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Development of a defined synthetic medium to mimic sugarcane molasses for ethanol fermentation

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DEDICATÓRIA

À minha família, que me apoiou incondicionalmente, nos bons, médios e maus momentos.

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RESUMO

A produção de etanol no Brasil ocorre através da fermentação do caldo ou melaço de cana-de-açúcar por leveduras da espécie *Saccharomyces cerevisiae*. O uso do melaço apresenta vantagens logísticas e econômicas em relação ao caldo, devido ao seu teor de umidade reduzido e alta concentração de açúcares. Entretanto, a qualidade dos melaços varia mesmo em uma mesma usina, em função de fatores como a qualidade da cana, fertilização e número de reciclos ao qual o melaço foi submetido para a etapa de cristalização de açúcar. Por isto, utilização de melaços para pesquisas torna-se problemática pois não é possível garantir que um determinado lote de melaço seja adequadamente representativo e assim, a reprodutibilidade de experimentos é prejudicada, dificultando sua utilização em diferentes laboratórios. Desta maneira, o objetivo deste trabalho é desenvolver um meio definido que seja capaz de reproduzir parâmetros fisiológicos e tecnológicos obtidos a partir de amostras de melaços reais. Como primeira iteração deste meio a ser proposto, uma formulação semidefinida recentemente publicada para simular mosto de melaço de cana foi testada em duas linhagens industriais e duas linhagens laboratoriais de *S. cerevisiae* e esta não foi capaz de reproduzir de maneira adequada os crescimentos em batelada para as quatro linhagens distintas em comparação aos melaços reais de usinas alcooleiras. Assim, o meio foi adaptado, gerando o meio sintético 1SMol, que teve sua composição dividida em sete grupos nutricionais que tiveram seus efeitos avaliados sobre o crescimento de uma levedura industrial (PE-2) e uma laboratorial (CEN.PK113-7D), através de ensaios de crescimento em batelada. Os grupos nutricionais que mais impactaram na velocidade específica de crescimento e na absorbância máxima foram as vitaminas e a fonte de nitrogênio. Entretanto, ao ter os resultados comparados com melaços reais, o meio 1SMol apresentou valores de máxima velocidade específica de crescimento menores que melaços reais. O meio foi então adaptado, gerando 2SMol, um meio definido, com resultados em bateladas simples similares aos de melaços industriais. Para este meio, foi observado que as concentrações de vitaminas, amônio e aminoácidos tiveram maior impacto sobre parâmetros de crescimento em batelada. O meio 2SMol foi então comparado a três melaços industriais em um experimento de batelada alimentada com reciclo de células, simulando o processo industrial brasileiro de produção de etanol. Resultados similares foram obtidos para os cultivos com a formulação sintética e os meios industriais com relação aos parâmetros viabilidade, pH de vinho, rendimento em etanol, perfil de produção de CO_2 e biomassa ao longo de cinco reciclos. Assim, o meio 2SMol se

apresenta como uma boa alternativa de meio de cultura padronizado para a realização de estudos envolvendo a fermentação de melaço de cana-de-açúcar por leveduras.

Palavras-chave: melaço; fermentação etanólica; *Saccharomyces cerevisiae*.

ABSTRACT

Ethanol production in Brazil occurs through the fermentation of sugarcane juice or molasses by yeasts of the species Saccharomyces cerevisiae. The use of molasses has logistical and economic advantages compared to sugar cane juice, due to its reduced moisture content and high concentration of sugars. However, the quality of the molasses varies even in the same mill, depending on factors such as the quality of the cane, fertilization and the number of recycling to which the molasses was submitted for sugar removal. For this reason, the use of molasses for academic research becomes problematic because it is not possible to guarantee that a given lot of molasses is representative and thus, the reproducibility of experiments is impaired, making its use difficult by research centers. Thus, the objective of this work is to develop a defined medium that is able to reproduce physiological and technological parameters from real molasses. As the first iteration of this medium to be proposed, a published semi-synthetic medium intended to simulate sugar cane molasses must was tested in two industrial lines and two laboratory strains of S. cerevisiae and not able to adequately reproduce the batch growths for the four distinct strains compared to real molasses from sugarcane mills. Thus, the medium was adapted, generating the 1SMol synthetic medium, which had its composition divided into seven nutritional groups that had their effects on the growth of an industrial yeast strain (PE-2) and a laboratory strain (CEN.PK113-7D) in microplate batch growth experiments. The nutritional groups that most impacted the specific growth rate and maximum absorbance were vitamins and the nitrogen source. However, when compared to real molasses, 1SMol presents high specific growth velocity values lower than real molasses. The medium was then adapted, generating 2SMol, a defined medium, with results in simple batches similar to industrial molasses. For this medium, it was observed that the options of vitamins, ammonium and amino acids have had a greater impact on growth parameters in batches. The 2SMol medium was then used in a batch-fed experiment with cell recycling, simulating the Brazilian industrial process of ethanol production, obtaining results similar to the three industrial molasses regarding viability parameters, wine pH, ethanol yield, profile of CO2 and biomass production over five cycles. Thus, the 2SMol medium is presented as a good alternative as a standard medium for carrying out studies involving the fermentation of sugarcane molasses by yeast.

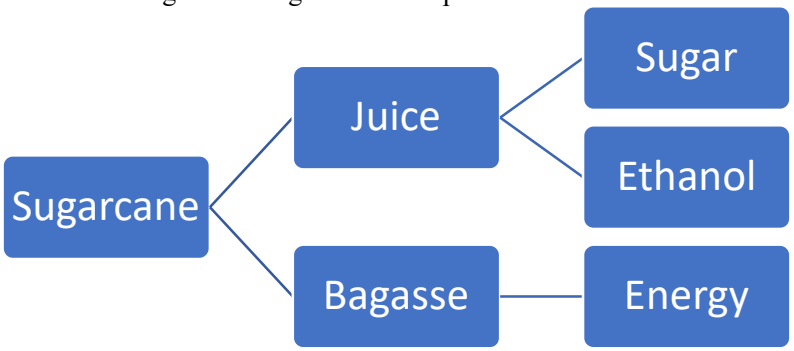
Keywords: molasses; ethanolic fermentation; *Saccharomyces cerevisiae*.

SUMARY

1 INTRODUCTION

Sugarcane comprises several species belonging to the *Saccharum* genus, characterized by tall perennial grasses that can accumulate sucrose in its fibrous stalks. Brazil is the biggest sugarcane producer accounting for 39% of the world production with 8.4 million hectares and presenting a productivity of 74.217 $kg.ha^{-1}$, yielding 622 million tons of sugarcane each year (CONAB, 2019). This crop is used to produce edible sugar, ethanol, and renewable energy (Figure 1). Sugar is obtained from sugarcane juice concentration and crystallization processes, ethanol by yeast fermentation of the carbon sources available in the juice and/or in the molasses (sucrose and minor amounts of glucose and fructose), and energy from the burning of the sugarcane bagasse to produce steam and generate electricity.

Figure 1 – Sugarcane main products



Source: The author

The processing steps prior to sugar and ethanol production involves several operations. After the harvesting, sugarcane stalks are transported to factories where they are cleaned, chopped, crushed, and washed several times for separation of sugarcane juice from the bagasse. The juice, which still contains solid materials, is then clarified to remove fibres and sand. This procedure involves heating the juice to 70 °C, calcium oxide (CaO) addition, heating up to 105 °C and flocculant addition. This results in a clarified juice and a mud that can be filtrated for further extraction. For sugar production, the clarification step requires more CaO and sulphur dioxide (SO_2) if white sugar is the final product. Upon the mill's demands, this juice can either be concentrated and sent straight to fermentation, or concentrated and crystallized, yielding sugar and molasses as a coproduct (Dias et al., 2015). The crystallization steps may be repeated several times at different conditions to remove more sugar from the molasses, yielding different qualities of molasses regarding their sugar concentration. Such high sugar concentrations (735-875 $g.kg^{-1}$) inhibit bacterial degradation and allows its storage (Amorim, Basso and Lopes, 2009).

The fermentation step is conducted using a substrate, known as sugarcane or fermentation must, that contains around 200 $g.L^{-1}$ total reducing sugars (TRS). This can be achieved by diluting molasses with either sugarcane juice or water, or simply concentrating a clarified juice to these levels, although the latter might exhibit nutritional deficiencies. The final substrate is then a result of several factors, including the sugarcane variety, fertilization levels, the proportion of sugarcane juice and molasses used, and the steps involved in the concentration of the molasses. As a result, there are large variations in the composition in these substrates, even in the same mill along same the year (L.C. Basso, Basso, and Rocha, 2011).

The fermentation process in Brazil is robust and fluctuations in quality of the fermentation must are usually addressed to avoid large losses in fermentation performance. However, for research purposes it is of paramount importance to ensure results homogeneity and reproducibility. For so, this work proposes the development of a standardized fully defined synthetic media that successfully reproduces the parameters obtained during molasses fermentations which would fill an important gap in ethanol production research.

Throughout this work the terms “synthetic medium” and “defined medium” will be used. The term synthetic will be used as direct opposite to the media one would classify as “natural”, or as having origin on a “natural” source, such as molasses and grape juice. The term “defined” refers only to the chemical composition of the medium, for instance, one could formulate a “non-defined synthetic molasses” using, for example, a commercially available vegetal peptone or yeast extract. On the other hand, a defined medium will always be formulated from “pure” components and will be necessarily “synthetic”. The redundancy in “defined synthetic” is purposeful to encompass both classifications highlighting both characteristics of the medium.

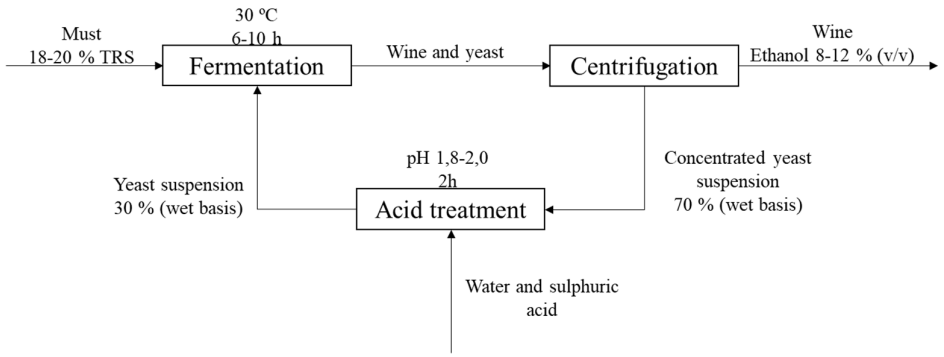
2 LITERATURE REVIEW

2.1 Ethanol production in Brazil

The main feedstock for ethanol production in Brazil is sugarcane, which is crushed to extract the sucrose from its stalks, yielding a juice can be concentrated and undergo a crystallization process for sugar production, with molasses as by product. The substrate, or must, for fermentation may be composed of concentrated juice, or molasses diluted with water or juice (Dias et al., 2015).

Most distilleries run a fed-batch process with cell recycle for the conversion of the sugars in the must to ethanol. The first inoculum for the fermentation process is prepared by the addition of 2 to 12 tons of baker's yeast and 10 to 300 kg of a selected yeast strain to water, yielding a 30 % yeast suspension on a wet basis. This suspension is used to fill about a third of the fermentation vat volume that is then fed with the must, which contains 18 % to 20 % (w/w) total reducing sugars (TRS), mostly comprised of sucrose, a non-reducing sugar that can be hydrolysed by yeast in its reducible monomers, glucose, and fructose. The feeding time lasts between 4 – 6 hours and the fermentation ceases within 6 – 10 hours. The vat contents are then centrifuged, yielding the "wine" or "beer" with ethanol titres between 8 and 12 % (volume/volume) and a concentrated yeast cell suspension with 60 to 70 % cells on a wet basis. The wine is sent to distillation for ethanol recovery and the concentrated yeast cell suspension is diluted with water and undergoes an acid treatment at pH 1.8-2.0 by the addition of sulphuric acid, to reduce bacterial contamination. After the acid treatment step, the resulting cell suspension is recycled to fill another fermentation vat (L. C. Basso et al., 2011). The fermentation and cell recycle process is illustrated on Figure 2.

