.

Key Points:

- Acetic acid tolerance of *Kluyveromyces marxianus* is strain dependent.
- Decreasing pH value correlated to prolongation of lag-phase for all strains in the presence of acetate.
- There is correlation between β-galactosidase localization and acetic acid tolerance on lactose medium.

Keywords: *Kluyveromyces marxianus*, β-galactosidase, acetate stress, lactose, non-conventional yeast

Introduction

Bioconversion of renewable resources is a sustainable method for production of bioethanol, microbial biomass and secondary metabolites. Yeasts have been traditionally used in biotechnology. Although *S. cerevisiae* is the most common eukaryotic organism used in bioconversion, many non-conventional yeasts, such as *Kluyveromyces marxianus*, are capable of consuming a wider spectrum of substrates. The yeast *K. marxianus* is well known due to it's high growth rate, thermotolerance and ability to convert lactose. It possesses high activities of β-galactosidase, inulinase, invertase and xylose reductase (Fonseca et al., 2008, Lachance, 2011). *K. marxianus* is suitable for producing simple metabolites, such as ethyl acetate (Löser et al., 2015), ethanol, succinate, 2-phenylethanol, for producing biomass and proteins, as well for removal of lactose from food (Lane and Morrissey, 2010).

Yeasts from genus *Kluyveromyces* are isolated from a wide variety of habitats, resulting in high levels of metabolic diversity and intraspecific polymorphism (Lane and Morrissey, 2010; Fonseca et al., 2008). Genetic analysis of *K. marxianus* strains from different isolates and geographic origins shows a rich intraspecific polymorphism (Belloch et al., 1998, Fasoli et al., 2016 and Suzzi et al., 2000). Genetic analysis of *K. marxianus* isolates and population structure shows high degree of variation in ploidy and heterozygosity. The most popular *K. marxianus* trait is the ability to ferment lactose, but it is not universal in all *K. marxianus* strains. This phenotype is due to polymorphisms in the lactose transporter *LAC12* gene (Varela et al., 2017). Lactose transport protein is an integral membrane protein, member of the major facilitator superfamily and works via a proton-symport mechanism. There are three haplotypes of *K. marxianus* yeast: A and C are non-dairy types, and B is dairy haplotype. Non-dairy isolates can grow on lactose, but cannot ferment this sugar, in turn dairy haplotype can ferment above mentioned disaccharide (Ortiz-Merino et al., 2018).

Lactose is hydrolysed to glucose and galactose by enzyme β-galactosidase (EC 3.2.1.23). Optimal conditions for *K. marxianus* β-galactosidase activity are at pH 6.2 and temperature range from +45°C to +54°C (Goncalves and Castillo, 1982). There are also requirements on reaction cofactors, for *K. marxianus* β-galactosidase they are Mn^{2+}, Mg^{2+} and K^{+} cations, but for its sister species *K. lactis* it is Mn^{2+} and Na^{2+} ions (Jurado et al., 2002). In some microorganisms β-galactosidase enzyme is intracellular like in the *K. lactis* (Dickson et al., 1979), or both intracellular and extracellular like in *Neurospora crassa* (Bates et al., 1967). There are contradictory data about β-galactosidase localization in different *K. marxianus* strains. The analysis of twelve lactose-assimilating strains of the yeast species *Kluyveromyces marxianus* and its varieties showed the differences between strains regarding to extracellular lactose hydrolysis (Carvalho and Spencer, 1990; Bacci et al., 1996).

However, several limitations have been reported that hamper a wider industrial usage of Crabtree-negative *K. marxianus* yeast. For example, relatively low ethanol tolerance (Rosa and Sa-Correia, 1992), impossibility to grow under strictly anaerobic conditions (Van Dijken et al., 1993) and intolerance to weak acids has been reported (Martynova et al., 2016).

Acetate is a short-chain weak organic acid and one of the fermentation by-products with pKa 4.76. During fermentation medium pH value decreases and concentration of protonated acetate increases. Protonated form of acetate diffuses into the cell cytosol and hampers cell growth and metabolism (Piper et al., 2001; Martynova et al., 2016).

We have observed that acetic acid's effect on *K. marxianus* growth and metabolism is substrate dependent. Acetic acid inhibition effect was most severe in the cells growing in lactose media (Martynova et al., 2016). In our previous experiments we used *K. marxianus* DSM 5422 which can be classified as a good lactose consumer (Varela et al., 2017). Due to metabolic and physiology diversity among the yeast species, the mechanism of the cell stress to elevated acetic acid concentration can differ between the good and poor lactose assimilating strains. At low pH when protonated acetate diffuses in the cells, the maintenance of an intracellular pH is expensive in terms of ATP due to the acidification

of cytosol (Piper et al., 2001). A decreased proton gradient could slow down proton symport dependent lactose transport. Experimental data on β-galactosidase localization in different *K. marxianus* strains is scarce and somewhat contradictory. We performed research on strain acetate tolerance when growing on lactose at low pH and correlated these effects to β-galactosidase localization.

Material and methods

Organisms and cultivation conditions

Three yeast *Kluyveromyces marxianus* strains (DSM 5422, DSM 4906 and DSM 5418) were obtained from the Leibniz-Institute Collection of Microorganisms and Cell Cultures (Germany) and yeast *Kluyveromyces marxianus* strain NCYC 2791 was obtained from the National Collection of Yeast Cultures (UK). All *K. marxianus* strains were maintained on YPD agar. YPD contained (per litre of distilled water) 10 g of yeast extract (Biolife, Italy), 20 g of peptone (Biolife, Italy), 20 g of glucose (Sigma) , and 20 g of agar (Biolife, Italy). For acetate stress experiments, acetate concentration of 40 mM was chosen from Martynova et al., 2016.

For 96-well plate and flasks experiments, preculture and main culture were grown on a liquid full semisynthetic medium: carbon source (glucose, galactose, and lactose (Sigma)) 20.0 g/L, yeast extract 5.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.7 g/L, KH_2PO_4 1.0 g/L, K_2HPO_4 0.1 g/L, $(NH_4)_2SO_4$ 5.0 g/L. Cultures in flasks were cultivated at 30°C with an agitation speed of 180 rpm.

For 96-well plate experiments, fresh overnight culture inoculated from a single colony was used. Cells from preculture were washed with sterile distilled water and reinoculated in a cultivation medium with initial OD_{600} 0.15.

Determination of acetate effect on growth rate

We used micro cultivation in a 96-well plate for *K. marxianus* strain growth rate measurements at different pH and substrates with four biological replicates. Medium pH was maintained by acetic or citric buffers (sodium acetate, acetic acid, sodium citrate, and citric buffers with final acetate or citrate molarity 40 mM). Plates were incubated in multimode reader (Tecan infinite 200 M Pro). The following cultivation protocol was set up: incubation temperature 30°C, shaking with amplitude 3.5 mm, and optical density (600 nm) readings were taken every 10 min followed by a minute-long pause, then cycle repeats. Specific growth rate and doubling time were calculated from the microcultivation data as described by Toussaint et al. (2006).

Determination of cell protein

Bredford protein assay was used for protein concentration determination. The results were calculated by comparison with bovine serum albumin (Sigma) as standard.

Cell disruption

Cell disruption was performed by 2-times stirring (1min 30 times per second) and cooling cycles with 0.3 ml ⌀ 0.45 glass beads (Sigma) in phosphate buffer with protease inhibitors 20 µl/ml (Sigma). The cell extract was prepared by centrifugation (30 min 13000 rpm at 4°C).

Yeast cells permeabilization

Cells were washed twice with 0.1 M potassium phosphate buffer (pH 7.0), centrifuged at 8000 rpm +4°C 10 min. 20 µg/mL protease inhibitor (Complete Protease inhibitor cocktail, Roche CO-RO) was added to cell suspension (OD_{600} = 0.5). Permeabilization was carried out by incubation with 10 µL/mL 2 mM digitonin (Sigma). Method modified from Bacci et al., 1996. Uncoupler CCCP (carbonyl cyanide-m-chlorophenylhydrazone, Sigma) 10 µg/mL 200 µM was used to disrupt proton gradient.

β-galactosidase activity measurement

β-galactosidase activity was measured by modified Domingues et al. 1997 method. For β-galactosidase activity measurements in cell extract and permeabilized cells 50 µL/mL and 10 mM X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used. The colourful 5,5'-dibromo-4,4'-dichloro-indigo – 2 produced was quantified by absorbance at 650 nm. To ensure optimal conditions for β-galactosidase activity 1 mM $MgCl_2$ and 1 mM $MnCl_2$ were added.

Results

β-galactosidase localization in different *K. marxianus* strains

Literature shows differences in growth parameters when various *K. marxianus* strains were cultivated in lactose medium. Carvalho-Silva and Spencer-Martins (1990) have demonstrated that lactose hydrolysis can occur both in the periplasm and cytosol. We wanted to find out where lactose hydrolysis occurs in four strains that were used in this research. To induce β-galactosidase, we cultivated all *K. marxianus* strains (DSM 5422, DSM 5418, DSM 4906 and NCYC 2791) in a full semisynthetic medium containing 50 g/L lactose. Then we incubated cells with CCCP and digitonin. CCCP causes an uncoupling of the proton gradient across the cell membrane (Park et al., 1997). It is known that lactose transport to the cell occurs via proton-symporter (Varela et al., 2017). In the presence of CCCP lactose hydrolysis can occur just in periplasmic space (since lactose cannot be transported inside the cell). Digitonin associates with membrane cholesterol molecules and forms insoluble compounds which result in membrane permeabilization (Sperry and Webb, 1950). Total β-galactosidase (both cytosolic and periplasmic) activity can be measured when cells are treated with digitonin or disintegrated. For each strain we measured the periplasmic or total β-galactosidase activity using CCCP and digitonin treated cells. We calculated periplasmic or cytosolic β-galactosidase activity as a percentage from total β-galactosidase activity. There was not a significant difference in total β-galactosidase activity for all four strains (Fig.1A). Lactose hydrolysis occurs both in periplasm and cytosol, but distribution between periplasmic and cytosolic activity varies between different strains. Highest percentage of cytosolic β-galactosidase activity (from total activity) was found in the NCYC 2791 strain where 80% of β-galactosidase activity was located in cytosol (Fig.1B)

The negative effect of protonated acetic acid on *K. marxianus* growth on lactose is strain dependent

Our previous studies have shown that the most pronounced negative effect of 40 mM acetic acid at pH 4.5 was when *K. marxianus* DSM 5422 strain was cultivated in lactose containing medium (Martynova et al., 2016). Since lactose assimilation ability within *K. marxianus* species is strain dependent we tested the effect of 40 mM acetic acid on growth parameters of four different strains cultivated on semi synthetic lactose medium over a range of pH values from 4.5 to 6.0. Specific growth rates are shown in Figure 2.

By observing the results from cultivation of all four *K. marxianus* strains in the media where pH was maintained by citrate buffer one can see that pH does not influence growth rate, however if the same pH is reached by acetate buffer, reduction of growth rate towards more acidic pH is easily seen. The difference between growth rates if the pH was maintained by

different buffers was the most pronounced in strain NCYC 2791 ($\Delta\mu = 0.32\ h^{-1}$) and the least in DSM 5418 ($\Delta\mu=0.09h^{-1}$).
We observed that the differences in the inhibitory effect of acetic acid for tested strains depends on location of β-galactosidase activity. A direct linear correlation between percentage of cytosolic β-galactosidase in different strains and strength of inhibition of acetic acid on *K. marxianus* cell growth in lactose medium at 4.5 pH value was found. Correlation between cytosolic β-galactosidase % from total β-galactosidase activity and growth rate difference (Δμ) of strains between medium buffered with citrate and acetate buffers at pH value 4.5 is shown on Figure 3.

Acetic acid stress effect on *K. marxianus* growth on different carbon sources

To further explore phenomena of acetic acid stress effect on *K. marxianus* growth, we prepared three types of carbon substrates - lactose, glucose and mixture of glucose and galactose. The latter mimics hydrolyzed lactose. By this experiment we wanted to test if acetic acid effect relates to hydrolysis of *K. marxianus* specifically or it also stops metabolism of glucose and/or galactose (therefore has general negative effect on the cells).

We prepared 1% glucose + 1% galactose, 2% lactose or glucose containing semisynthetic medium with 40 mM acetic acid concentrations at pH 4.5. Cultivations were carried out in the 96-well microplate, cultivated and measured by TECAN multimode reader. Typical growth curves of four different *K. marxianus* strains depending on the source of carbohydrate at 40 mM acetate concentrations at pH 4.4 are depicted in Fig4. Glucose and lactose YPD containing mediums also were used as positive controls.

We compared the growth of *K. marxianus* by considering two parameters - growth rate - μ and maximal OD. All strains were able to grow on glucose and galactose + glucose equimolar mixture in the semisynthetic media in presence of 40 mM acetate at pH 4.5. The ability to grow on lactose differs in the presence of 40 mM acetic acid. In the case of DSM 5422, DSM 5418, DSM 4906, the difference in specific growth rates is not as striking as in the case of NCYC 2791 (Table 1). In the case of the first three strains, despite differences in specific growth rate and lag-phase length, they achieve the same optical culture density when grown on lactose (OD_{600} = 0.6) in the presence of 40 mM acetic acid. A significantly different growth pattern we see in the case of NCYC 2791, where we see a long lag phase (40 hours) and a slow specific growth rate (μ = 0.13). The OD_{600} for strain NCYC 2791 reached only 0.18 at 45 hours. (Fig. 4)

If *K. marxianus* DSM 5422 strain is cultivated in a medium with glucose as a carbohydrate source, the μ in the medium with and without acetate addition does not differ, but biomass maximal optical density OD_{600} is reduced by 0.4 units. When using lactose in a 40 mM acetate medium, the specific growth fell 1.7 times and maximum optical density of the culture is reduced by half compared to cultivation without acetic acid.

During *K. marxianus* DSM 5418 growing in glucose-containing medium, the difference in specific growth rate (μ) in the presence of acetate is minimal, but a decrease in maximal biomass density (OD_{600}) is noted. The specific growth rate decreases 1.6 times when lactose is used as a carbon source in acetate-containing medium compared to medium without acetate.

Compared with *K. marxianus* DSM 5422 and DSM 5418, in the case of DSM 4906 and NCYC 2791 there is a greater drop in μ and maximal biomass density (OD_{600}) between cultivations with and without the addition of acetate in glucose-containing medium. In the case of lactose medium, the DSM 4906 has 1.7 times decrease of specific growth rate and the maximum

culture OD fell by a factor of two. In turn, in the NCYC 2791 strain μ dropped by a factor of 3.9 and the onset of the exponential phase did not begin until after 40 hours.

An interesting growth pattern is observed when a mixture of glucose and galactose is used as a carbohydrate source in a 40 mM acetate medium. In all four strains the μ in this case is higher than with lactose and even slightly higher than with glucose, except in the case of NCYC 2791 where in the mixture of glucose + galactose specific growth rate by 1.6-times higher than in glucose containing medium in the acetic acid presence. However, when comparing the maximum density of the obtained biomass, the decrease in this parameter compared to glucose is more perceptible than with lactose (Table 1). Compared to other growth curves, using lactose and glucose as a carbohydrate source, a small increase in biomass is observed after an exponential growth phase in the case of glucose + galactose.

When all four strains were cultivated in medium with reduced pH and in the presence of 40 mM acetate, a reduction in specific growth rate and a decrease in maximal OD_{600} obtained during the growth was observed compared to cultivation in medium without acetate. Despite this general trend among the strains used, differences in the degree of negative effect of acetate in combination with reduced pH were also observed depending on the substrate and strain used.

Disccusion

Literature shows that β-galactosidase localization and the capability of lactose assimilation in *K. marxianus* is strain dependent (Carvalho and Spencer, 1990). We compared our data with previously published data. We found that in four of tested strains lactose hydrolysis occurs both in periplasm and cytosol, but distribution between cytosolic and periplasmic localization is strain dependent. Our results show that the highest percentage of cytosolic β-galactosidase is in NCYC 2791 strain. Cytosolic location of β-galactosidase in NCYC 2791 was also observed by Bacci et al., 1996. However, in the β-galactosidase localization experiment Carvalho-Silva and Spencer-Martins (1990) observed extracellular lactose hydrolysis in NCYC 2791 strain and possible periplasmic location in DSM 5422. Unfortunately, it is difficult to compare and discuss our results with Carvalho and Spencer due to the lack of description of the experiment. Furthermore, Carvalho and Spencer did not add magnesium and manganese salts to the reaction mixture, although Mn^{2+} and Mg^{2+} ions are required in maintenance of β-galactosidase enzyme active site conformation (Hermanson, 2013).

We have previously shown negative effects of acetic acid on *K. marxianus* DSM 5422 strain growth during lactose fermentation at low pH (Martynova et al., 2016). Here we had four strains with varying effects of acetic acid on growth that correlated with localization of β-galactosidase. For all strains total β-galactosidase activity was similar. We show that low pH itself is not causing growth inhibition and the effect is acetate specific.

We found that strain ability to grow in lactose in the presence of acetic acid has correlation to the localization of β-galactosidase. The specific growth rate of the strain decreases as the amount of cytosolic β-galactosidase increases. Protonated acetic acid disrupts the proton gradient of the cell membrane and thus uptake of the lactose. If β-galactosidase is located in the cytoplasm and the lactose transport via sugar-proton symport is interrupted by weak acid action, lactose as a carbon source becomes unavailable. Whereas if lactose hydrolysis occurs extracellularly glucose and galactose enter the cell using hexose transporters. It is clearly shown in literature that lactose gets to the cell using H+ symport transporters (Varela et al.,

2017). Glucose and galactose are taken up by the cells using low and high affinity transporters (Varela et al., 2019). Unlike glucose and galactose, lactose can only be transported into the cell through the proton symport transport system. In turn, the above-mentioned monosaccharides can be transported into the *K. marxianus* cell by alternative transporters that do not use the proton symporter. The growth of this strain in lactose medium seems to be unaffected by the presence of protonated acetic acid. Obviously, the lactose hydrolysis occurs outside the cell and then glucose and galactose are taken up and metabolized.

The cultivation of the four strains used in the study in medium with 40 mM acetate at pH 4.5 and without acetate addition show that the negative effect of acetate is shown as a decrease of the specific growth rate and a decrease of the maximal optical density of the cultures. This can be explained by the cells' need to adapt to adverse environmental conditions, which is an energy-consuming process. However, the degree of negative effect of acetate in combination with a reduced medium pH on the above-mentioned growth parameters depends both on the strain used and on the carbohydrate source in the growth medium. Keeping in mind *K. marxianus* yeast's high metabolic diversity and a substantial degree of intraspecific polymorphism (Lane et al., 2011) acetic acid tolerance could also be strain dependent. The greatest decrease in specific growth rate and maximal optical density of the culture was observed when lactose was used as a carbohydrate source. This could be due to the inhibition of the lactose transport system into the cell, as the transport of this saccharide is a proton symporter. The greatest decrease in specific growth rate was observed in strain NCYC 2791. Although this strain is a poor consumer of lactose compared to the other three, a comparison of the specific growth rate of a particular strain when cultured in the presence of acetate versus when cultured without the addition of acetate shows the greatest drop (more than three-fold) in the specific growth rate. This is the largest drop in this parameter among all the strains tested. According to the results obtained in this publication we explain this by the fact that in this strain most of the β-galactosidase is located in the cytosol (80%). When these strains are cultivated in a medium containing acetate in combination with pH 4.5 and with a mixture of glucose and galactose as a carbohydrate source, such growth parameter as specific growth rate, remains close to that of identical growth conditions but with glucose. There is also a decrease in the maximal optical density of the culture. This can be explained by the fact that when the glucose + galactose mixture is used, due to catabolite repression, glucose enters the cell first and is metabolized (Beniwal et al., 2017; Fonseca et al, 2013). Therefore, firstly we observed a fast growth phase after which a stationary phase does not follow, and a slow biomass growth is observed. It is shown in the literature, using different strains of *K. marxianus*, that this slow growth is presumably because after glucose assimilation, induction is required for the cells to start utilizing galactose.

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Data Availability Statements: All data generated or analysed during this study are included in this published article

Competing interests:
Jekaterina Martynova declares that she has no conflict of interest.
Kristiana Kovtuna declares that she has no conflict of interest.
Janis Liepins declares that he has no conflict of interest.
Agnese Kokina declares that she has no conflict of interest.
Armands Vigants declares that he has no conflict of interest.

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Ethical statement: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure captions.

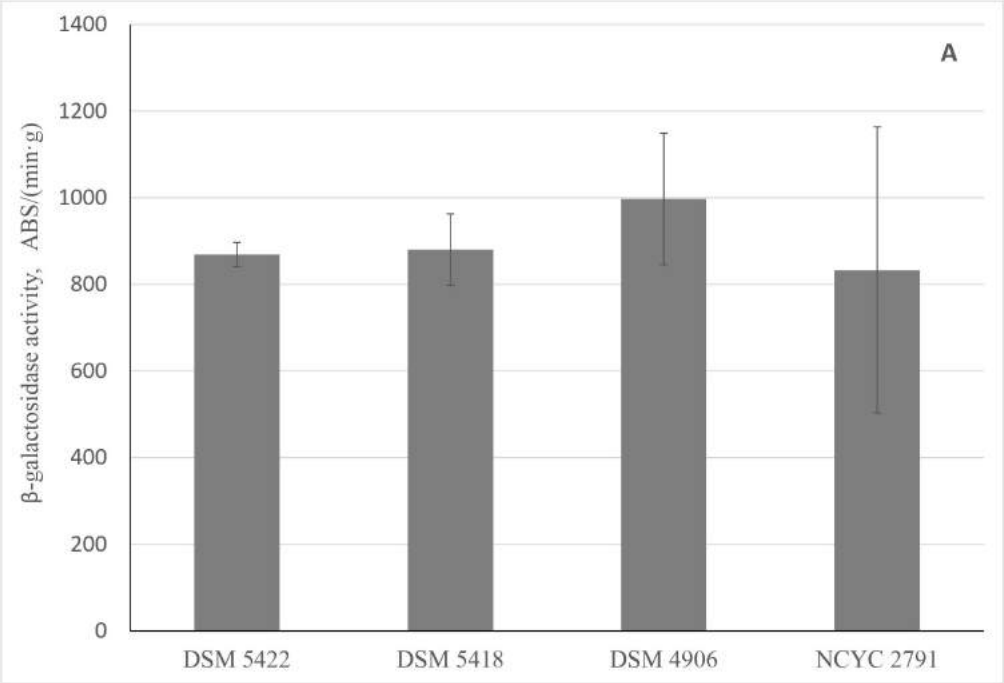
Fig.1. **(A)** Total specific β-galactosidase activity of *Kluyveromyces marxianus* strains DSM 5422, DSM 5418, DSM 4906, NCYC 2791 grown in batch process on semisynthetic 2% lactose medium (30 °C, 24 h). **(B)** Localization of lactose hydrolysis within *Kluyveromyces marxianus* strains DSM 5422, DSM 5418, DSM 4906, NCYC 2791. Results are average of three numbers of replicates; β-galactosidase activity was determined quantifying hydrolysis of lactose analogue chromogenic substrate X-Gal and absorbance measurement at 650 nm.