Figure 2 – Simplified scheme of the fed-batch fermentation with cell recycle for ethanol production in Brazil.



Source: The author with data from L. C. Basso et al. (2011

This process configuration holds several advantages: lower equipment cost and simpler operation, when compared to continuous fermentations; the cell recycling process is able to recover 90 – 95 % of the yeast cells, biomass increase of 5 – 10 % in each fermentation cycle recovers the losses in centrifugation step, allowing high cell densities during fermentation (10 – 17 % wet basis); such high cell density promotes fast fermentation (6 – 10 hours) and high ethanol titres; although the process is non-aseptic, the acid treatment step is able to decrease bacterial contamination (Della-Bianca et al., 2013).

However, this process imposes several stress factors to the yeast cells, such as low pH during acid treatment, high ethanol and sugar concentrations, high osmotic pressure, bacterial contamination, and possible presence of inhibitors, demanding the use of yeast strains able to cope with these conditions. L. C. Basso et al. (2008) conducted a study between 1995 and 2003 to select strains isolated from Brazilian distilleries, collecting 1160 yeast cream samples. This allowed the selection of yeast strains with desirable features, such as low foaming and flocculating levels, high ethanol yield, low residual sugar, high intracellular trehalose and glycogen levels, maintenance of high viability and low glycerol formation. Strains presenting these characteristics were implanted in distilleries and three strains, PE-2, CAT-1, and BG-1 were able to compete with indigenous strain and dominate the industrial fermentations. In the 2007-2008 season, PE-2 and CAT-1 strains were used in 150 distilleries and were responsible for about 60 % of the ethanol produced in Brazil.

2.1.1 The molasses as fermentation substrate

Molasses is a broad term used for concentrated solutions of sugar cane, beet, or citrus which undergo various processes for removal of sugars in various degrees (Jacques et al., 2003). This section will focus on molasses produced from sugarcane, focusing on the varieties used for ethanol production in Brazil.

Sugarcane industry in Brazilian model is based on the joint production of sugar, ethanol, and electricity generation (from the bagasse burning). As of 2019, 67 % of the ethanol and 95 % of the sugar production in Brazil was done in sugar factories with annexed distilleries. In these factories, must for fermentation is prepared by diluting molasses with water or sugarcane juice, achieving yields of 260 litres of ethanol per ton of molasses. This strategy decreases commercial risks from price fluctuation of both sugar and ethanol allowing

production flexibility. Fermentation of molasses is also preferred as sugarcane juice can become heavily infected with bacteria and cannot be stored, being available only during the harvest season. (Carioca & Leal, 2019).

Molasses can be classified based on the extension of sucrose removal for sugar production. In the mills, the juice is concentrated, forming massecuite, a viscous liquid containing sucrose crystals, which is centrifuged for separation of the crystals, yielding A-sugar and A-molasses. The A-molasses can be recycled for further sucrose removal resulting in different molasses qualities (A, B or C) with progressively lower sucrose content, parameter used to define its "quality" (Amorim et al., 2009; Dias et al., 2015).

As a result of the sucrose removal, lower quality molasses' have higher salt to sugar ratio, resulting in musts with elevated salts concentration, which impose an osmotic stress to the yeast cells. Additionally, the high temperatures demanded for concentration promote hydrolysis of sucrose to glucose and fructose, which are prone to react with amino acids in Maillard reactions, as well as dehydration, fragmentation, and condensation reactions that give the molasses its dark brown colour. Some products of these reactions may affect negatively yeast metabolism, such as hydroxymethylfurutal (HMF) and formic acid from dehydration and fragmentation of fructose (Eggleston & Amorim, 2006). Maillard reaction products, on the other hand, are believed to chelate toxic metals such as aluminium and cadmium alleviating their effects negative effects on the fermentation (L. C. Basso et al., 2011).

Another remarkable feature of molasses is its low free amino nitrogen (FAN) content. In the previously described fed-batch process for ethanol production, the low FAN content is usually sufficient to support 5 to 10 % biomass increase between cycles, replacing the cell loss during centrifugation and acid treatment steps (Della-Bianca et al., 2013). When molasses is used as a carbon source for cell propagation, nitrogen might be supplied with the addition of urea, ammonium sulphate, ammonium phosphate or ammonia (Russel, 2003).

Sugarcane juice and consequently molasses elemental composition is affected by the crop variety and maturity, climatic conditions, and fertilization levels. Thus, molasses is a heterogeneous substrate group, presenting wide ranges of concentration for each element. (Palmonari et al., 2020) analysed the chemical composition of 16 sugarcane molasses, showing wide concentration ranges for several elements among the samples. Although this study assessed molasses used for animal feed produced outside Brazil, it illustrates the

variability in molasses composition. Compiled data of sugarcane base substrates by L. C. Basso et al. (2011) also demonstrates such heterogeneity. In this case, however, additional variation might be the result of different proportions of sugarcane juice and molasses used. The data for the most relevant elements for the fermentation processes are presented on Table 1, illustrating the large concentration ranges found for most elements.

Table 1 – Range of concentration in sugarcane-based substrates.

	Source	
Element	L. C. Basso et al. (2011)[1]	Palmonari et al. (2020)[2]
Nitrogen (NH_4+ and R-NH_2)	70-350	-
Phosphorus	20-200	0.7-2.97
Potassium	300-12,000	0.31-7.99
Magnesium	80-3,900	0.19-0.63
Sulphur	80-3,900	0.27-1.36
Calcium	150-2,000	0.82-3.13
Zinc	0,45-9	-
Copper	0.20-8	-
Manganese	2-8	-
Aluminium**	2-500	-

[1]:In $mg.L^{-1}$ for a 200 $g.L^{-1}$ TRS must.

[2]:In percentage of dry mass

Source: Adapted from L. C. Basso et al. (2011) and Palmonari et al. (2020)[2]

Additionally, a plethora of organic compounds can be found on molasses, originated from the secondary metabolism of sugarcane, increasing its complexity. Among them are organic acids, including aconitic, malic, citric, oxalic, and glycolic acids, which are believed to provide buffering effect to the musts, as well as other compounds such as waxes, sterols and gums (Clarke, 2003). Important compounds for yeast cell metabolism like biotin, pantothenic acid, inositol, thiamine, pyridoxine and nicotinic acid are also reported to be present in molasses (Kampen, 2014). Polyphenols, a large natural product group, are also reported to be found on molasses. Deseo, Elkins, Rochfort, & Kitchen (2020) analysed the ethanolic extract of sugarcane molasses, detecting the presence of 13 polyphenols and 13 glycosylated polyphenols, which highlights the chemical complexity of molasses.

2.2 Culture media classification

Culture media used in biotechnological processes can be classified into two basic groups: defined and complex media. The use of digests of plant, animal, and/or microbial

products defines a medium as complex, while defined media are formulated using purified inorganic and organic compounds added to purified water (Madigan et al., 2012).

The digests used to formulate complex media (e.g., yeast extract, bovine serum albumin, and molasses) contain several nutrients required for microorganisms and usually promote fast growth and product formation. Often, a digest might be added to a defined base to satisfy a specific nutritional requirement, or several digests might be used to satisfy different needs, such as the case of yeast extract-peptone-dextrose media, used for routine yeast cultivations. In this case, the yeast extract (YE) provides growth factors, macro, and micronutrients; peptone (whether vegetal or animal) provides mainly amino acids; and pure glucose is added as a carbon source for cell growth (Walker, 2014).

However, the chemical complexity of such media may interfere with purification steps after fermentation and the quantification of key components for a process might be hindered. Jinyou Zhang, Reddy, Buckland and Greasham (2003) assessed the performance of 40 lots of yeast extract (YE) from three producers on the production of an antigen by auxotrophic *Saccharomyces cerevisiae* strain for adenine. Both biomass production and antigen production varied up to 2-fold and 3-fold, respectively. Although adenine was a known limiting nutrient in the cultivations, supplementation of the nucleobase to some of the lots did not translate into larger antigen production. Through analysis of chromatographic data, the authors found that trehalose and lactate concentrations on YE were also linked to antigen production, evidencing that several components of a complex media might limit growth and product yield.

Defined media, on the other hand, are prepared by the addition of highly purified inorganic and organic compounds to purified water thus, minimal variation is expected between lots, leading to minimal variability, consequently. Besides production consistency, the main advantages of defined media in commercial fermentation are better fermentation control and monitoring, facilitated scaling up, simplified downstream processes, and easier process validation by regulatory authorities, nonetheless its cost is higher when compared to complex media (J. Zhang & Greasham, 1999). For research activities, the qualitative and quantitative precision of defined media provide reproducibility and allows the conduction of experiments that demand rigid composition control. For instance, defined media have been used to characterize the transcriptional responses of *S. cerevisiae* in chemostats to growth limitation by zinc (De Nicola et al., 2007) and carbon, nitrogen, phosphorus, or sulphur (Boer et al., 2003).

2.3 Yeast nutrition

Media composition plays a major role in any bioprocess, as each organism has unique nutritional requirements to enable growth and product formation, which might not be directly correlated. Apart from oxygen, which is usually fed through aeration to the media, the nutrients found in media, either solid or liquid, can be categorized as: carbon sources; nitrogen sources; minerals; and growth factors (Walker, 2014).

Due to the central role *S. cerevisiae* yeasts have in ethanol production, their interaction with each nutritional group will be covered, relating to the composition of sugarcane molasses.

2.3.1 Carbon source

Carbon sources include carbohydrates, oils and fats, hydrocarbons, alcohols, and proteins. Most industrially relevant microorganisms are chemoorganotrophs, which means that their carbon source is also the energy source for cell activity. Carbohydrates are certainly the most common carbon source in both laboratory and industrial fermentations. Notably, the ethanol industry is based on the fermentation of either starch from corn or sucrose from sugarcane (Stanbury et al., 2016).

Sugarcane substrates present the carbohydrate sucrose as the main carbon source, followed by its monomers, glucose, and fructose, which derive from hydrolysis of sucrose during the treatment steps of the sugarcane juice. In *S. cerevisiae* strains, the monomers enter the yeast cells through facilitated diffusion via hexose transporters and are metabolised via the Embden-Meyerhof-Parnas glycolytic pathway. Sucrose can be hydrolysed in the extracellular environment by invertases secreted by the yeast cell or imported to the cytosol and be hydrolysed intracellularly. Different yeasts strains may exhibit different patterns of sucrose consumption (extracellular hydrolysis or transport followed by intracellular hydrolysis) based on their genetic traits (Marques et al., 2017). For instance, the genome sequencing of Brazilian ethanol producing strains (BG-1, CAT-1, PE-2, SA-1 and VR-1) did not show amplification of the genes encoding extracellular invertase (Stambuk et al., 2009). According to the authors, this suggests that extracellular invertase activity does not limit sucrose fermentation in the Brazilian industrial setting.

2.3.2 Nitrogen sources

Nitrogen sources are either organic or inorganic. Inorganic sources include ammonia gas, ammonium salts, nitrates, and nitrites, although few organisms can assimilate the latter. Organic nitrogen sources are urea, amino acids, or proteins. Usually, by-products from agricultural and food industries, such as corn steep liquor, peptones, and milk proteins, are used as supplement in industrial fermentation media. These materials contain, apart from other nutrients, complex organic nitrogen sources, therefore decreasing the number of compounds that cells would have to synthesize, and increasing growth rates (Kampen, 2014).