Fig 2. The specific growth rates of *K. marxianus* **(A)** DSM 5422; **(B)** – DSM 5418; **(C)** – DSM 4906; **(D)** – NCYC 2791 strains during cultivation in lactose (20g/l) full YPD medium with acetic acid/sodium acetate or citric acid/sodium citrate buffering at different pH, t=30°C

Fig 3. Correlation between cytosolic β-galactosidase % from total activity and Δμ (odds between strain specific growth rate in citric buffer and acetic buffer) at pH value 4.5.

Fig 4. *K. marxianus* strains growth on different carbon sources (glucose, glucose+galactose and lactose) in acetic acid/sodium acetate buffered medium at 4.5 pH and on lactose and glucose YPD medium. Initial carbohydrate concentration 20 g/l, t=30°C. **(A)** DSM 5422, **(B)** DSM 5418, **(C)** DSM 4906, **(D**) NCYC 2791

Fig.1 (A)


A
β-galactosidase activity, ABS/(min·g)
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1000
800
600
400
200
0
DSM 5422
DSM 5418
DSM 4906
NCYC 2791

Fig. 1 (B)

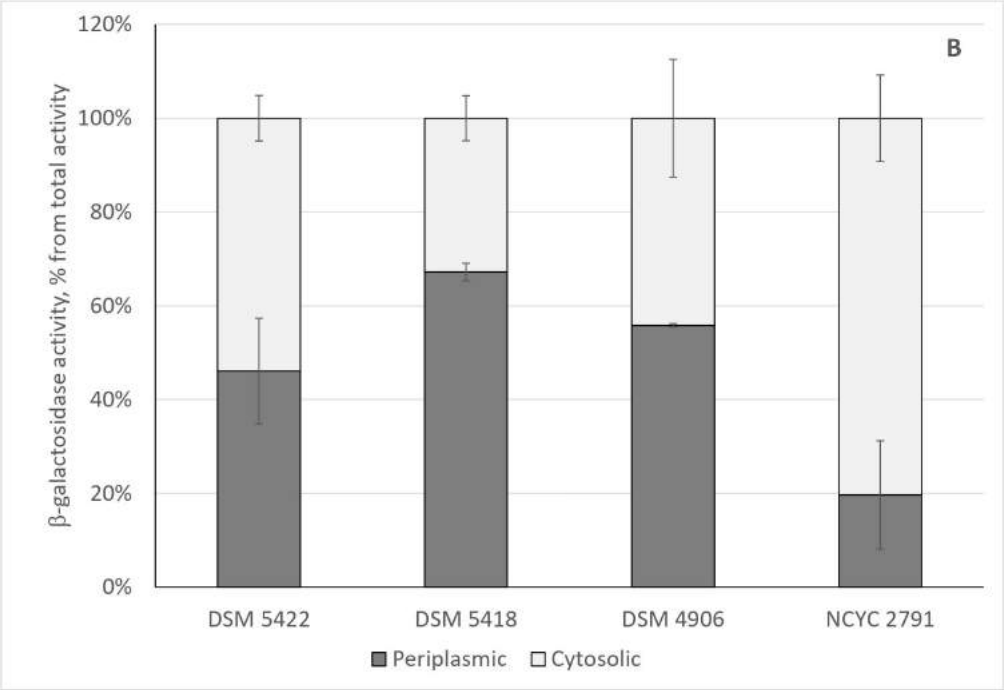
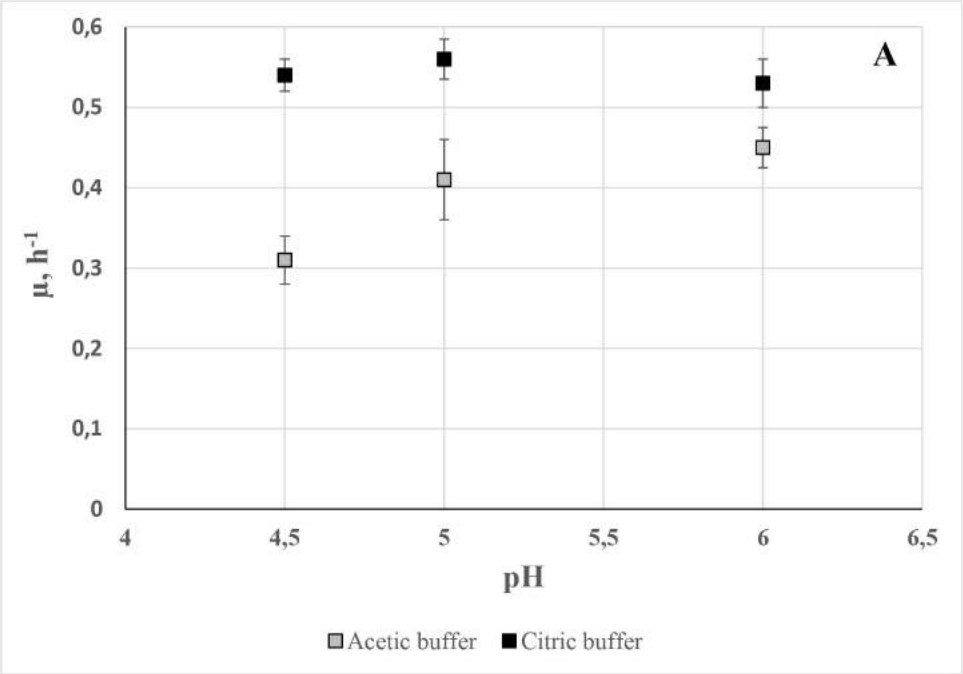

B
120%
100%
80%
60%
40%
20%
0%
β-galactosidase activity, % from total activity
DSM 5422
DSM 5418
DSM 4906
NCYC 2791
Periplasmic
Cytosolic

Fig. 2 (A)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0,4
0,3
0,2
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μ, h-1
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4,5
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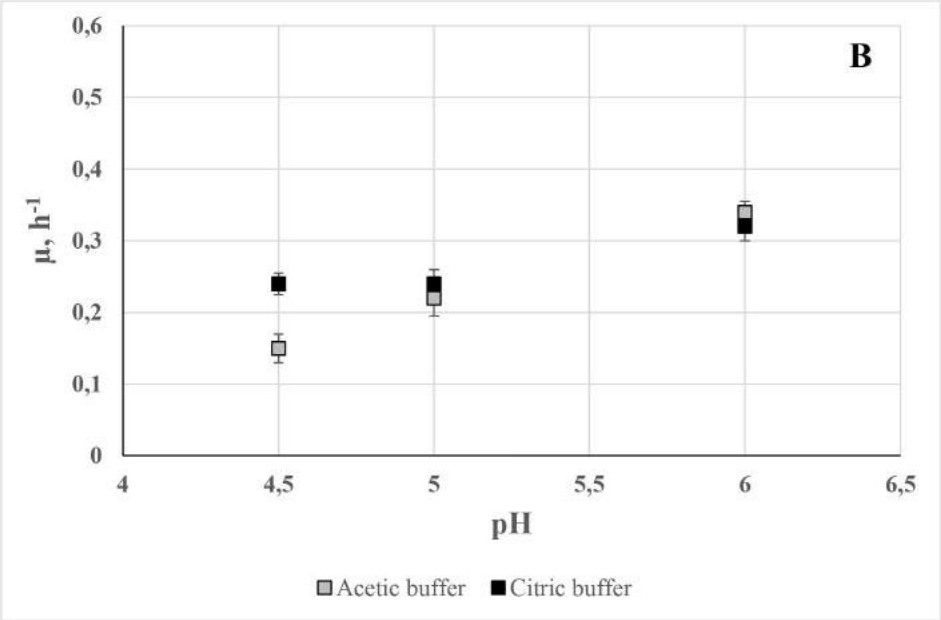
5,5
6
6,5
pH
Acetic buffer
Citric buffer

Fig.2 (B)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0,4
0,3
0,2
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μ, h-1
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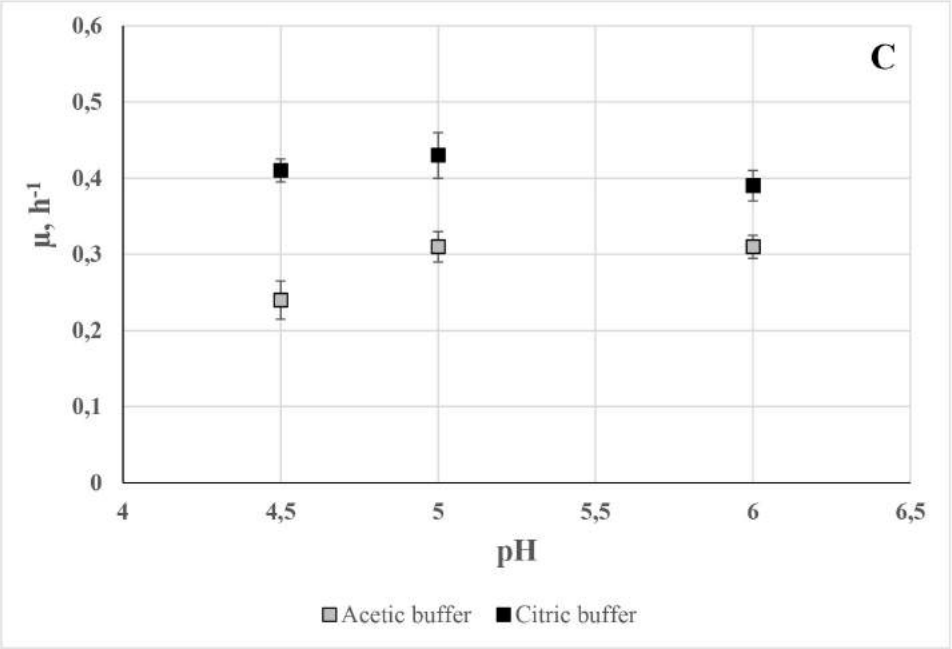
6
6,5
pH
Acetic buffer
Citric buffer