The *S. cerevisiae* strains utilize use ammonium ions, urea, amino acids, and small peptides as nitrogen sources, with remarkable effects to the cell metabolism. For instance, Albers, Larsson, Lidén, Niklasson and Gustafsson (1996) observed varying effects on the cell growth and on the extracellular metabolite production parameters in anaerobic batch cultivations using either ammonium salts, glutamic acid or a mixture of amino acids as nitrogen sources. The results for specific growth rate when a mixture of amino acids (0,52 ± 0,04 h^{-1}) was supplied was higher than for ammonium (0,45 ± 0,04 h^{-1}) or glutamic acid (0,33 ± 0,04 h^{-1}). The authors also found differences in the product yields for ethanol, glycerol, acetic acid, succinic acid, α-ketoglutaric acid and fumaric acid. Particularly for ethanol and glycerol yields, important parameters in the industrial ethanol production, were higher when ammonium was the sole nitrogen source.

Molasses and sugarcane based substrates, on the other hand, contain low nitrogen organic levels (Della-Bianca et al., 2013), which are available as proteins, amino acids, pyrrolidine carboxylic acids and other unidentified nitrogenous compounds (Clarke, 2003) and limited data is available on the composition of molasses regarding yeast assimilable nitrogen.

2.3.3 Minerals

Minerals include both some macronutrients such as phosphorous, sulphur, potassium, and magnesium, in a millimolar scale, and micronutrients such as iron, cobalt, molybdenum, copper, manganese, nickel, sodium, chlorine and many other elements, in a micromolar scale.

Usually, these components are found in the feedstocks used for media preparation in sufficient concentrations, however, it may be needed to supplement the media with the appropriate salts to ensure maximal fermentation performance (Walker, 2014).

Among the most important minerals for fermentation processes by yeasts are sulphur, phosphorous, magnesium, potassium, zinc, manganese, copper, calcium, and iron, which have their main roles in yeasts cell metabolism briefly presented on Table 2.

Table 2 – Important minerals and their role in yeast metabolism

Mineral	Role in yeast metabolism
Sulphur	Required for the biosynthesis of sulphur amino acids, such as methionine.
Phosphorous	Present in nucleic acids. Orthophosphate is substrate for many enzymes.
Magnesium	Most abundant intracellular divalent cation, acting primarily as enzyme cofactor. Protective effect on yeast cultures subject to stress conditions such as temperature, osmotic pressure, and high alcohol concentration.
Potassium	Most prevalent cation in yeast cytoplasm.
Zinc	Cofactor for alcohol dehydrogenase
Manganese	Essential for yeast growth and metabolism.
Copper	Cofactor for enzymes involved in electron transfer reactions, such as the pyruvate metabolism
Calcium	Involved in membrane structure and function. Kept at low concentration inside cells
Iron	Cofactor for enzymes involved in cysteine biosynthesis

Source: Adapted from Berterame et al. (2018); Chen et al. (2021); Russel (2003),

The source of these minerals in sugarcane molasses are salts that normally present wide concentration ranges depending on several factors, as previously stated. Lower quality molasses, i.e., more exhausted and containing a higher salt/sucrose ratio, upon dilution to yield the fermentation must – aimed for 200 $g.L^{-1}$ TRS – will contain high dissolved salt concentrations. Ultimately, these high salt concentrations pose an osmotic stress to the yeast cells (Della-Bianca et al., 2013). Additionally, some musts might contain toxic concentrations of metals. For instance, in acidic conditions, aluminium can be converted to the Al^{3+} ion, that negatively affect yeasts, decreasing fermentation rates, ethanol yield, viability and cellular trehalose levels (L. C. Basso et al., 2011).

2.3.4 Growth factors and vitamins

Growth factors are organic compounds such as purines, pyrimidines, nucleosides, nucleotides, amino acids, fatty acids, and sterols. These compounds have catalytic or structural roles in microorganisms which may or may not be able to *de novo* synthesize them (Walker, 2010).

The vitamin requirements and biosynthesis in *S. cerevisiae* growth have been recently reviewed by Perli, Wronska, Ortiz-Merino, Pronk and Daran (2020). Among the most commonly used defined media in yeast research laboratories are Yeast Nitrogen Base (YNB) and a synthetic medium described by Verduyn, Postma, Scheffers, & van Dijken (1992), both media contain at least seven vitamins to support yeast growth: biotin, thiamine, pyridoxine, inositol, nicotinic acid, pantothenate and para-aminobenzoic acid. The YNB also features riboflavin and folic acid as growth factors. The concentration of each component in the media are different, whereas YNB concentrations were defined empirically without quantitative assessment, Verduyn media was optimized to maximize yeast biomass yield in aerobic glucose-limited chemostats under fully respiratory metabolism (Perli, Wronska, et al., 2020). These compounds roles in yeast metabolism, as well as their concentration in YNB and Verduyn media are presented on Table 3.

Molasses is regarded as rich in some of these vitamins. Piggot (2003) states that among these, biotin is present in molasses in great excess, pantothenate in near adequate concentrations and inositol may be deficient. Data presented by Walker (2014) for the composition of an average cane molasses indicate the presence of all the vitamins mentioned above except for para-aminobenzoic acid. However, these data are given for an average molasses sample and as observed for all the other components of molasses, large concentration ranges for some of these compounds is expected.

2.4 Synthetic media

Generally, the development of a chemically defined media involves obtaining maximum values for important fermentation parameters (e.g., specific growth rate, product concentration or productivity), by providing all the necessary nutrients in the perfect ranges to enable cell activity. However, the fermentation of natural substrates does not necessarily achieve these maximum values, thus the development of a synthetic media to simulate a natural substrate should consider these “imperfect” results.

Table 3 – Concentration of the seven growth factors needed.

	Concentration (mg. L^{-1})		Roles in the metabolism and bioavailability
	Verduyn	YNB	
Pyridoxine	0.82	0.4	Cofactor for enzymes involved in amino-acid, glucose, and lipid metabolism.
Thiamine	0.79	0.32	Thiamine biosynthesis and regulation. May also be available as pyridoxal, pyridoxamine and their phosphorylated derivatives.
Biotin	0.05	0.002	Thiamine diphosphate derivative is cofactor for several enzymes. Thiamine monophosphate and triphosphate can also be found intracellularly
Pantothenic Acid	1	0.4	Coenzyme in carboxylases involved in sugar and amino acids metabolism and in fatty acid synthesis.
Para-aminobenzoic acid	0.2	0.2	Precursor for synthesis of coenzyme A and acyl carrier protein.
Nicotinic Acid	1	0.4	Intermediate in the biosynthesis of folates, which are cofactors involved in amino-acid metabolism and nucleic acid formation.
Inositol	25	2	Precursor for nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP).
			Precursor for a main constituent of phospholipid membrane (phosphatidylinositol) and anchor proteins (glycosylphosphatidylinositol). There are 8 inositol stereoisomers, myo-inositol is the most common.

Source: Perli, Wronska, et al. (2020)

An example of the development of synthetic media simulating a natural substrate is the synthetic grape juice. Bely, Sablayrolles and Barre (1990) proposed a synthetic medium to simulate a standard grape juice, which composition is listed on Table 4. The effect of different levels of assimilable nitrogen (the sum of ammoniacal and alpha amino nitrogen) on fermentation duration, maximum CO_2 production rate, maximum cell concentration and the specific CO_2 production rate were assessed using a standard grape juice and a synthetic composition. The results obtained for the synthetic media were similar to the natural and this composition has since been used with different assimilable nitrogen content and amino acid distribution.

The establishment of a basal synthetic media that mimics a natural media used for wine fermentation facilitates researchers to study the effect of compositional changes in the process. For instance, Alegre, Culleré, Ferreira and Hernández-Orte (2017) used different amino acid compositions and concentrations, simulating different grape varieties, to evaluate the consumption of odourless polyfunctional mercaptans precursors by yeasts. Using this approach, they were able to identify amino acid profiles that alter yeast metabolism during alcoholic fermentation and promote conversion of odourless to aromatic mercaptans.

Table 4 – Synthetic medium simulating a standard grape juice proposed by Bely, Sablayrolles and Barre (1990)

Nutritional group	Compound and concentration
Carbon source (g)	Glucose (200),
Organic Acids (g)	Citric Acid (6), D-L Malic acid (6),
Nitrogen Sources (mg)	NH4Cl (18.6), L-Proline (20.5), L-Glutamine (16.9), L-Arginine (1.25), L-Tryptophan (6), L-Alanine (4.9), L-aspartic acid (4), L-valine (2.6), L-phenylalanine (1.3), L-isoleucine (1.1), L-histidine (1.1), L-methionine (1.1), L-tyrosine (0.6), L-glycine (0.6), L-lysine (0.6), L-cysteine (0.4)
Minerals (mg)	KH_2PO_4 (750), KH_2SO_4 (500), $MgSO_4.7H_2O$ (250), $CaCl_2.2H2O$ (155), NaCl (200), $MnSO_4.H_2O$ (4), $ZnSO_4$ (4), $CuSO_4.5H_2O$ (1), KI (1), $CoCl_2.6H_2O$ (0.4), H_3BO_3 (1), $(NH_4)_6Mo_7O_{24}$ (1)
Vitamins and anaerobic growth factors(mg)	Myo-inositol (20), Nicotinic Acid (2), Calcium Pantothenate (1.5), Thiamine Hydrochloride (0.25), Pyridoxine Hydrochloride (0.25), Biotin (0.003), Ergosterol (15), Sodium Oleate (5), Tween 80 (0.5 ml)

Source: Bely, Sablayrolles and Barre (1990)

Contributions of this media to the understanding of wine fermentation include its use on studies using labelled nitrogen (^{15}N) and carbon (^{13}C). Crépin et al. (2017) conducted

parallel fermentations using the same media, labelling a single nitrogen compound with either a ^{15}N or ^{13}C. This enabled the author to determine the fate of the amino acids for the synthesis of either protein or higher alcohols. The same approach was used by Rollero et al. (2019) to compare the metabolic fluxes of *S. cerevisiae* and *Kluyveromyces marxianus.*

However, the effects of the chemical complexity of a natural substrate and the effects of the treatment processes they are submitted to should not be underestimated. Viana, Loureiro-Dias and Prista (2014) found that changing concentration of organic acids in the media (malic from 6 to 3 $g.L^{-1}$, citric from 6 to 0.3 $g.L^{-1}$ and tartaric from 0 to 3 $g.L^{-1}$) in the media were necessary to replicate the residual sugar levels obtained in the fermentation of grape juice from the Arinto variety. They also added potassium metabisulfite to the medium, to replicate the process usually employed on natural grape juices.

2.4.1 Synthetic molasses

There have been attempts to establish a synthetic defined medium to mimic the conditions imposed by sugarcane molasses on the ethanolic fermentation. Chandrasena, Walker and Staines (1997) proposed a "synthetic molasses" medium while studying the effects of potassium, calcium, magnesium, and zinc concentration on ethanol production by a distiller's strain and fitted the data obtained using quadratic response surface models. However, the results obtained from the fermentation of authentic molasses samples were not within the values predicted by the models. The authors credited the errors to the possibility that the models could not be valid for molasses with high levels of some cations, and to the presence of other compounds in molasses could directly affect ethanol production.

More recently, a semi-defined molasses medium was proposed by Lino, Basso and Sommer (2018) adapted from the composition reported by Chandrasena et al. (1997). The most prevalent organic acids, aconitic and malic acids, were added to the media in proportions usually found in molasses and citric acid concentration was left unchanged. The total nitrogen amount was adjusted by the addition of peptone and reduction of ammonium sulphate concentration, achieving a ratio between ammoniacal and organic nitrogen usually found in sugarcane molasses. Concentrations of magnesium, potassium and calcium were adjusted to match the concentration of an average sugarcane molasses, and consequently, the sulphate and iodide concentrations. The carbons sources were adjusted, substituting 20 % of the sucrose content for the addition of equal amounts of fructose and glucose to match the average composition of molasses. Another feature of this medium was the simulation of the formation of Maillard reaction products. To perform this step, the authors proposed the

preparation of a "concentrated sugar stock", formulated with the following concentrations: sucrose (432 $g.L^{-1}$), glucose (54 $g.L^{-1}$) and fructose (54 $g.L^{-1}$), glutamine (57 $g.L^{-1}$), aspartic acid (33 $g.L^{-1}$) and asparagine (432 $g.L^{-1}$). This solution was then heated to 121 ºC for 15 min in an autoclave, yielding a solution that contained Maillard reaction products. The compositions proposed by Lino et al.(2018) and Chandrasena et al. (1997) are listed on Table 5.