Fig.2 (C)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μ, h-1
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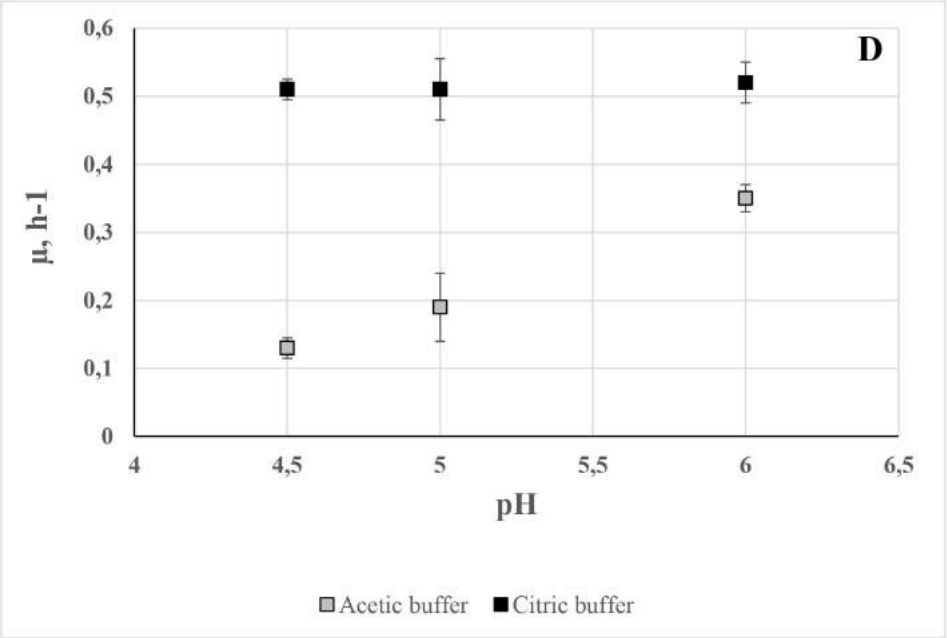
6,5
pH
Acetic buffer
Citric buffer

Fig.2 (D)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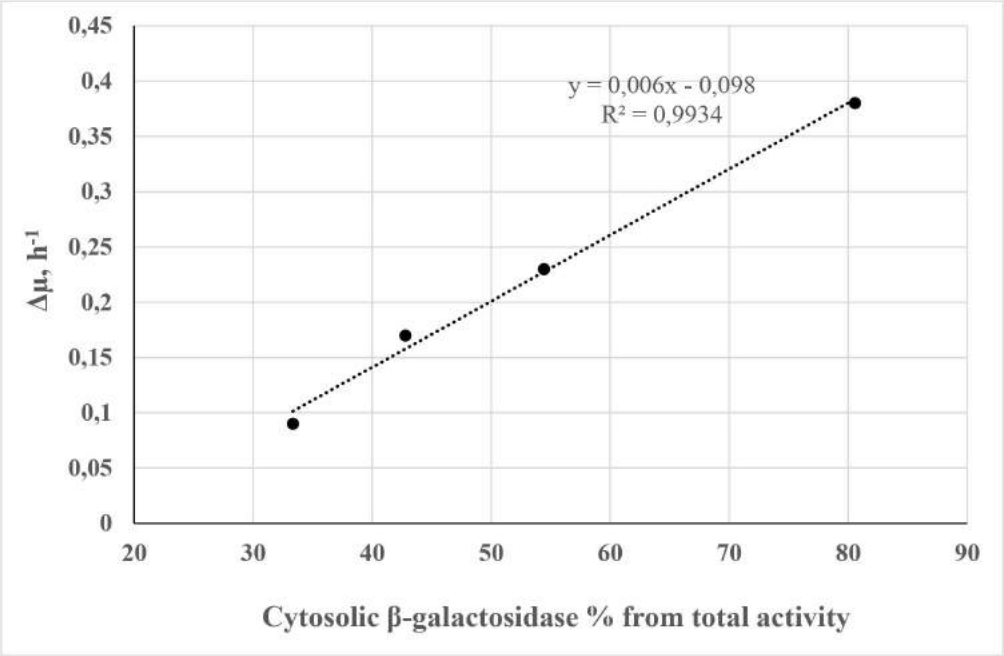
6,5
pH
Acetic buffer
Citric buffer

Fig.3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0,15
0,1
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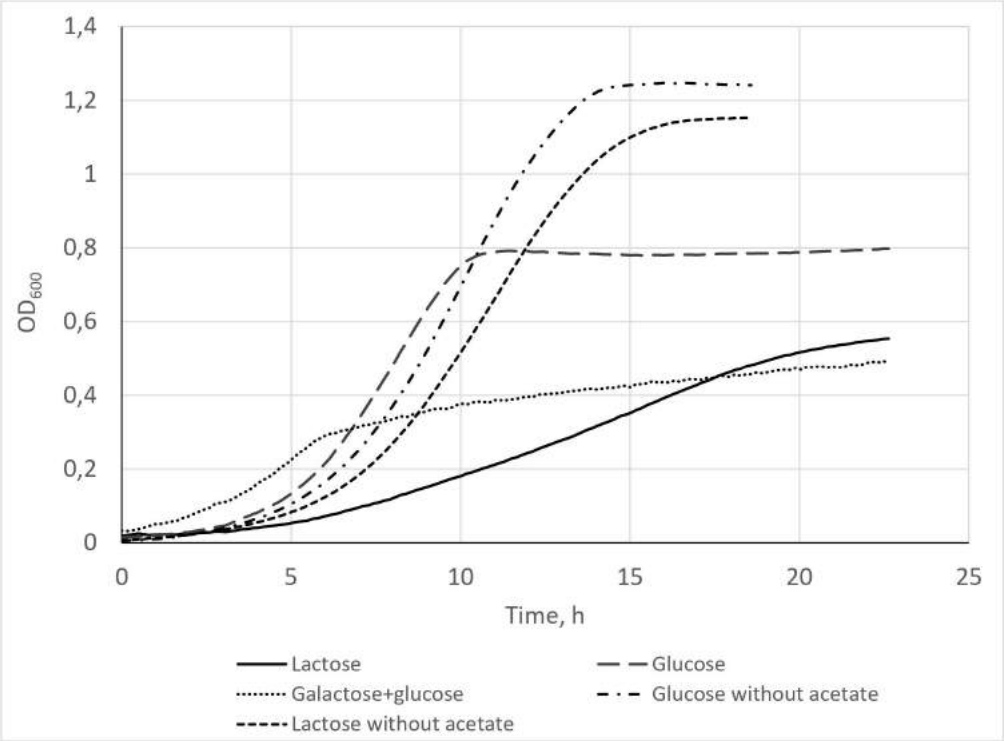
30
40
50
60
70
80
90
Cytosolic β-galactosidase % from total activity

Fig.4 (A)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OD600
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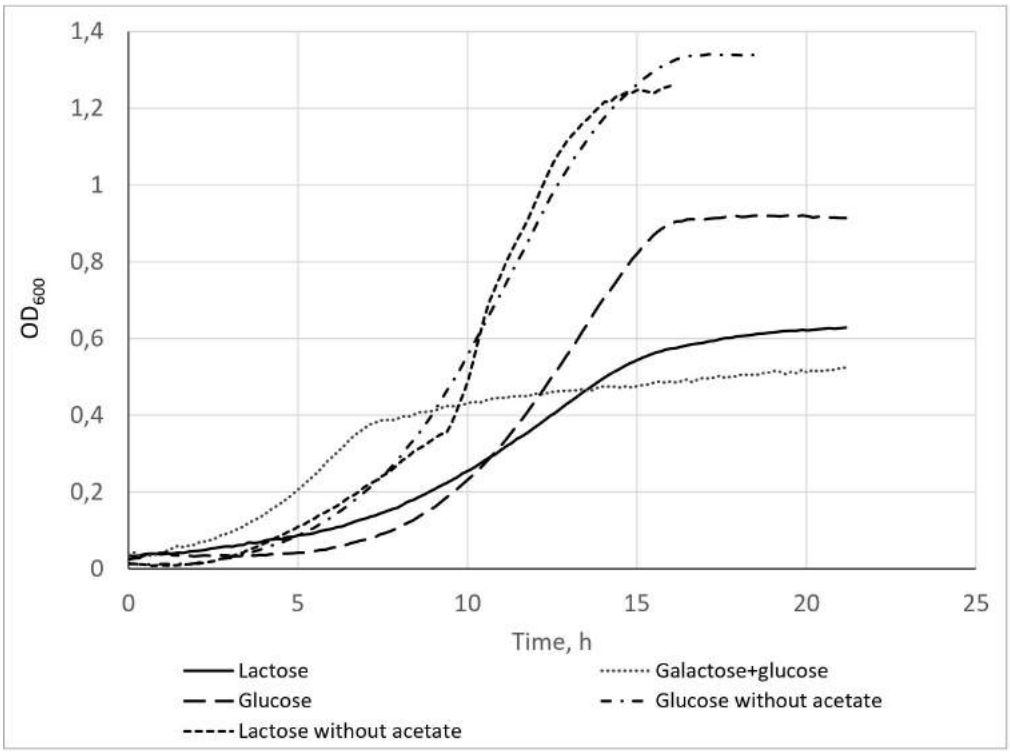
20
25
Time, h
Lactose
Glucose
Galactose+glucose
Glucose without acetate
Lactose without acetate

Fig.4 (B)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0,2
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OD600
0
5
10
15
20
25
Time, h
Lactose
Glucose
Lactose without acetate
Galactose+glucose
Glucose without acetate

Fig.4 (C)

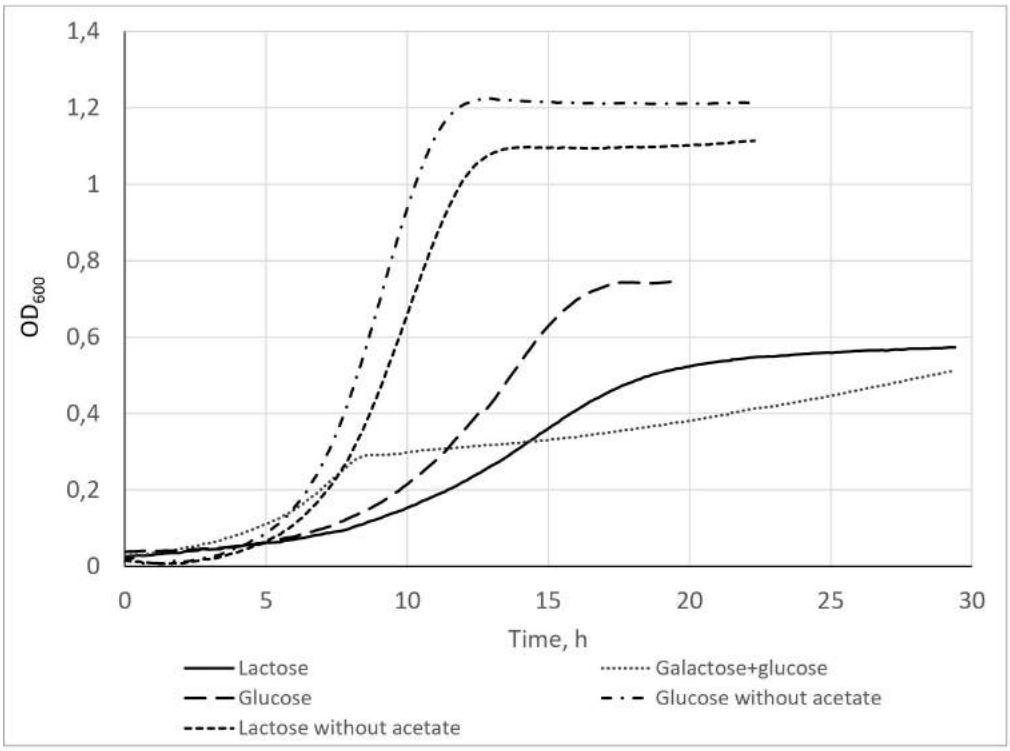
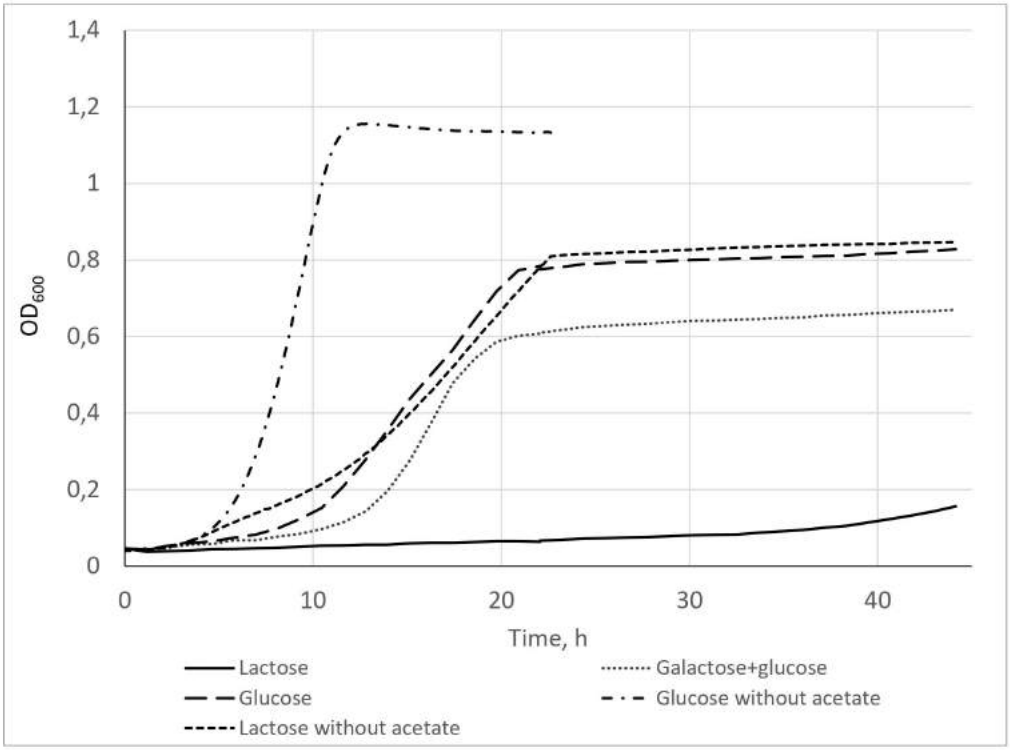

1,4
1,2
1
0,8
0,6
0,4
0,2
0
OD600
0
5
10
15
20
25
30
Time, h
Lactose
Galactose+glucose
Glucose
Glucose without acetate
Lactose without acetate

Fig.4 (D)


1,4
1,2
1
0,8
0,6
0,4
0,2
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OD600
0
10
20
30
40
Time, h
Lactose
Galactose+glucose
Glucose
Glucose without acetate
Lactose without acetate

Table 1. Specific growth rate of four *K.marxianus* strains in YPD medium (pH 4.5, t=30°C) containing 20 g/l glucose, lactose or equimolar mix of glucose and galactose with and without addition of 40 mM acetate.

	Specific growth rate (μ)				
	With 40 mM acetate			Without acetate	
K.marxianus strain	Glucose	Lactose	Glucose + galactose	Glucose	Lactose
DSM 5422	0.44±0,02	0.31±0,03	0.38±0,025	0.48±0,03	0.54±0,02
DSM 5418	0.36±0,015	0.15±0,02	0.38±0,02	0.41±0,03	0.24±0,015
DSM 4906	0.25±0,01	0.24±0.025	0.3±0,04	0.57±0,01	0.41±0,015
NCYC 2791	0.18±0,01	0.13±0,01	0.28±0,04	0.46±0,04	0.51±0,015

4 DISCUSSION

Non-conventional food-grade yeast *K. marxianus* is an excellent candidate for use in industrial biotechnology for biomass, ethanol, enzymes etc producing from cheap raw materials like lactose and inulin containing substrates. But there are several limitations that hamper a wider industrial usage of this Crabtree-negative yeast.

4.1 Application of modelling techniques to increase the biotechnological potential of K. marxianus

Considering the wide range of sugars that the yeast *K. marxianus* can metabolize and its other features, it is a promising microorganism for use in industrial biotechnology. Its ethanologous nature and the presence of enzymes such as β-galactosidase and inulinase make *K. marxianus* an attractive candidate for the production of ethanol and yeast biomass using renewable sources containing such carbohydrate as lactose and inulin (Morrissey et al., 2015; Fonseca et al., 2008; Lane et al., 2011). The development and application of the kinetic model can help to develop and optimise biotechnological processes for obtaining ethanol and other valuable products from renewable resources. Keeping in mind the phenotypic diversity in growth parameters between different *K. marxianus* strains (Lane et al., 2011), depending on the physico-chemical conditions of cultivation and the composition of the nutrient media, problems may arise in applying the same kinetic model to different strains. There are several kinetic models have been developed (Zafar et al., 2005; Ariyanti et al., 2013; Parrondo et al., 2009). These kinetic models have been developed to describe the ethanol production of various *K. marxianus* strains from lactose-containing substrates, including the strain DSM 5422. The use of these models is limited by differences in structure and by disregard the possibility of inhibition effects by product and/or substrate.

The unstructured kinetic model obtained in our study was developed on the basis of parameters derived from a series of batch fermentations (Martynova et al., 2017). Obtained parameters and statistical indexes demonstrates that different substrates for the ethanol fermentation by *K. marxianus* do not affect the structure of the model, which follows from identical parameter sets being eligible for the system of relevant ordinary different equations. However, the numerical values of the parameters are noticeably affected. Thus, the markedly reduced *Ks* (half-saturation constant) value corresponding to whey permeate (WP) fermentation over the entire substrate concentration range indicates the high affinity of *K. marxianus* to this substrate. In turn, the low substrate inhibition constant ($K_{I,S}$) compared to the other substrates (lactose and inulin) indicates a marked non-competitive substrate

inhibition, which can be explained by the complex composition of whey permeate with a wide range of osmolytes (Dale et al., 1994). This feature of WP may also cause the apparent increase in the value of the maintenance coefficient (m_s), which reflects the effect of metabolic costs on osmotic regulation and as a consequence, a relatively reduced yield coefficient for biomass on substrate. It is also important to note that within the scope of these studies (Martynova et al., 2017), despite significant differences between the parameter values, the maximum growth rate remains high and almost unchanged for all three substrates. This agrees well with the notion of *K. marxianus* as the fastest-growing eukaryote on Earth (Rocha et al., 2010). Furthermore, in all cases, ethanol formation can be described according to Luedeking-Piret kinetics (Luedeking and Piret et al., 2000) as an almost exclusively growth-linked process, where the specific rate of product formation is proportional to the specific growth rate of *K. marxianus* yeast. This is indicated by significantly higher values of the growth related parameters (α). However, WP fermentation can also result in partially mixed growth-associated ethanol formation, as indicated by the slightly higher value of non-growth associated term (β). Highly significant R-squared values show that the model adequately simulates actual changes in biomass, substrate and product concentration during ethanol fermentation of whey permeate, lactose or inulin by *K. marxianus* yeast, as only a relatively small proportion (0.79-3.66 %) of the total variance remains unexplained. This is also confirmed by the relatively low RMSE (Root-Mean-Square-Error) value of the model.

Based on this model, the general pattern is that with increasing substrate concentration, there is a proportional increase in biomass growth and ethanol concentration. However, there is a notable difference in biomass and ethanol concentrations which can be obtained using different carbohydrate sources with identical initial concentrations in the cultivation media. It is worth noting that in media containing pure lactose and inulin *K. marxianus* DSM5422 produces a higher concentration of biomass compared to WP-containing media. This is also reflected in the biomass productivity (*Qx*) and yield (*Yx/s*) per unit of the substrate consumed. This can be explained by differences in the composition of the nutrient media, e.g. the presence of osmolytes in the whey permeate and possible nitrogen deficiencies, as opposed to substrates with pure lactose and inulin (Dale et al., 1994; Moreira et al., 2015). In turn, the ethanol concentration with WP is higher than with pure lactose or inulin. In the case of WP, the volumetric ethanol productivity and the specific rate of product formation are not significantly different from those of the other two substrates. Particularly noteworthy is that medium containing whey permeate was the most suitable for achieving the

highest ethanol yield per unit of substrate consumption ($Y_{p/s}$ = 0.460 g/l), that is 90.2% of the theoretical yield, significantly higher than 79.2% and 64.5% for pure lactose and inulin respectively. The data obtained from this model indicate the prospects for the technological use of *K. marxianus* DSM 5422 to produce bioethanol from a lactose-containing renewable resource such as dairy permeate.

Another approach to enhance the biotechnological potential of *K. marxianus* is to use a biomass-associated stoichiometric model of central metabolism. Keeping in mind the wide range of metabolites and products that *K. marxianus* is able to produce from various substrates, the above-mentioned approach can be used not only to optimise biotechnological processes but also can help to significantly accelerate the analysis of the metabolic effects and limitations of the microorganism's metabolism, including the ability to predict the phenotype of recombinant strains (Kalnenieks et al, 2014; Kerkhoven et al., 2014). Successful use of medium-scale stoichiometric models of the central metabolism of microorganisms such as *E. coli* (Trinh and Srienc, 2009; Trinh et al., 2008; Unrean et al., 2010), *Z. mobilis* (Pentjuss et al., 2013) and *P. pastoris* (Tortajada et al., 2010) are known. Recent active attempts at genetic engineering of *K. marxianus* also point to the relevance of this model (Hong et al., 2015; Kim et al., 2015; Kim et al., 2014; Zhang et al., 2016; Zhang et al., 2015a). Such main carbon fluxes as substrate uptake, CO_2, ethanol, glycerol, acetate and biomass production were used for our model validation (Pentjuss et al., 2017). The sufficiency of these fluxes for validation of the model is confirmed by the fact that in the case of batch cultivations of *K. marxianus* under oxygen-limited conditions these fluxes can account for up to 100% of the total carbon (Sansonetti et al., 2011). Also, a similar set of fluxes was successfully applied to validate the medium-scale carbon metabolism of *P. pastoris* (Tortajada et al., 2010). In the our model validation process, using lactose as a substrate, it was assumed that this substrate is transported into the cell with subsequent hydrolysis by β-galactosidase. The model was able to achieve a steady-state solution for all experimentally measured flux distributions.

By controlling the oxygen availability in the nutrient medium it is possible to orient the fermentation process either towards biomass formation or ethanol synthesis. In both cases, however, the formation of ethanol as well as glycerol and acetate as main by-products is observed, although with a difference in the quantities obtained. A similar trend is noted in a scientific publication by Sansonetti et al., 2011 where the fermentation of lactose under self-aerobic conditions achieved an ethanol yield of 3.33 per unit of lactose, but the growth rate

was low and glycerol was the main by-product. On the other hand, fermentation of *K. marxianus* under aerobic conditions shows a rapid increase in biomass with a relatively low ethanol flux (Longhi et al., 2004).

In the case of our model validation using glucose, sucrose and inulin as substrates for fermentation, it was assumed that hydrolysis of the latter two occurs outside the cellular space and the resulting monosaccharides enter the cell as fructose and glucose. A large amount of free fructose after inulin hydrolysis has been shown also by other authors (Yuan et al., 2013). All fermentation results using glucose, fructose and inulin were replicated by the model.

When validating our model using xylose, it was taken into account that in *K. marxianus* the enzyme xylose reductase is NADPH dependent (Zhang et al., 2011). From the available literature data three scientific papers were selected and in all three a slow growth of biomass was observed (Delgenes et al., 1986; Margaritis and Bajpai, 1982; Signori et al., 2014). Interestingly, the value of experimental μ correlates with oxygen supply; an increase in oxygen leads to an increase in μ (Signori et al., 2014).