Table 5 – Composition of synthetic molasses compositions proposed by Lino et al. (2018) and Chandrasena et al. (1997)

		Author	
		Lino et al. (2018)[1]	Chandrasena et al. (1997)
Carbon sources (g)	Glucose	18	-
	Fructose	18	-
	Sucrose	144	180
Organic Acids (g)	Trans-aconitic acid	2	
	Citric Acid	0.001	0.001
	D-L Malic acid	1	
Minerals (mg)	$MgSO_4.7H_2O$	1002	Variable
	$CaCl_2.2H_2O$	67.12	Variable
	NaCl	500	500
	$MnSO_4.H_2O$	0.4	0.4
	$ZnSO_4$	0.4	0.4
	$CuSO_4.5H_2O$	0.04	0.04
	KCl	12	Variable
	KI	0.1	0.1
	H_3BO_3	0.5	0.5
	$FeCl_3 \cdot 6H_2O$	0.17	0.2
	$H_2Mo_4.H_2O$	-	0,16
	$Na_2MoO_4.2H_2O$	0.31	-
Vitamins and anaerobic growth factors(mg)	Myo-inositol	10	10
	Nicotinic Acid	10	10
	Calcium Pantothenate	1	1
	Thiamine Hydrochloride	0.04	0.04
	Pyridoxine Hydrochloride	0.04	0.04
	Biotin	0.01	0.01
	Para-aminobenzoic acid	2	2
	Ergosterol	2 mL*	2 mL*
Nitrogen Sources (mg)	$(NH_4)_2HPO_4 \cdot 4H_2O$	1420	-
	$(NH_4)_2SO_4$	100	5000
	$(NH_4)_2HPO_4$	-	1420
	Peptone	4900	-

*6% w/v ergostel in ethanol: [1]: The quantified values reported solely regard the nutrients added as pure salts. Source: Adapted from Lino et al. (2018) and Chandrasena et al. (1997)

To test the suitability of this medium, the synthetic molasses was used in small scale fermentations mimicking the fed-batch process with cell recycle used for ethanol production in Brazil (with 4 cycles), using the yeast strains PE-2 and Ethanol Red™. Two Brazilian and one Indian molasses were benchmarked against the proposed medium. The theoretical ethanol yield values were found to vary even between the industrial samples (from 78 % in the Indian to 95 % in one Brazilian molasses) and the synthetic molasses results were statistically similar to at least one of the benchmark media in each cycle. The biomass increase between cycles was kept below 10%, as in industrial fermentations (Della-Bianca et al., 2013). Results for yeast viability were also statistically similar between the synthetic and at least one industrial molasses. The major differences were found in the glycerol production, whereas the Indian molasses showed significantly lower values than both Brazilian molasses and the synthetic composition.

These attempts to reproduce industrial media with synthetic laboratory alternatives have several advantages, including the validation of yeasts in industrially relevant conditions, the evaluation of the influence of specific nutritional groups on cell metabolism. Thus, this work aims at the development of a defined synthetic medium more appropriate to represent sugar cane molasses, starting from formulations previously reported in the literature.

3 OBJECTIVES

This work objective is to develop a defined media that mimics sugarcane molasses in fermentation for ethanol production. For so, the specific objectives include:

- To investigate reported synthetic media (Lino, Basso and Sommer, 2018) in different strains of *S. cerevisiae* and compare it to industrial molasses samples in batch fermentations;
- To develop a defined media that appropriately mimic molasses-based media fermentations used in fuel ethanol production;
- To assess the impact of individual media components (such as nitrogen source, vitamins, trace-elements, etc.) in batch fermentations;
- To validate the defined medium in fed-batch fermentations, comparing its results to the results obtained by the fermentation of industrial molasses samples.

4 MATERIALS AND METHODS

4.1 Industrial molasses samples

All molasses samples used in this work were kept in 5 ºC refrigerators prior to use. The samples were diluted with reverse osmosis (RO) water to yield a 500 g molasses per litre solution, and then centrifuged at 2000 g for 15 minutes to remove insoluble materials that could interfere with spectroscopic and gravimetric methods used thereafter. A handheld refractometer was used to measure the refractive index of the supernatant and its value was adjusted to 20 ºBrix before sterilization at 121 ºC for 20 min (Raghavendran et al., 2017).

Five industrial molasses samples were used as a benchmark to compare the results obtained with the proposed synthetic molasses developed. Table 6 presents the 5 industrial molasses used, their origin sugarcane mill, and their collection date.

Table 6 – Molasses samples origins and collection dates

Mill of origin	Collection date
Raízen Univalem	June 2019
Pedra	June 2019
Iracema	September 2017
São José	October 2014
Lucélia	July 2017

Source: The author

4.2 Media preparation

4.2.1 Semi-defined synthetic molasses Lino et al. (2018) preparation

Lino et al. (2018) proposed the preparation of the media by mixing three stock solutions to phosphate buffer saline. A 3x concentrated *"Sugar Stock"* solution, containing sugars and amino acids to simulate the formation of Maillard reaction products by autoclaving, a 5x concentrated macro-nutrient solution containing all the components up to 0.1 $g.L^{-1}$ and a micronutrient solution 100x concentrated, containing components below 0.1 $g.L^{-1}$. However, to enable the evaluation of different synthetic molasses compositions, the macronutrient solution in the present work was split into Broth Stock 5x concentrated (containing peptone, $(NH_4)_2SO_4$, $(NH_4)_2PO_4.4H_2O$, NaCl, $MgSO_4.7H_2O$, $CaCl_2.2H2O$ and KCl) and Organic acids stock solution 5x concentrated (containing trans-Aconitic acid, L-Malic acid and citric acid). The micronutrient solution was also split into Vitamin stock

solution 100x concentrated (inositol, nicotinic acid, calcium pantothenate, biotin, pyridoxine hydrochloride, thiamine hydrochloride and para-aminobenzoic acid) and Trace elements stock solution 100x concentrated (H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $FeCl_3.6H_2O$, $Na2MoO_4.H_2O$, KI and $CuSO_4.5H_2O$). The *"Sugar Stock"* preparation was kept the same as described in the referred work.

4.2.2 Proposed medium preparation

The medium preparation was based on the mixing at different proportions of the various sterilized stock solutions. Sugar stock described by Lino et al. (2018) was mixed in a 1:4 ratio to a filter sterilized (0,22 µm pore diameter) 500 $g.L^{-1}$ solution to yield a new sugar stock solution, containing 80% sucrose that would be hydrolysed if heat sterilization was used. Vitamin and trace elements solutions were prepared according to Verduyn et al. (1992). The "Salt Stock" (K_2SO_4, $MgSO_4$), "Organic acids Stock" (trans-Aconitic, L-Malic, citric acids and KOH) and "Calcium Stock" ($CaCl_2$) solutions were heat sterilized at 121 ºC for 15 min. "Inorganic nitrogen Stock" solution was filter sterilized (0,22 µm pore diameter) to avoid thermal degradation.

The amino acids solution used was prepared by dissolving the amino acids (glutamine, aspartic acid and asparagine) in reverse osmosis water and had its pH adjusted to 5 prior to sterilization by vacuum filtration through 0,22 µm pore diameter sterile membranes.

4.3 Yeast strains

Industrial strains of *S. cerevisiae* PE-2 and SA-1 were kindly provided by Prof. Luiz Carlos Basso (ESALQ-USP, Brazil), and the laboratory *S. cerevisiae* strains CEN.PK113-7D, haploid, and CEN.PK122, diploid, were kindly provided by Prof. Andreas Gombert (Unicamp, Brazil) and Dr. Gleidson Teixeira (Unicamp, Brazil), respectively. The CEN.PK family was chosen for being reference strains for physiologic studies.

The strains were stocked under – 80ºC in YPD medium (1% yeast extract, 2% peptone and 2% glucose) containing 20 % glycerol. Each strain was grown overnight in YPD medium (1%yeast extract, 2% peptone and 2% glucose), and glycerol was added to achieve a final ~ 20% glycerol concentration and stocked in – 80 ºC freezer.

4.4 Growth kinetics assays

4.4.1 Microplate assays

The inoculum for microplate assays was prepared by inoculating a loopful of yeast cells from a – 80 ºC stock culture in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). The culture was then incubated at 30 ºC in an orbital shaker at 150 rpm overnight. Then, 0.1 mL of this cell suspension was added to a fresh YPD medium, incubated similarly as before, and growth was monitored by reading the optical density at 600 nm (OD_{600nm}). Upon reaching the exponential growth phase, cells were harvested and washed three times with sterile reverse osmosis water to avoid interference from micronutrients present in the YPD medium. A microplate reader (Tecan Infinite, model M200PRO) was used to monitor growth in 10 minutes intervals at 30 ºC in different media by incubating 20 µL of the washed cell suspension in 180 µL of each molasses media. Ten readings were done in each well and the OD_{600nm} values represent the mean of these readings. All experiments were done in triplicates.

4.4.2 Growth kinetics parameters calculation

To determine the maximum specific growth rate (µ), the natural logarithm of OD_{600nm} values were plotted against time and a linear regression model was adjusted to the region of the curve with highest slope. Deceleration time (t_{decel}) was determined as the difference between the time when maximum absorbance was reached minus the time of the last point in the exponential phase used to determine (µ). A script was developed on Python to allow the determination of the several µ, OD_{max} and t_{decel} values with minimal bias.

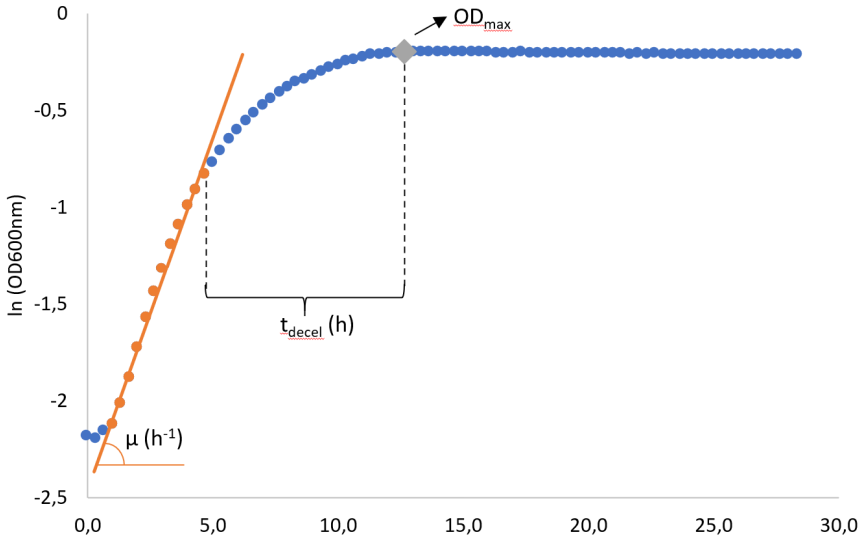
4.5 Scaled down fed batch fermentations

Fed-batch fermentations with cell recycle were conducted in 50 mL centrifuge tubes, as described by Raghavendran et al. (2017), reproducing conditions usually encountered in industrial sugarcane based substrate distilleries.