In the process of our model optimisation to assess the feasibility of metabolic control by oxygen supply control. Optimisation and variability analyses were carried out for two extreme respiration cases -low respiration (required for cells growth) and high respiration at fixed growth rate $\mu=0.4\ h^{-1}$. When comparing the carbon fluxes into the product under different culture oxygenation conditions it was observed that succinate was the only product with the highest yield which was obtained from xylose compared to lactose, inulin and glucose. It is also important to note that for all oxygen supply regimes this value was maximum for ethanol and acetate using lactose, inulin and glucose as substrate. In the case of lactate, this value decreases with increasing aeration.

In this study (Pentjuss et al., 2017), the predicted yields of products and by-products obtained using this model were compared with the theoretical yields of these metabolites. These comparisons also can help to estimate the necessity of applying metabolic engineering to increase yields of these metabolites. Our model suggests that metabolites such as ethanol, acetate, L-lactate and ethyl acetate can be synthesised by *K. marxianus* cells with yields close to theoretical if substrates such as lactose, glucose and inulin are used. Interestingly, when xylose was used as a substrate to produce ethanol, the model predicted that the yield of this product was 60% less than theoretical. This is most likely due to the carbon flux through the

glucose-6P dehydrogenase reaction to produce NADPH, which is required for the reduction of xylose. In addition, according to the model, a large proportion was directed towards acetate production. Also, the literature notes the need for oxygen supply for *K. marxianus* growth if xylose is the only source of carbon (Signori et al., 2014). Also, *K. marxianus* respiration mutants are unable to ferment xylose (Lertwattanasakul et al., 2013). This dependency can be due to three different causes. First, the cell requires sufficient resources of cytoplasmic NAD+ consumed by xylitol dehydrogenase for xylose metabolism. This NAD+ requirement can be satisfied by the reaction of alcohol dehydrogenase producing ethanol and NAD+ or by the activity of the mitachondrial malate-aspartate shuttle (Easlon et al., 2008; Inokuma et al., 2015). The model predicted that the latter is inactive in case of oxygen deficiency. Secondly, the model suggests an accumulation of acetate, up to 50% of the ethanol flux, under the condition of low oxygenation. A similar effect was demonstrated in the fermentation of *S. cerevisiae* with engineered xylose dehydrogenase, xylose reductase and xylose kinase (Klimacek et al., 2010; Pitkanen et al., 2003). According to our model, replacing the xylose reductase cofactor from NADPH to NADH reduces acetate production and the ethanol to xylose ratio coincides with the theoretical one. This in silico results complements the *in vivo* results of various authors who have constructed a xylose reductase cofactor in *S. cerevisiae* or investigated the cofactor specificity of wild-type xylose reductase of various yeast species (Klimacek et al., 2010; Bruinenberg et al., 1984). And the third reason why respiratory activity is crucial for xylose utilisation is the increased consumption of ATP to maintain cytoplasmic pH levels. As xylose transport is a symporter (Stambuk et al., 2003), each xylose molecule is imported into the cell with a proton, which in turn leads to lower pH levels inside the cell. To maintain pH levels, the cell membrane is facilitated by ATPase to export protons by hydrolysing ATP.

In terms of commercial use of *K. marxianus* for acetate production, these yeasts are estimated to have low potential. Consequently, the production and accumulation in the medium of this organic acid is considered undesirable, along with glycerol. Acetate production can take place without significant synthesis of by-products, but strong aeration is required. Based on our model, accumulation of acetate in the cytoplasm was observed in two cases - when additional NADH or cytoplasmic AcetylCoA was required.

Due to the large industrial application of lactic acid and considering the GRAS status and the high production rate, *K. marxianus* is considered as a promising producer of lactic acid. Taking into account the fact that yeast lacks the enzyme lactate dehydrogenase,

recombinant strains containing this enzyme should be used. However, the introduction of this enzyme alone is not sufficient to increase lactate production within theoretical limits under in vivo conditions. This is due to the distribution of carbon flux at the pyruvate level. Decarboxylation of pyruvate makes it impossible to produce lactate directly from pyruvate; instead, the carbon is redirected towards the formation of acetaldehyde, ethanol or acetate. This is confirmed by the model that there is an equimolar increase in CO_2 and ethanol fluxes when the lactate flux is simulated to decrease. Members of the genus *Kluyveromyces* spp, unlike *Saccharomyces* spp, have only one *pdc* gene, which makes it easier to obtain a *pdc* knockout mutant. A similar strategy with subsequent integration of the *ldh* gene was successfully applied to *K. lactis* (Porro et al, 1999). Similarly, another *K. lactis* strain with inactivated genes encoding pyruvate decarboxylase and pyruvate dehydrogenase was obtained in maximum to the theoretical lactate yield (Bianchi et al., 2001). Another strategy can be used to increase lactate dehydrogenase expression by increasing the lactate dehydrogenase gene copy number (Pecota et al., 2007).

K. marxianus is regarded as the most productive producer of an organic solvent such as ethyl acetate (Loser et al., 2014). Analysis of variability fluxes (AVF) indicates a strong effect of aeration on ethyl acetate formation. However, the most efficient formation of this metabolite was not observed during growth with maximum aeration. In addition, this analysis indicates an increase in glycerol production during aeration restriction, indicating the need for cytoplasmic NADH reoxidation to maintain acetate production. Keeping in mind the above mentioned, one strategy to maximise ethyl acetate production could be to accurately adjust the oxygen supply to the medium. A similar strategy was applied to limit *K. marxianus* access to metal ions as Fe, Cu, Zn, the presence of which negatively affects the production of ethyl acetate (Urit et al., 2012; Urit et al., 2013). The greatest effect was performed by limiting Fe, which is a cofactor of mitochondrial aconitase and succinate dehydrogenase it leads to the accumulation of acetylCoA, which leads to an increase in ethyl acetate production (Urit et al., 2012).

As for the metabolites such as succinate and phenylethanol, our model suggests that without metabolic engineering an increase in yield cannot be achieved. Succinate, with its wide range of applications, is one of the 12 most recognised sugar derivated chemical precursors. Despite the extensive use of bacteria for production on an industrial level (Werpy et al., 2004), there are several advantages to using yeast for this production (Cok et al., 2014). Compared with bacteria, yeasts are not strictly anaerobic, more resistant to acids, more

osmotolerant and have non-pathogen status (Raab et al., 2010). Based on the predictions of our model xylose is the most promising substrate for the production of succinate. Although few in vivo results on the use of xylose-based *Kluyveromyces* spp are mentioned in the literature, there is a claim that a mixture of xylose/ethanol is a promising substrate for the production of glyoxylate paired with succinate in recombinant *S. cerevisiae* and *K. lactis* strains with overexpressed isocitrate lyase (Koivistoinen et al., 2013). As succinate production is not a redox-neutral reaction our model suggests the accumulation of at least one by-product. The model also predicts that glycerol formation is observed independently of the substrate under conditions of low aeration. Compensation of NADH reoxidation by increasing glycerol production in *S. cerevisiae* strains optimised for succinic acid production was demonstrated under *in vivo* conditions (Raab et al., 2010). Our model indicates that phenylalanine can be accumulated as a by-product. But in the case of application, the biolimitation in nitrogen limits production of this amino acid, as our model is not nitrogen-limited. A strategy such as deletion of the succinate dehydrogenase subunit gene is a popular strategy for succinate production using yeast including *K. lactis* (Raab et al., 2010; Saliola et al., 2004). In turn, our model suggests that inactivation of aspartate malate shunt in combination with an increase in oxygen consumption should give the maximum yield of succinate, while inactivation of glyoxalate shunt together with inactivation of succinate dehydrogenase are recommended when xylose is used as a substrate.

The amino acid phenylalanine is a precursor of many industrial flavours as well as an ingredient for the artificial sweetener aspartame. Rose flavoured phenylethanol has a growing demand on the world market which cannot be satisfied by traditional extraction methods. The use of bacterial cells for the synthesis of this product could have a sustainable perspective (Stark et al., 2003). Traditionally, *S. cerevisiae* yeast has been seen as a main candidate for phenylethanol production, although other yeast strains, including *K. marxianus*, have also been attempted. In our model, phenylethanol production is represented as a chain of 11 reactions, where the final step was represented according to Uzunov et al., 2014. The model showed two cases of phenylethanol production by *K. marxianus* yeast. When sucrose and glucose were used as substrate, the product-to-substrate ratio was maximum and when other substrates were used, the ratio was lower. However, these ratios are still far from those obtained from in vivo experiments (Garavaglia et al., 2007; Wittmann et al., 2002).

The amino acids phenylalanine and glutamate were included in our model as desired products because the amino acid composition of yeast biomass is of practical interest. A

typical method for producing yeast strains with increased glutamate content is the random mutation method (Nakajo and Sano, 1998). Our model provides a theoretical analysis of possible scenarios for increasing glutamate yield in *K. marxianus*. In *K. lactis* and with high probability in *K. marxianus*, glutamate can be produced by one of two reactions. Our model suggested that the greater carbon flux would be via NADPH-dependent glutamate dehydrogenase. At the same time for substrates such as glucose and lactose, a high degree of aeration is required to maximise the glutamate yield. Interestingly, when xylose was used as a substrate at a lower degree of aeration, a high yield of this amino acid was achieved (80% of the theoretical yield), with the main carbon flow passing through the GOGAT reaction (Romero et al., 2000). It is worth noting that in *K. marxianus* the synthesis of glutamate is tightly regulated by products with feedback inhibition. Just as in *S. cerevisiae*, glutamate synthesis via NADPH dependent glutamate dehydrogenase is affected by nitrogen catabolites (de Morais-Junior, 2003). A kinetic model can be used to analyse this type of product-substrate interaction.

Our model can be used not only to analyse *K. marxianus* and their metabolic engineering, but is also a good basis for a large-scale model. Also, to improve the model, more accurate characterisation of the reactions associated with the transport system can be included to pair with the H+-ATPase system of the plasma membrane.

4.2 The effect of acetate on K. marxianus growth and metabolism

Metabolically active cells produce a wide range of metabolites that can inhibit their growth. During validation of our stoichiometric model during aerobic fermentation of lactose, it was observed that ethanol was the main product, while glycerol and acetate were the main by-products. With the exception of glycerol, a similar trend was shown when optimising the model to assess whether metabolism could be controlled by varying the oxygen supplies. At both minimum and maximum oxygen supply, the carbon flux to products such as ethanol and acetate is kept at a maximum to the theoretical one. There are many studies available in the scientific literature on the topic of ethanol tolerance of *K. marxianus*, and on the mechanisms of negative effects on the cell (Silveira et al., 2020; Mo et al., 2019). In turn, it is worth noting that while there has been extensive research on *K. marxianus* from a biotechnological perspective, less attention has been focused on basic physiology. In particular, very little is known about cellular responses to stress and the adaptation mechanisms induced by acetic acid. As our monitoring in fermentations using lactose as substrate without adjustment of the pH level in the medium has shown that acetate can accumulate at a concentration of 40 mM

and the pH level can drop to 4. Acetate is a weak organic acid with a pKa value of 4.76, this value is higher than previously mentioned level of pH that can be achieved without pH regulation. At these values of pH, yeast cells may be at risk of weak-acid stress caused by excessive acetate uptake.