For cell propagation, yeast colonies grown on YPD agar plates were inoculated into 250 mL Erlenmeyer flasks containing 100 mL YPD broth (with 40 $g.L^{-1}$ glucose) and were incubated at 200 rpm, 30 ºC. After 24 h, the resulting cell suspension was transferred to 2 L reagent flask containing 1L diluted molasses (100 $g.L^{-1}$) and incubated at 30 ºC without agitation, expect for occasional stirring for gas release. After 48 h, the flask contents were centrifuged, yielding a wet biomass and wine. Approximately 4g of wet biomass were

resuspended in 2 mL of wine and 6 mL of water into 50 mL centrifuge tubes, emulating the yeast cream from continuous centrifugation employed in industrial plants. The resulting cell suspension was then used as inoculum for the first fermentation cycle.

Figure 3 - Visual representation of the calculated parameters.



Source: The author

The fermentations were conducted at 32 ºC without agitation with three additions (9,25 mL) of fermentation medium, either industrial molasses or synthetic molasses, containing 180 $g.L^{-1}$ TRS at 0h, 2h and 4h of fermentation. Fermentation was monitored by weighting the tubes every hour for 10 h and then left at 25 ºC overnight. In the following day, the tube contents were homogenised, 1 mL samples were collected for microbiological analysis and the contents of the centrifuge tubes were centrifuged at 2000 g for 15 min, yielding the first cycle wine and a wet biomass. This biomass was resuspended with 2 mL of wine from the first cycle and 6 mL water, and the resulting suspension underwent acid treatment, by lowering its pH to 2.5, by the addition of H_2SO_4 0.5 M, for 1 hour. After this treatment, the second cycle was started by the addition of fresh fermentation medium as previously described for the first cycle and the same procedure was repeated 4 times, totalling 5 cycles. Wines from each cycle were frozen at -20 ºC for metabolite analysis.

The evaluated parameters after each cycle were: yeast mass; wine pH; yeast viability; glycerol concentration, and ethanol yield, as % of the theoretical maximum, calculated by Equation 1 (Raghavendran et al., 2017).

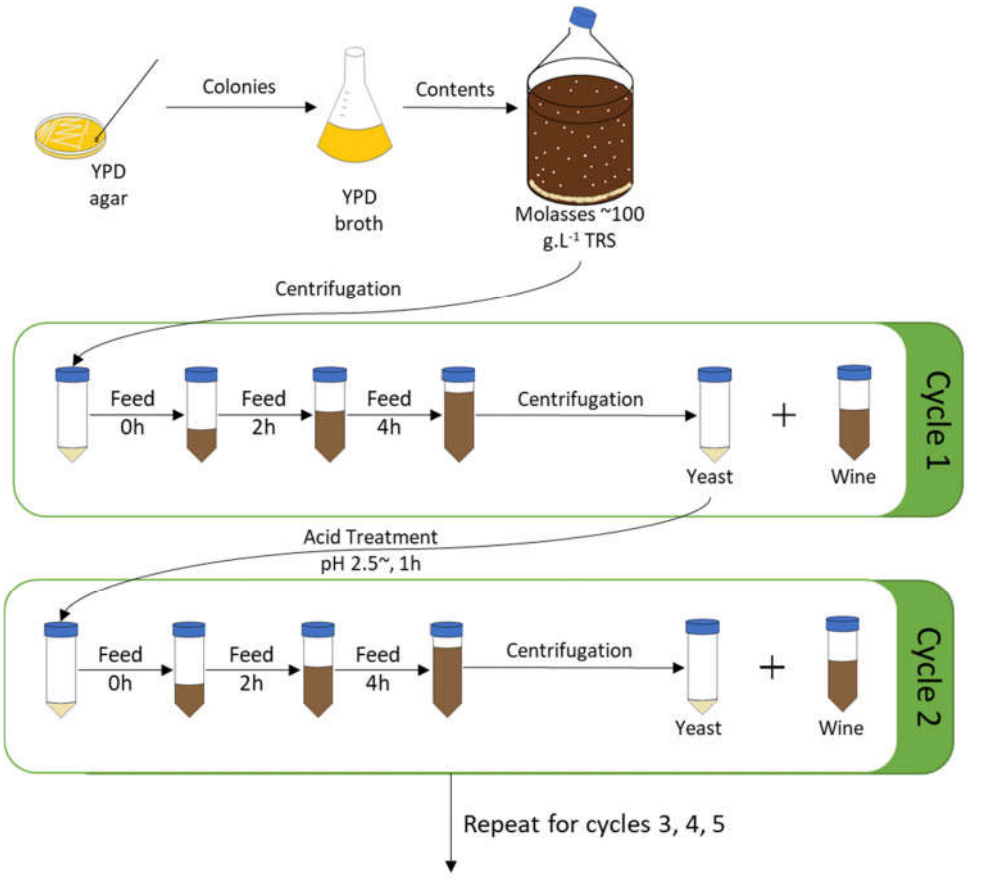
$$Et\ \ anol\ yield_{cycle\ n}\ (\%) = 100.\frac{\left(V_{wine} + 0.7M_{pellet}\right).C_{eth}\quad\left(2 + 0{,}7M'_{pellet}\right).C'_{eth}}{0{,}511.\left(27{,}75.10^{-3}.C_{TRS}\right)} \qquad (1)$$

In which V_{wine} represents volume of wine from cycle n [L], M_{pellet} is the biomass from cycle n [g], M'_{pellet} is the biomass from cycle n 1 [L], C_{eth} is the ethanol concentration of wine from cycle n [g.L^{-1}], C'_{eth} is the ethanol concentration of wine from cycle n 1 [g.L^{-1}], 0.7 represents an estimate of ethanol concentration in the biomass, 2 represent the volume of wine from cycle n 1 used to resuspend the biomass at the beginning of cycle n. C_{TRS} is the total reducing sugar concentration in the fermentation medium, 27,75 is the volume of wine added to the tubes, and 0,511 represents the maximum mass conversion of hexoses to ethanol. All fermentations were done in triplicates, a simplified representation of the experiment is presented on Figure 4.

4.6 Substrate and extracellular metabolites quantification

Liquid samples filtered through 0,22 µm syringe filter were used to determine concentration substrate in culture media and metabolites produced in fermentation. A high-performance liquid chromatography (HPLC) system (Shimadzu Prominence) with refractive index detector. Glucose, fructose, glycerol, and ethanol in fermentation samples, and trehalose in TCA extracts were separated using a Bio-Rad HPX-87H column at 60 ºC with H_2SO_4 5 mM as eluant with 0.6 mL.min^{-1} flow rate. A Bio-Rad HPX-87C column kept at 85 ºC with ultrapure water (Milli-Q) as eluant with 0.6 mL.min^{-1} flow rate was used to separate and determine concentrations of sucrose, glucose and fructose on molasses and medium samples. For both columns' injection volume was 10 µL.

Figure 4 – Simplified schematic representation of scaled down fed-batch fermentation with cell recycle.



Source: The author

4.7 Statistical analysis

All statistical analyses were done using Minitab®, United States of America.

4.7.1 Analysis of variance

One-way ANOVA and Tukey honest significant difference were used to differentiate mean values when variances statistically not different (Bartlett test, $p > 0.05$) and Welch's Test and Games–Howell Tests were used when variances were statistically different (Bartlett test, $p \leq 0.05$). Differences were considered statistically significant at the level of 0.05.

4.7.2 Response surface

A 3^3 full factorial design with replicates with factors vitamin, ammonium and amino acids concentration were used to obtain a second-order polynomial function for maximum

specific growth rate, OD_{max} and deceleration time. Coefficients with p-value <0.05 were considered not significant but were not removed from the model.

5 RESULTS AND DISCUSSION

5.1 Adaptation of the semi-defined synthetic molasses proposed by Lino et al. (2018)

5.1.1 Defining the sugar composition in the proposed synthetic medium

Sugarcane molasses usually contain 45 to 60 % w/w sucrose and 5 to 20 % of fructose and glucose (Della-Bianca et al., 2013). Table 7 displays the sugar concentrations of fermentation media prepared by diluting five real molasses samples to yield approximately 20 $g.L^{-1}$ total reducing sugars (TRS) solutions. All molasses samples were obtained from Brazilian sugarcane mills, and confirming previously reported data, sucrose represented 74.5-88.6 % of the total sugar content, with glucose and fructose as the remaining sugars, with their sum ranging from 11.4 to 25.5 %. On the other hand, in the semi-defined synthetic molasses (LSM) previously reported (Lino et al., 2018), sucrose content was significantly lower (10.3 %) with increased fructose and glucose values (48.8% and 40.9 %, respectively), as shown on Figure 3.

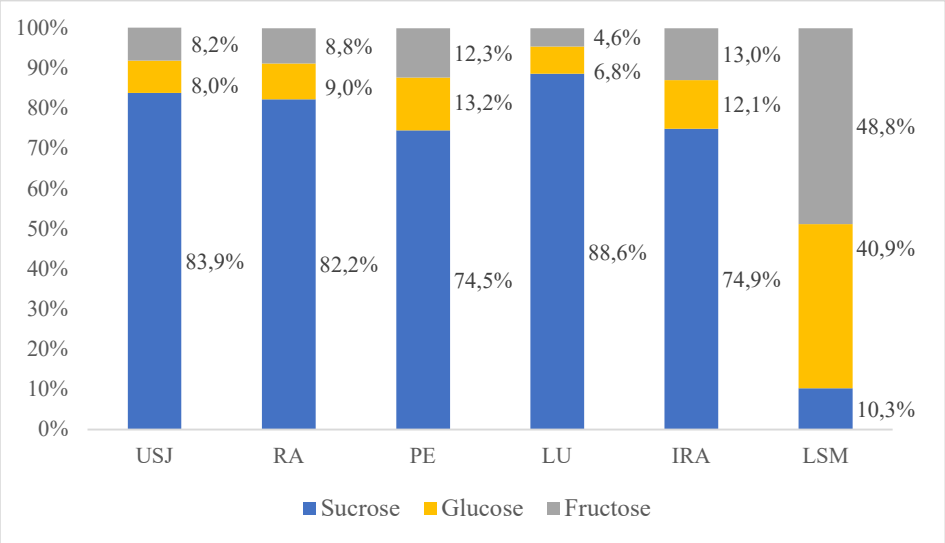
Table 7 - Sugar concentrations ($g.L^{-1}$) of different molasses and the LSM

Sample Name	Sucrose	Glucose	Fructose
São José	15.15	1.52	1.55
Raízen	16.05	1.84	1.81
Pedra	14.40	2.68	2.50
Lucélia	15.38	1.25	0.84
Iracema	14.33	2.44	2.63
LSM	1.65	6.89	8.22

Source: The author

As the sugar stock preparation procedure in LSM involves heating the sugar and amino acid mixture for 15 minutes at 121 ºC in autoclave, it should be expected that sucrose would undergo inversion, yielding glucose and fructose (Eggleston & Amorim, 2006). Thus, an alternative preparation of synthetic molasses should consider adjustment of the sucrose composition to match that of real molasses, since these sugars act differently in yeast metabolism and may have technological impacts on the fermentation process (T. O. Basso, 2011).

Figure 5 – Sugar content of each molasses sample- Usina São José (USJ), Raízen (RA), Pedra (PE), Lucélia (LU), Iracema (IRA), semi-defined synthetic molasses (LSM)



Source: The author

Therefore, the main features to be considered when preparing the sugar stock solution are the following:

- Sugar stock solution concentration is 432 $g.L^{-1}$ of sucrose, 54 $g.L^{-1}$ of glucose, and 54 $g.L^{-1}$ of fructose Lino et al. (2018). As this sugar stock is further diluted with the other stock solutions, decreasing its concentration would decrease upper TRS concentration of the final prepared media.
- Sucrose inverts at high temperatures, thus heat sterilizing should lead to different concentrations of sucrose. Filter sterilizing up to 500 $g.L^{-1}$ of TRS is a possible alternative to avoid sucrose inversion.
- The heating process leads to the presence of Maillard reaction in sugarcane juice processing, its products lead to many compounds that are supposed to negatively affect the metabolism of yeasts and contribute with color to the final medium.

In this case, the proposed solution to match the composition of sugars (in terms of sucrose, glucose, and fructose) in industrial molasses is to use a filter sterilized 500 $g.L^{-1}$ sucrose stock solution supplemented with the “autoclaved” stock solution. This step was performed in order to the sugar composition of industrial molasses, setting as target a composition of: 80% (w/w) sucrose, 10% (w/w) glucose, and 10% (w/w) fructose, in terms of

relative sugar fraction. Using this approach, the new sugar stock solution would be a mixture of 80% of filter sterilized sucrose (500 g/L) solution and 20% of the stock solution proposed by Lino et al. (2018) which simulates the formation of Maillard reaction products.

5.1.2 Issues of the "Broth stock" solution

The "Broth Stock" solution used in the LSM formulation includes the components shown on Table 8, from the protocol provided by the author (Lino et al., 2018). A precipitate was formed during the preparation of this solution, most probably due to the presence of phosphate from the diammonium hydrogen phosphate (DAP) and calcium and/or magnesium salts, as all phosphates are insoluble except those of sodium, potassium, and ammonium. In addition, some hydrogen phosphates, such as $Ca(H_2PO4)_2$, are soluble (Vogel, 1957). The formation of a precipitate of any sort in a liquid medium is undesirable, affecting both the homogenization and the final concentration of the salts in the liquid phase. In this case, phosphorous, magnesium and/or calcium concentrations might not be within the values proposed for the final medium.

Table 8 – Broth stock proposed by Lino et al (2018)

Component	Concentration ($g.L^{-1}$)
Peptone	24.5
$(NH_4)_2SO_4$	0.5
$(NH_4)_2PO_{4.4}H_2O$	8
NaCl	2.5
$MgSO_{4.7}H_2O$	5.01
$CaCl_{2.2}H2O$	0.335
KCl	0.06

Source: Adapted from Lino et al. (2018)

To avoid the precipitation of phosphates, concentrated solutions containing this anion should not be mixed with any solution containing bivalent cations (Kampen, 2014), at least until the final preparation, when values should be below the precipitation threshold.

Additionally, the broth stock also contains peptone, which is a class of product made from the chemical or enzymatic hydrolysis of either animal or plant tissues, containing sources of nitrogen such as amino acids, peptides, and proteins (Stanbury et al., 2016). As a result, these products are subject to variation in composition depending on the feedstock used,

making their use unfavourable when the exact composition of the media is a desirable feature. Thus, in the preparation of a "defined synthetic molasses" the substitution of peptone for other organic nitrogen sources, such as a defined mixture of amino acids or urea, would be more appropriate.

Another feature of this solution is the combination of both of nitrogen sources and inorganic salts in the same stock solution. Each of this class of nutrients have very distinct effects on yeast metabolism (Walker, 1998) and their concentration on molasses are not expected to be directly correlated, as low-nitrogen molasses and high-nitrogen molasses can yield musts with the same sugar concentration. Then, it would be convenient to split the "Broth stock" solution into a "Nitrogen stock" solution, containing the inorganic nitrogen source of the medium, and another solution or solutions which would contain the other macronutrients (mainly magnesium, potassium, and sulphur) in the broth stock. Two different solutions might also enable physiological studies accounting the effect of different ratios between inorganic and organic nitrogen sources.

It is important to highlight that phosphate buffer saline (PBS), used to dilute the stock solutions of LSM, also contain phosphate salts. Nevertheless, no visible formation of precipitate occurs when the stock solutions are added to the buffer. Regardless of that, it would be more practical if all the stock solutions were diluted directly in reverse osmosis water to increase accuracy in mineral concentration.

5.1.3 Evaluation of vitamin concentration

Given the harsh temperature conditions and the wide pH variations which sugarcane juice is submitted during sugar processing to yield molasses (Dias et al., 2015), it would be expected that some vitamins, which are prone to thermal and chemical degradation (Combs & McClung, 2017), might be deficient in industrial molasses. Consequently, it was hypothesized that the vitamin concentration in the LSM would be superior to the average of real molasses.

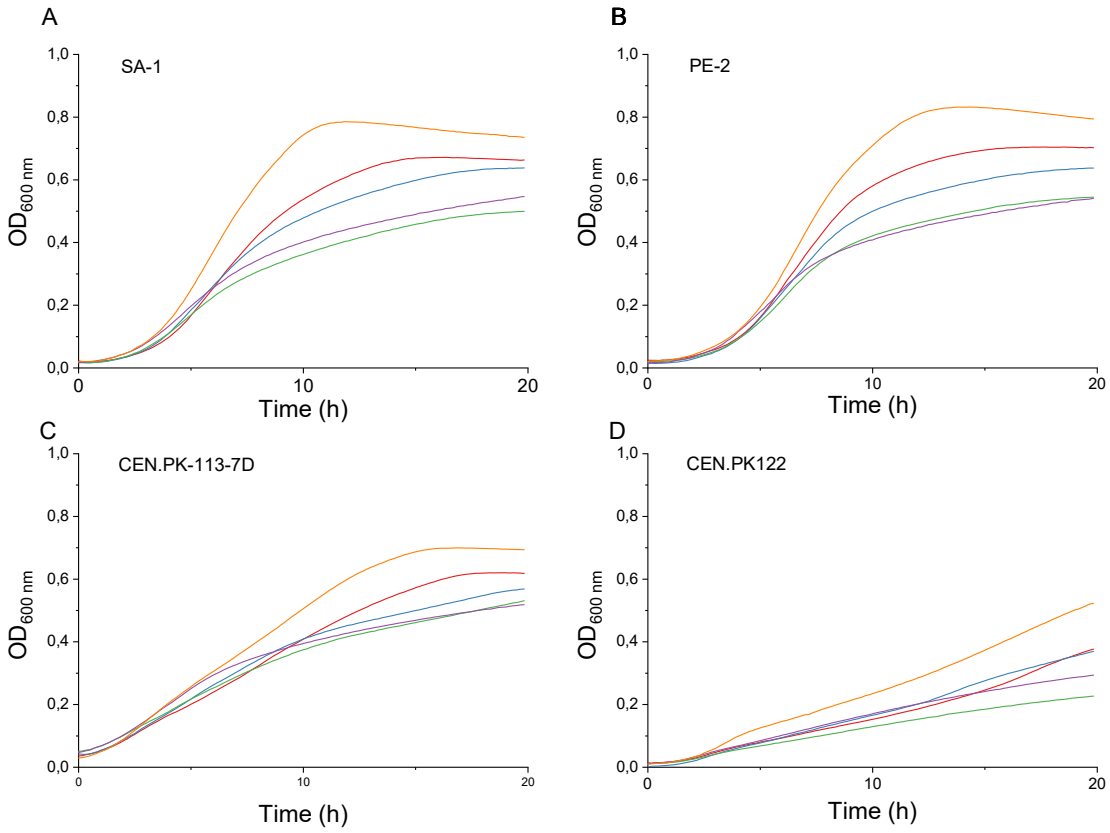
To assess whether this hypothesis was true, batch fermentations of CEN.PK122, CEN.PK113-7D, SA-1 and PE-2 strains were conducted on 96-wells microplates in a microplate reader using the five industrial molasses and the LSM medium, both diluted to 20 $g.L^{-1}$ TRS, without vitamin supplementation. In additional experiments, the industrial molasses were supplemented with a stock solution of vitamins based on the Verduyn defined media (Verduyn et al., 1992), and the vitamin stock added to the LSM medium was diluted 10-fold. Therefore, the impact of vitamin deficiency in these strains was evaluated, since two

of them are reference laboratorial strains and two were isolated from industrial environments, vitamin requirements could be different among them.

The results obtained for unsupplemented molasses show different growth behaviour of industrial and laboratory strains. The later showed an almost linear growth as opposed to the presence of an better defined exponential growth phase on the industrial strains (Figure 6). Also, the figure shows the different behaviour in molasses to each strain, indicating the fluctuation of composition in molasses. For all strains, Iracema molasses promoted a higher final OD_{600} values, followed by Raízen, Pedra, Lucélia and São José, as the sugar concentration for all molasses were within a close range, a nutrient other than the sugars might be limiting cell growth.

To futher investigate this observation, vitamins were added to the industrial molasses media in concentrations proposed by Verduyn et al. (1992) in its classical yeast defined medium. Interestingly, vitamin supplementation showed no apparent effect on the growth curves of the industrial strains, PE-2 and SA-1 (Figure 7A and 7B). On the other hand, vitamin supplementation promoted faster growth of the laboratory strains (Figure 7C and 7D) as compared to the not supplemented molasses media (Figure 6), making their growth curves similar to that of the industrial strains on pure molasses. The shape of the growth curves in supplemented molasses was also closer to the well-characterized yeast growth behaviour with clear lag, exponential, and deceleration phases (Walker, 1998).

Figure 6 - Growth curves for strains SA-1 (A), PE-2 (B), CEN.PK113-7D (C) and CEN.PK112 (D) media prepared with molasses from different sugar mills. ( ) Iracema, ( ) Raízen, ( ) Pedra, ( ) Lucélia, ( ) São José.

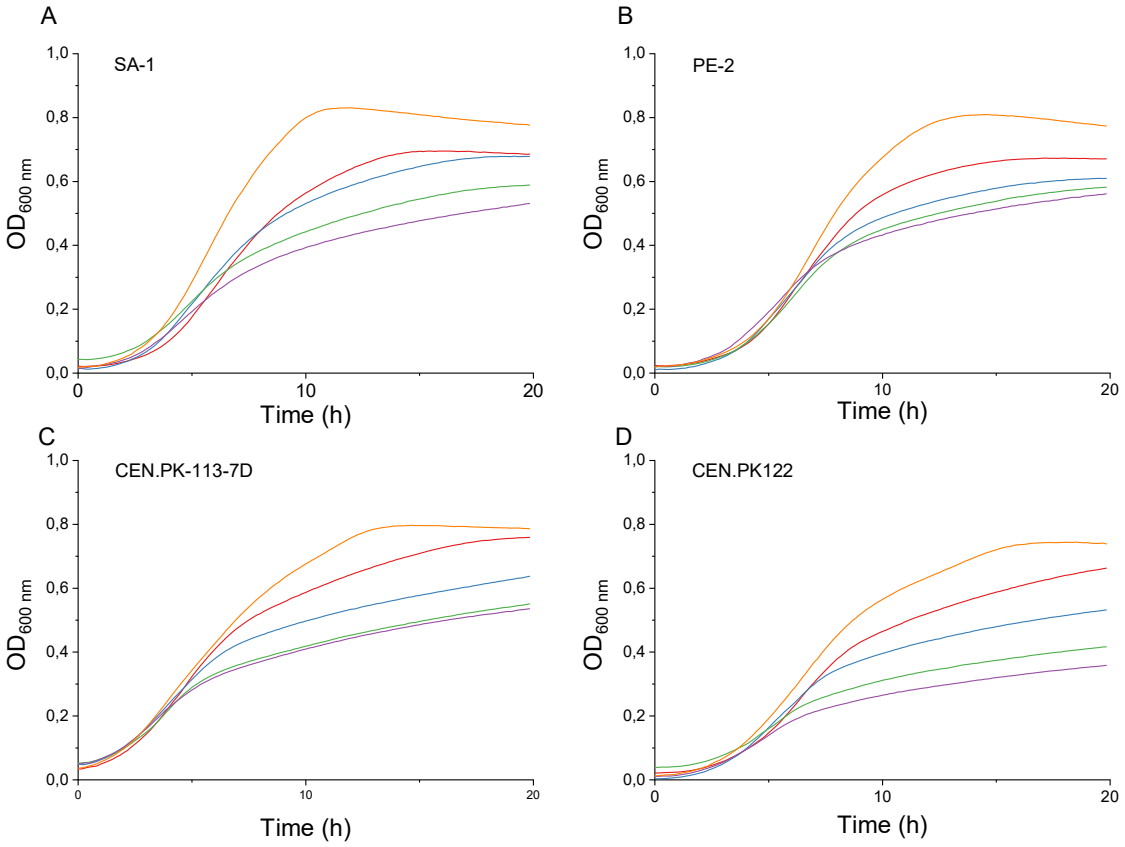


Source: The author

Although unexpected, these set of results may indicate that industrial yeast strains isolated from sugarcane-based ethanol plants might have a lower vitamin requirement as compared to laboratory strains. It also points to the conclusion that vitamins might be deficient in molasses media, at least for these two laboratory strains. In fact, (Stambuk et al., 2009) reported that industrial fuel ethanol strains CAT-1, PE-2, VR-1, BG-1, and SA-1 contain amplification of gene pairs *SNO2/SNZ2* and *SNO3/SNZ3*, involved in the biosynthesis of thiamine and pyridoxine (vitamins B_1 and B_6, respectively), two of the vitamins present in the vitamin stock solutions used (Verduyn et al, 1992). A more recent study clarified the interaction between the *SNZ* gene paralogs, demonstrating that *SNZ2* and *SNZ3* were sufficient to generate growth on a minimal vitamin media (only biotin and D-pantothenic acid hemicalcium salt were added as vitamins) (Paxhia & Downs, 2019). Therefore, eventual

genetic modifications, such as the increased copy number of *SNZ2/SNZ3* genes, could explain industrial strains faster growth on non-supplemented molasses.