Acetate accumulation, which coincides with a reduction in growth rate and ethanol production, during poorly aerated fermentation with *K. marxianus* has been noted by various authors (Loser et al., 2013; Signori et al., 2014). Acetate accumulation at concentrations of 10 mM (Sanonetti et al., 2013), 24 mM (Loser et al., 2015) and up to 100 mM (Signori et al., 2014) was observed. Also, research data on this topic may be useful for obtaining more rebust strains of *K. marxianus*. Due to the advantages previously described, these yeasts are promising organisms for use in such biotechnological processes where aggressive media, such as lignocellulose hydrolysate, are used. Dilute acid or enzymatic hydrolysis converts lignocellulose into hydrolysate, which contains not only monosaccharides available to fermenting microorganisms, but also inhibitors harmful to fermentation, including weak organic acids, among which acetic acid is also present (Rugthaworn et al., 2014; Palmqvist and Hahn-Hagerdal, 2000). This knowledge can also be useful in the food industry. Acetic acid has a long history of use as a preservative, and keeping in mind the wide range of *K. marxianus*, including in food (e.g. milk, yoghurt and more exotic products), this research can be applied to limit the growth of this yeast species in food.

Bearing in mind the ability of *K. marxianus* to utilise different carbon sources and the role of the pH level in the medium on the ability of weak acid to enter the cell, the growth ability of *K. marxianus* DSM5422 was tested in the range of pH 4-6 in the presence of different carbon sources. Our results indicate that, depending on the medium pH, an increased concentration of acetate in the medium leads to lower cell growth. Using citrate buffer, the effect of acetate addition was distinguished from the effect of pH itself, because the pH of the medium had to be lower than 3.5 to achieve an increase in the concentration of undissociated citric acid. Since the level of pH determines the amount of undissociated acid, which in turn can diffuse freely through the lipid bilayer, these results indicate that the undissociated acetate is the agent that causes the effects in yeast cell growth. Thomas et al. (2002) also showed that it is not only the undissosiated acetic acid concentration that determines the degree of growth inhibition during cultivation of *S. cerevisia* in the presence of elevated concentrations of acetate but also the total acetic acid concentration. In our case, the concentration of undissociated acetate seems high enough to explain the reduced growth rate

at biotechnologically inhibited concentrations of acetate. In the cases of *K. marxianus* and *S. cerevisiae*, different cellular mechanisms regarding acetic acid at different values of pH. It is worth noting an interesting fact that the effect of acetate on the growth doubling time of DSM 5422 culture is dependent on the carbon source used. The most dramatic effect of the combination of low pH and 40 mM acetate was observed when the culture was grown on lactose and galactose. In the case of glucose this effect is not so pronounced. This fact leads us to look for common traits and pathways in lactose and galactose metabolism, as these elements can be influenced by acetate accumulation and cytosolic acidification. The first key step in the metabolism of any substrate is transport. In the case of glucose, galactose and fructose, two variants of transport are possible, one related to the proton symporter and the other independent of the symporter (Gasnier, 1987; de Bruijne et al., 1988; Carvalho-Silva and Spencer-Martins, 1990). In turn, the only mode of transport of lactose into the cell is proton symporter, which means that cell growth on lactose may be limited at this step (de Bruijne et al., 1988; Varela et al., 2017). Maintaining a proton gradient between the extracellular space and the cytosol by ATP when acetate enters the cell freely at low pH levels is expensive because of cytosol oxidation (Piper et al., 2001). A decrease in the proton gradient slows down lactose transport due to proton symporter. Growth rate can be limited by insufficient lactose uptake in combination with high energetic costs. However, it is unclear whether lactose uptake is limited by the effect on the proton gradient and the uptake mechanism itself or whether it is due to reduced lactose uptake in subsequent catabolic reactions. The results show that the presence of acetate within the test range of pH does not affect the activity of extracellular inulinase. Furthermore, if glucose and galactose uptake is provided by facilitated diffusion (Carvalho-Silva and Spencer-Martins, 1990) and proton symporter (de Brujne et al., 1988), it is possible that during acetate stress the capacity of facilitated diffusion transporters cannot provide sufficient sugar amount to satisfy the energetic needs of the cell. The conversion of galactose into glucose-6-phosphate occurs with the help of five enzymes of the Leloir pathway, which can be further utilised in glycolysis (Sellick et al., 2008; Fonseca et al., 2013). In turn, fructose is phosphorelated into fructose-6-phosphate by the enzyme hexokinase, and then enters glycolysis. So most likely the negative effect of acetate on culture growth rate is due to direct or indirect intervention in the Leloir pathway. But due to the scarce information on the enzymes of this pathway in *K. marxianus*, none of these speculations about the physiological or molecular effects of acetate stress on the growth of DSM 5422 cultures on lactose and galactose can be neither confirmed nor disproved. When comparing the maximum optical density of the biomass obtained by

cultivations in fermenters and in a 96-well plate, a difference is noted. In the latter case, the lower value can be attributed to a worse degree of aeration compared to the first case, resulting in a decrease in the optical density of the biomass, which may indirectly indicate the influence of the degree of aeration. Inhibition of yeast culture growth and the consequent decrease in cytoplasmic pH levels due to increased acidity of the medium is well described in *S. cerevisiae*. The functional effects of acid stress include a decrease in specific growth rate and inhibition of mitochondrial proliferation (Orij et al., 2012) as well as inhibition of substrate uptake (Ding et al., 2013). Induction of cytoplasmic and vacuolar ATP-ases, which export cytoplasmic protons at the expense of ATP, especially in the presence of succinate and acetate at pH below 3.5, is observed during acid stress (Carmelo et al., 1997). The prolongation of the lag-phase in *K. marxianus* cultivation at pH 4 and 4.5, during which the cell growth decreases and ethanol accumulates, can be explained by the similar principle as in *S. cerevisiae*. Growth in the acetate-rich medium is expensive for the cell energy because of consumption of ATPase, which in turn inhibits fast growth and mitochondria functioning.

Stratford et al. (2013) demonstrated that a small fraction of the *Z. bailii* population is tolerant to high concentrations of acetic acid as well as to other weak organic acids. We obtained similar results in *K. marxianus* DSM 5422, where the population is heterogeneous with respect to acetic acid. This can be observed when DSM 5422 culture is inoculated into acetate-rich medium during the prolonged lag-phase with a constant amount of biomass and the number of acetate-tolerant cells increases. It seems likely that non-tolerant cells are more likely to become tolerant to the increased acetate concentration than non-tolerant cells are to die and tolerant cells continue to grow. Differences in lag-phase length indicate that when the concentration of undissociated acetate increases, the time required for adaptation increases. The fact that after re-inoculation of the adapted culture into fresh medium containing acetate, growth of the culture is observed without slowing down, indicates that the key to adaptation is adaptation of the cell itself, not changes in the medium. A similar growth pattern in the way of an extension of the adaptation period and an immediate continuation of growth after reinoculation in fresh medium has been observed in other weak acids in *S. cerevisiae* (Viegas et al., 1998; Cabral et al., 2001; Teixeira and Sa-Correira, 2002) and *Z. bailii* (Stratford et al., 2013). The results obtained by van Heerden et al. (2014) suggest that random metabolic variations at the cellular level determine which of the cells will eventually begin to grow under such conditions. It is also worth noting that cultures that have been cultured in medium without the presence of added acetate become more tolerant to acetate when diauxia is

reached. It appears that slow growing or stationary phase cells achieve tolerance to acetate as they become resistant to many environmental stress factors such as desiccation (Klosinska et al., 2011). Schuller et al. (2004) showed that in *S. cerevisiae* sorbate-activate genes and genes of the common stress response pathway share common regulators. If the same is true for *K. marxianus* and other weak acids like acetic acid, this could at least partly explain why tolerance to acetate is acquired along with general stress tolerance during the post-diauxic growth phase. Also the idea that cells become tolerant to acetate along with general stress tolerance when the growth rate decreases and the cells reach a stationary growth phase is supported by experiments on the measurement of respiratory activity. The observed decrease in respiratory activity of the culture in the post-diauxia phase, when the medium concentration of acetate is zero, can be attributed to a general slowing down of growth and metabolism at the time of nutrient depletion in the medium after the diauxia. In turn, the sensitivity of cells in the exponential growth phase can also be explained by the fact that fast-growing cells are more sensitive to various types of stress than stationary cells (Klosinska et al., 2011). However, in spite of the results obtained in this study, the mechanisms of *K. marxianus* adaptation remain unclear. Further research is needed to clarify the exact processes behind the growth inhibition by acetate. Compared to other yeasts, *K. marxianus* DCM 5422 is less tolerant to elevated concentrations of acetate (Thomas et al., 2002; Stratford et al., 2013). Understanding the mechanisms of inhibition and adaptation could provide a strategy for developing detoxification methods or obtaining new yeast strains that will combine the superior biotechnological abilities of *K. marxianus* with its high tolerance to acetic acid and similar inhibitors present in the substrate and as by-products of yeast metabolism.

Previous studies have shown that the greatest negative effect of acetic acid is observed in the medium with lactose as a carbon source at pH 4.5 (Martynova et al., 2016). We hypothesised that acetic acid in its undissociated form at low pH could pass through the cell membrane, thereby inhibiting lactose transport through the membrane for strains that would rely on the intracellular activity of β-galactosidase. It can be supposed that in *K. marxianus* strains with a periplasmic β-galactosidase the negative effect of acetate will be less pronounced than in strains with a cytoplasmic β-galactosidase. Data on the localization of galactosidase in *K. marxianus* are contradictory. Literature data indicate that the localization of β-galactosidase is strain dependent (Carvalho-Silva and Spencer-Martins, 1990). Our findings indicate that both periplasmic and cytosolic β-galactosidases are present

in the four tested strains, while the total β-galactosidase activity is similar, but the distribution between them is strain specific. The NCYC 2791 strain has the highest percentage of cytosolic β-galactosidase, which was also noted by Bacci et al. 1996. However, in an experiment to determine β-galactosidase localization, Carvalho-Silva and Spencer-Martins (1990) observed extracellular lactose hydrolysis in strain NCYC 2791 and a possible periplasmic localization in DSM 5422. Unfortunately, due to the lack of a detailed description of the experiment and the absence of the necessary for β-galactosidase cofactors in the form of Mg^{2+} and Mn^{2+} ions in the reaction mixture, it is difficult to compare our data with Carvalho-Silva and Spencer-Martins, 1990. We found a correlation between the ability of the strain to grow on lactose medium and the distribution between the localization of β-galactosidase in the strain in the presence of acetic acid. The specific growth rate of the strain decreases as the amount of cytosolic β-galactosidase increases. This correlation can be explained by the specific transport of lactose, glucose and galactose. The literature clearly shows that lactose enters the cell via H^+ symport transporters (Varela et al., 2017), while glucose and galactose are taken up by cells via low- and high-affinity transporters (Varela et al., 2019a). Thus, in the case where lactose is hydrolysed in the extracellular space, the released monosaccharides can be transported into the cell by hexose transporters, which are independent of the proton movement across the cell membrane.