Figure 7 - Growth curves for strains SA-1 (A), PE-2 (B), CEN.PK113-7D (C) and CEN.PK112 (D) on media prepared with molasses from different sugar mills supplemented with vitamins from Verduyn media (10x diluted to match the range of concentration in LSM). ( ) Iracema, ( ) Raízen, ( ) Pedra, ( ) Lucélia, ( ) São José.

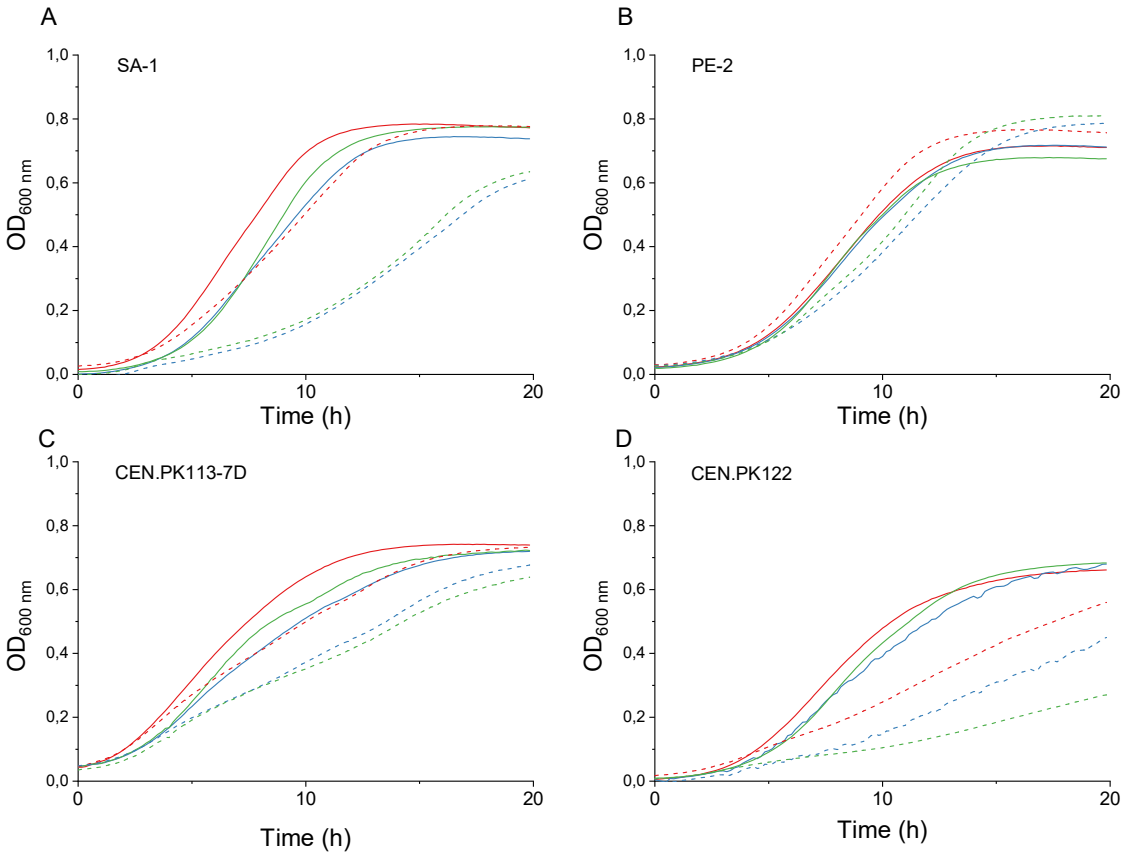


Source: The author

To assess whether the growth on the LSM was similar to that observed on molasses, the same strains were grown on LSM without modifications, LSM without peptone (LSM-peptone) and LSM without peptone and with vitamins and trace elements from Verduyn media (LSMverduyn-peptone). The removal of peptone was proposed in the previous section as an effort to eliminate a source of variation in the media and the use of vitamin and trace element solution from Verduyn et al. (1992) was done as they are largely used by research groups studying *S. cerevisiae*, facilitating the formulation of the media. In addition, these three media were also prepared with a 10-fold reduction of vitamin concentration, as

compared to the defined medium proposed by Verduyn et al. (1992), in order to evaluate the effect of reduced vitamin content on the growth pattern of these strains (Figure 8).

Figure 8 - Growth curves for SA-1 (A), PE-2 (B), CEN.PK113-7D (C) and CEN.PK112 (D) on ( ) LSM, ( )LSM-peptone, ( )LSMverduyn-peptone. Dashed lines represent media with vitamins diluted 10-fold.



Source: The author

As the main hypothesis was that vitamin content of LSM media was superior to that of industrial molasses, it was expected that LSM would promote well defined growth, with a pronounced exponential phase for the laboratory strains, similar to that of vitamin supplemented molasses and the reduction of the vitamin content would lead to the “linear” growth observed on industrial molasses samples. Indeed, laboratory strains showed a near linear growth curves on LSM compositions with 10-fold reduction in vitamin concentration, indicating that such lower amounts of vitamin might be more appropriate to simulate sugarcane molasses. Nonetheless, SA-1 growth behaviour was not as expected for an industrial strain, having its growth more affected for the versions of LSM without peptone

with decreased vitamin content, indicating that this strain could not deal with both the reduced vitamin and nitrogen concentrations, as opposed to the behaviour of industrial strains and the previous results observed in Figure 6.

5.1.4 Elemental composition

Molasses substrates may contain large amounts of minerals that may result in stress responses in the fermenting yeast (Della-Bianca et al., 2013). Table 9 presents the elemental composition of the LSM (Lino et al., 2018) medium (apart from C, O and H) and additional data reported in the literature, for musts containing 200 $g.L^{-1}$ TRS. Table 9 presents the elemental concentration, not including the addition caused by any acid or base present in the stock solution to adjust pH, nor the addition caused by PBS buffer used to dilute the media, as they were disregarded in the LSM. In addition, it is important to mention that the values obtained from L. C. Basso et al. (2011) refer to "sugarcane-based substrates", which includes mixtures of molasses and sugarcane juice. The concentration of "not-sugar components" in the juice are lower than that of molasses, as it is does not undergo processing steps to remove sugar, then it is expected that the range values of a pure molasses substrate would be closer to the upper limits presented by L. C. Basso et al. (2011).

By analysing literature data (Table 9) it was concluded that the LSM composition may not be adequate to represent the elemental composition of sugarcane molasses. Notably, the concentration of potassium, magnesium, and sulphur were far below the lower concentration range for such elements. The sum of all elements, only as an estimate, also yielded a much lower value in comparison to the literature L. C. Basso et al. (2011). Thus, the elemental LSM composition might not reflect the "high salt concentration" usually reported in molasses, as well as the high osmotic pressure often pointed as one of the main features of sugarcane molasses (Amorim et al., 2009).

Thus, a new medium was formulated, considering all the points mentioned in this section, in order to formulate a defined composition for a synthetic molasses.

5.2 Initial proposition of the synthetic defined molasses

Based on the considerations bellow, the LSM medium had the concentration of several of its components altered to match the elemental composition of sugarcane-based media reported in the literature (Table 10). This initial formulation is based on the results obtained from the previous sections. As more iterations could be made throughout this work, this first

version will be regarded as 1SMol, the following modifications will then be named 2SMol, 3SMol and so on.

Table 9 – Elemental composition of LSM media, and concentration range of sugarcane-based media and Molasses in $mg.L^{-1}$

	Lino et al. (2018)	L. C. Basso et al. (2011)		INRA-CIRAD-AFZ Feed Tables (n.d.)	
		Lower	Upper	Lower	Upper
K	9.12	300	12000	2902	25142
S	154.77	80	3900	1293.38	4447.95
Mg	98.79	80	3900	725.55	1956
Ca	18.30	150	200	1861	4416
P	215.45	20	200	31.55	441.64
Cl	341.47			2113.56	16782.33
N	215.98	70	350		
Na	196.76			123	1940
Zn	0.09	0.45	9	1.26	24.29
Mn	0.13	2	8	6.94	38.17
Cu	0.10	0.20	8	0.63	6.94
I	9.17			1.89	1.89
Mo	0.13			0.32	0.95
B	0.09				
Fe	0.01			38.80	87.38
Al	0.00				
Co	0.00			0.32	0.32
Total	1260	703	20575	9100	55286

Source: Lino et al. (2018), L. C. Basso et al. (2011), INRA-CIRAD-AFZ Feed Tables (n.d.)

Table 10 - Initial composition proposed by this work

Class	Compound	Concentration	Stock solution for medium preparation
Carbon sources ($g.L^{-1}$)	Sucrose	160	Sugar stock - 2.5x Concentrated
	Glucose	20	
Maillard reaction products simulation ($g.L^{-1}$)	Fructose	20	
	Glutamine	3.8	
	Aspartic Acid	2.2	
	Asparagine	1.42	
Inorganic nitrogen source ($g.L^{-1}$)	$(NH_4)_2HPO_4$	1.00	Inorganic Nitrogen Stock - 100x Concentrated
Organic acids ($g.L^{-1}$)	Trans-aconitic acid	2.00	Organic Acids Stock Solution - 20x Concentrated
	l-malic acid	1.00	
	Citric acid	0.01	
	KOH	1.96	
Trace elements[1] ($mg.L^{-1}$)	EDTA.Na_2	150.00	Trace Elements Stock Solution - 100x Concentrated
	$CaCl_2.2H_2O$	45.00	
	$MnCl_2.4H_2O$	10.00	
	$ZnSO_4.7H_2O$	45.00	
	$CuSO_4 \cdot 5H_2O$	3.00	
	KI	1.00	
	$CoCl_2.6H_2O$	3.00	
	H_3BO_3	10.00	
	$FeSO_4.7H_20$	30.00	
	$Na_2MoO_4.2H_20$	4.00	
Vitamins[2] ($mg.L^{-1}$)	Inositol	25.00	Vitamin Stock Solution - 1000x Concentrated
	Nicotinic acid	1.00	
	Calcium pantothenate	1.00	
	Biotin	0.05	
	Pyridoxine hydrochloride	1.00	
	Thiamine hydrochloride	1.00	
	Para-aminobenzoic acid	0.20	
Magnesium and potassium ($g.L^{-1}$)	K_2SO_4	5	Mg & K Stock Solution - 50x Concentrated
	$MgSO_4.7H_2O$	10	
Calcium ($g.L^{-1}$)	$CaCl_2.2H_2O$	0.5	Calcium Stock - 500x Concentrated