The cultivation of four *K. marxianus* strains (DSM 5422 (= CBS 397), DSM 5418, DSM 4906 and NCYC 2791 (= CBS 712)) used in our study in the medium with 40 mM acetate at pH 4.5 and without the addition of acetate showed that the negative effect of acetate is represented by a decrease in the specific growth rate and reducing the maximum optical density of the culture. This can be explained by the need for cell adaptation to adverse environmental conditions, which is an energy demanding process. However, the degree of negative effect on the above mentioned growth parameters depends on both the carbon source in the medium and the strain that is used. Due to the high metabolic diversity of *K. marxianus* yeasts and the substantial degree of intraspecific polymorphism (Lane et al., 2011), tolerance to acetic acid may also be strain dependent. Cultivation of the four *K. marxianus* strains in this study in media with 40 mM acetate at pH 4.5 using different carbohydrate sources (lactose, glucose and glucose + galactose) showed that the greatest reduction in growth rates was observed with lactose. This can be due to the above mentioned characteristics of the transport systems. The greatest decrease in specific growth rate was observed for strain NCYC 2791 (more than 3-fold compared to this parameter obtained by cultivation without

the addition of acetate) . This is the largest decrease in this parameter among all tested strains, according to our data of that strain, it has the highest percentage (80%) of galactosidase is in the cytosol. In turn, when these strains were cultured in acetate medium using an equimolar mixture of glucose and galactose, the growth rate remained close to that under identical growth conditions but using glucose. There is also a decrease in the maximum optical density of the culture, which can be explained by the repression of catabolites. Since when *K. marxianus* is cultured on a mixture of glucose + galactose, glucose is transposed and metabolised primarily. This is why we observe a rapid growth phase followed by no stationary phase but a slow growth in biomass. It has been shown in the literature in various strains of *K. marxianus* that this slow growth is presumably due to the fact that after glucose assimilation, induction is required for the cells to start transport and catabolism of galactose (Beniwal et al., 2017; Fonseca et al, 2013).

5 CONCLUSIONS

1 A simple unsegregated and unstructured kinetic model was developed and experimentaly validated for the batch production of bioethanol by *K. marxianus* DSM 5422 from the renewable sources of agricultural and food processing origin, such as whey permeate or inulin, which includes the terms of both substrate and product inhibition. There is a reverse correlation between biomass yield and ethanol yield on such substrates as lactose, whey permeate and inulin.

2 *K. marxianus* shows the highest ethanol yield (90.2 % of the theoretical yield) on whey permeate as substrate, while ethanol and biomass productivity were lower compared to semi-synthetic media with lactose or inulin due to nitrogen deficiency in whey permeate. Whey permeate is a suitable raw material for the fermentation of bioethanol by *K. marxianus*.

3 The developed stoichiometric model of the central carbon metabolism of *K. marxianus*, balanced by mass and charge, including biomass production, is able to reproduce the experimentally observed mixture of industrially valuable products, as well as to explain the formation of undesirable by-products (acetate and glycerol).

4 The results of the stoichiometric model show that oxygen control can be used to influence product yield and flux distributions.

5 Replacing cofactors (NADPH to NADH and/or vice versa) can significantly improve xylose conversion to products.

6 Xylose has proved to be a biotechnologically promising substrate for *K. marxianus* with the unused potential linked to fine-tuning the redox engineering.

7 The stoichiometric model predicted that ethanol, acetate, L-lactate and ethyl acetate could be produced in near to theoretical yields and without the need for gene engineering, using substrates such as lactose, glucose and inulin, but a high proportion of the theoretical yields of phenyl ethanol and succinate could not be achieved without metabolic engineering.

8 Acetate inhibits growth in a pH-dependent manner and has pronounced effects if *K. marxianus* yeast is grown on lactose or galactose.

9 Negative effect of acetate on the *K. marxianus* growth parameters (maximal growth rate and maximal optical density) depends on both the carbon source in the medium and the strain that is used.

10 When challenged with acetate, *K. marxianus* culture extends lag phase, during which cells adapt to elevated acetate concentrations, and growth reoccurs, albeit at slower rate, when majority of the population is acetate resistant. Acetate resistance is maintained only if acetate is present in the media or if the *K. marxianus* culture has reached end of active growth phase.

11 In four of tested *K. marxianus* strains (DSM 5422, DSM 5418, DSM 4906 and NCYC 2791) both periplasmic and cytoplasmic β-galactosidases activity is similar, but the distribution between them is strain dependent. There is a correlation between the ability of the *K. marxianus* strain to grow on lactose medium and distribution between the localization of the β-galactosidase in the strain in the presence of acetic acid. The specific growth rate of the *K. marxianus* strain decreases in the acetic acid presence as percentage of cytosolic β-galactosidase in total enzyme activity increases.

6 MAIN THESES FOR DEFENCE

1 The kinetic and stoihometric models allows to predict biotechnological potential of *K. marxianus* for bioconversion of substrates containing different carbon sources to valuable products and to evaluate necessity for metabolic engineering.

2 *K. marxianus* is suitable microorganism for bioconversion of lactose containing diary industry by-product – cheese whey permeate to bioethanol.

3 Acetate tolerance of *K. marxianus* is strain dependent and varies from carbon source used and medium pH.

4 One of the factors influencing acetate tolerance of *K. marxianus* on lactose is distribution of β-galactosidase between cytosol and periplasm.

7 LIST OF ORIGINAL PUBLICATIONS

Martynova J, Kokina A, Kibilds J, Liepins J, Scerbaka R, Vigants A. (2016) "Effects of acetate on *Kluyveromyces marxianus* DSM 5422 growth and metabolism" ***Applied Microbiology and Biotechnology*** Vol. 100, N 10 (2016), p. 4585 – 4594. DOI: 10.1007/s00253-016-7392-0

Martynova J, Mednis M, Vigants A, Zikmanis P. (2017) "Kinetic modeling of ethanol fermentation by yeast *Kluyveromyces marxianus* from lactose- and inulin- containing substrates" ***Engineering for Rural Development***: 16th International Scientific Conference Proceedings Jelgava: Latvia University of Agriculture, 2017 Vol. 16, p. 88 – 97 DOI: 10.22616/ERDev2017.16.N016

Pentjuss A, Stalidzans E, Liepins J, Kokina A, Martynova J, Zikmanis P, Mozga I, Scherbaka R, Hartman H, Poolman MG, Fell DA, Vigants A. (2017) "Model-based biotechnological potential analysis of *Kluyveromyces marxianus* central metabolism" ***Journal of Industrial Microbiology & Biotechnology***, 2017 Aug; 44 (8): 1177 – 1190 DOI: 10.1007/s10295-017-1946-8

Jekaterina Martynova, Kristiana Kovtuna, Janis Liepins, Agnese Kokina, Armands Vigants (2022) "Acetic acid stress hampers *Kluyveromyces marxianus* growth on lactose" Under review in **Yeast** Manuscript ID is YEA-Oct-22-0081

OTHER PUBLICATIONS:

1. J. Martynova, A. Kokina, J. Liepins, A. Vigants (2016) "Utilization of different carbohydrates by various *Kluyveromyces marxianus* strains" **Journal of Biotechnology**, Vol. 231, Supplement, 2016, p. 62

2. A. Vigants, J. Martynova, K. Kovtuna, A. Kokina, J. Liepins (2016) "Whey lactose bioconversion to valuable products by non-conventional yeasts *Kluyveromyces marxianus*" **Journal of Biotechnology**, Vol. 231, Supplement, 2016, p. 7

3. A. Patetko, R. Silins, R. Scherbaka, J. Martinova, A. Vigants (2016) “2-Phenylethanol production by *Kluyveromyces marxianus* on glucose and lactose substrates*”* **Journal of Biotechnology**, Vol. 231, Supplement, 2016, p. 62 – 63

4. J. Martynova, K. Kovtuna, M. Grube, A. Vigants (2015) “Enhancement of protein content in *Kluyveromyces marxianus* biomass produced on cheese whey lactose” **Journal of Biotechnology**, Vol. 208, Supplement, 2015, p. 76

5. A. Vigants, J. Lukjanenko, M. Grube, J. Liepins (2014) “Influence of fermentation conditions on biomass composition during ethanol biosynthesis from cheese whey lactose concentrate by *Kluyveromyces marxianus*” **Journal of Biotechnology**. Vol.185, Supplement 2014, p. 122

6. J. Lukjanenko, K. Kovtuna, R. Scherbaka, A. Vigants (2014) “Bioethanol and biomass production by *Kluyveromyces marxianus* during lactose fermentation at different salts and substrate concentrations” **Journal of Biotechnology**. Vol.185, Supplement, 2014, p. 122

8 APPROBATION OF THE RESEARCH

8.1 International

7th conference on physiology of yeasts and filamentous fungi. "**Impact of carbon source on acetic acid stress in different *Kluyveromyces marxianus* strains**" (Jekaterina Martynova, Kristiana Kovtuna, Janis Liepins, Agnese Kokina, Armands Vigants) June 24-27, 2019, Milan, Italy

4th Congress of Baltic Microbiologists. "**Impact of carbon source on acetic acid stress in different *Kluyveromyces marxianus* strains**" (Jekaterina Martynova, Kristiana Kovtuna, Janis Liepins, Agnese Kokina, Armands Vigants) September 10-12, 18, Gdansk, Poland

International Conference, Non-conventional Yeasts: from Basic Research to Application "**Acetic acid tolerance of different *Kluyveromyces marxianus* strains**" (Jekaterina Martynova, Kristiana Kovtuna, Janis Liepins, Agnese Kokina, Armands Vigants), May 15-18, 2018, Rzeszow, Poland

10th NIZO Dairy Conference, Innovations in Dairy Ingredients. "**Biosynthesis of galactooligosaccharides from milk permeate concentrate by different β-galactosidases**" (Armands Vigants, Ansis Zauers, Kristiana Kovtuna, Rita Scerbaka, Jekaterina Martynova) October 1 – 3, 2017, Papendal, Netherlands

33rd International Specialised Symposium on Yeasts – Exploring and Engineering Yeasts for Industrial Application. "**The growth and adaptation of yeasts *Kluyveromyces marxianus* in the presence of acetate**" (Jekaterina Martynova, Kristiana Kovtuna, Janis Liepins, Agnese Kokina, Armands Vigants) June 26 – 29, 2017, Cork, Ireland

3rd Congress of Baltic Microbiologists. "**Effects of acetate on *Kluyveromyces marxianus* growth and metabolism**" (Jekaterina Martynova, Agnese Kokina, Janis Liepins, Kristiana Kovtuna, Armands Vigants) October 18 – 21, 2016, Vilnius, Lithuania

32nd International Specialized Symposium on Yests "Yest Biodiversity and Biotechnology in the twenty-first century". "Impact of acetate on lactose bioconversion by non-conventional

yeasts *Kluyveromyces marxianus*" (J. Martynova, J. Kibilds, A. Kokina, J. Liepins, A. Vigants) September 13 – 17, 2015, Perugia, Italy

8.2 National

Kristiāna Kovtuna, Jekaterīna Lukjaņenko, Jānis Liepiņš, Agnese Kokina, Juris Ķibilds, Armands Vīgants "**Rauga *Kluyveromyces marxianus* acetāta tolerance**" 74 th Scientific Conference of the University of Latvia, February 10, 2016. Riga, Latvia

Kristiāna Kovtuna, Jekaterīna Lukjaņenko, Agnese Kokina, Armands Vīgants "**Rauga *Kluyvermyces marxianus* biomasas producēšana laktozi saturošos substrātos**" 73 th Scientific Conference of the University of Latvia, February 11, 2015. Riga, Latvia

Jekaterīna Lukjaņenko, Juris Ķibilds, Agnese Kokina, Jānis Liepiņš, Armands Vīgants "**Acetāta ietekme uz raugu *Kluyveromyces marxianus* biomasas augšanas parametriem**" 73 th Scientific Conference of the University of Latvia, February 11, 2015. Riga, Latvia

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