Source: The author

[1] As described by Verduyn et al. (1992), 10-fold increase in each component concentration

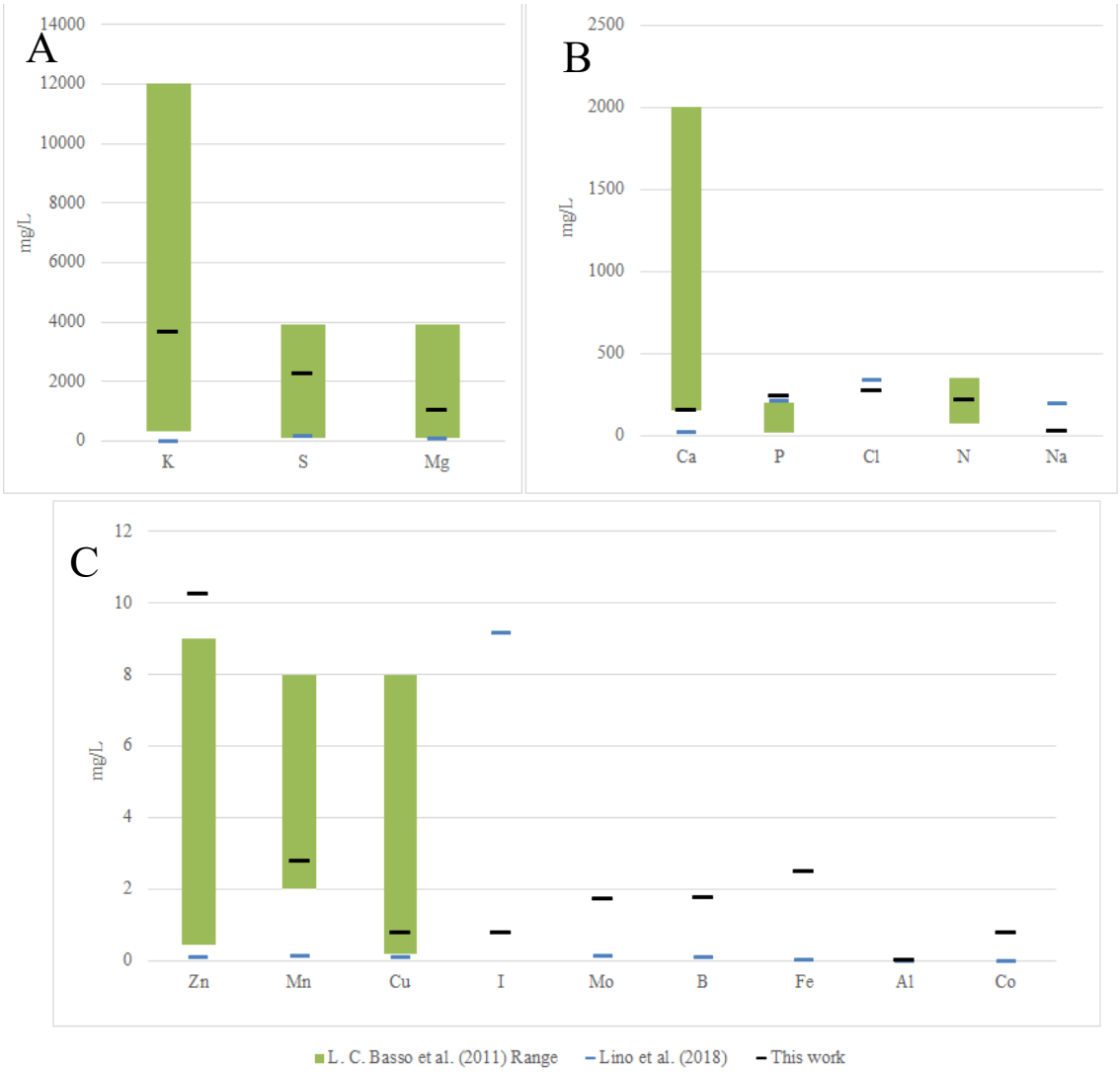
[2] As described by Verduyn et al. (1992)

As mentioned above, the detailed reasoning for the alterations in each class of nutrient and the main characteristics are listed below:

a) The proportions of sucrose, glucose and fructose were corrected. To achieve this, a new sugar stock was created by mixing a filter sterilized 500 $g.L^{-1}$ sucrose solution and LSM "sugar stock" in a 4:1 ratio, yielding a final solution containing 400 $g.L^{-1}$ sucrose and approximately 54 $g.L^{-1}$ glucose and 54 $g.L^{-1}$ fructose;
b) Vitamin concentrations were changed to that described by Verduyn et al. (1992) which is largely used in yeast research. Nonetheless, as previous experiments suggested, vitamin content of molasses may be lower than traditional media and fine tuning might be necessary;
c) Trace elements solution were used as described by Verduyn et al. (1992) increased 10-fold to match literature data regarding mineral concentration of molasses;
d) Organic acids stock solution had its concentration factor increased to 50, citric acid was also included in this solution. Solubilization of the organic acids in such concentrations yielded a pH 1,96 solution, addition of 1,96 g KOH increased the solution pH to 4.96;
e) The concentration of K, Na, Mg, Ca, ammoniacal N, Cl, S (as sulphate) and P (as phosphates) were heavily elevated to match literature data for sugarcane bases substrates used in Brazilian mills.

A visual representation of the elemental concentration range reported in the literature (L. C. Basso et al., 2011) and the values for both LSM and 1SMol is shown in Figure 9. According to L. C. Basso et al. (2011), the most abundant elements (besides C, H, O) on molasses are K, Mg and S, thus, its concentrations on the media proposed in this work were increased in relation to LSM (Figure 9A). Calcium ions are considered detrimental to fermentation performance, acting as a facilitator for flocculating phenomena and competing with magnesium uptake and supressing magnesium dependent enzymes, additionally (Walker, 2004), so its concentration was kept low on this initial iteration of the medium development (Figure 9B). Zn, Cu and Mn show that concentrations of such elements in LSM were below the ranges reported (L. C. Basso et al., 2011), then it was assumed that other "trace elements" are present in the molasses in similar concentration ranges, the use of Verduyn trace elements 10-fold concentrated in 1SMol increased the overall concentration of this compound class (Figure 9C)

Figure 9 - Elemental concentrations in LSM, the values proposed this work and ranges found in sugarcane-based musts. Absent bars for not reported elements.



Source: The author with data from L. C. Basso et al. (2011) and Lino et al. (2018)

5.3 Evaluation of each component group in the 1SMol medium

To assess the effect of each component group on the specific growth rate and the maximum OD_{600}, several versions of the 1SMol were prepared either increasing or decreasing each component group while maintaining the TRS concentration. The microplate reader was used to enable the analysis of multiple conditions in a single run, nonetheless it is not possible to run experiments using such equipment with 200 $g.L^{-1}$ TRS, as most conditions would reach a yeast concentration that would saturate the OD readings. Thus, each media was diluted 10-

fold, achieving 20 $g.L^{-1}$ TRS as was done for industrial molasses samples for the experiments described in section 5.1.3. The ratio of each component class in relation to 1SMol is presented on Table 11, the composition was coded using either +1 and -1 to represent increased or decreased concentrations, respectively. Previous experiments have shown that the effect from vitamin group effect can be pronounced at low concentrations (section 5.1.3), thus it had two additional lower levels, -2 and -3.

Table 11 - Coded names for compositions tested for microplate essays.

Coded composition name	Ratio in relation to 1SMol Composition
Original Composition	
$(NH_4)_2HPO_4$ Stock -1	0
$(NH_4)_2HPO_4$ Stock +1	2
Organic Acids Stock -1	0
Organic Acids Stock +1	2
Trace Elements Stock -1	0.1
Trace Elements Stock +1	10
Vitamins Stock +1	2
Vitamins Stock -1	0.5
Vitamins Stock -2	0.1
Vitamins Stock -3	0
Mg & K Stock -1	0.2
Mg & K Stock +1	2
Calcium Stock -1	0
Calcium Stock +1	2

Source: The author

Two parameters were obtained from these microplate assays, the maximum specific growth rates (μ), as described in materials and methods section, and the maximum optical density reading at 600 nm wavelength (OD_{600max}). From the analysis of these parameters and the shape of each growth curve, we expected to evaluate the sensitivity of two yeast strains (PE-2 and CENP.PK 113-7D) to each component group.

5.3.1. PE-2 strain

The specific growth rate during the exponential growth phase was determined and the results were compared using Tukey test to determine which conditions were significantly different with 95% confidence. Table 12 presents the mean values and standard deviations for calculated μ values, as well as Tukey test results, in which means that do not share a letter are

significantly different. An individual plot of the triplicates was also constructed to facilitate data visualization (Figure 10).

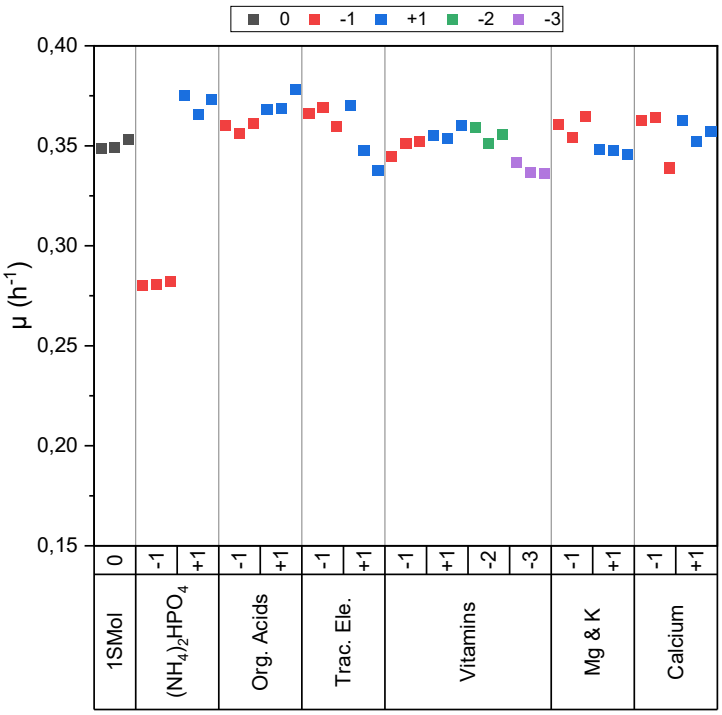
Table 12 - Specific growth rate for PE-2 strain grown on original 1SMol composition and conditions with increased or decreased composition of each compound group

	μ (h^{-1})	Tukey Test groups			
Original	0.350 ± 0.003		B	C	
$(NH_4)_2HPO_4$ -1	0.281 ± 0.001				D
$(NH_4)_2HPO_4$ +1	0.371 ± 0.005	A			
Org. Acids -1	0.359 ± 0.003	A	B		
Org. Acids +1	0.372 ± 0.006	A			
Trac Ele -1	0.365 ± 0.005	A	B		
Trac Ele +1	0.352 ± 0.017	A	B	C	
Vitamins +1	0.356 ± 0.003	A	B	C	
Vitamins -1	0.349 ± 0.004		B	C	
Vitamins -2	0.355 ± 0.004	A	B	C	
Vitamins -3	0.338 ± 0.003			C	
Mg & K Stock -1	0.360 ± 0.005	A	B		
Mg & K Stock +1	0.347 ± 0.001		B	C	
Calcium -1	0.355 ± 0.014	A	B	C	
Calcium +1	0.357 ± 0.005	A	B	C	

Source: The author

The results obtained by the application of the Tukey test with 95% confidence separate the values calculated for specific growth rate into 7 groups that are not statistically different. The result for the original composition for 1SMol belongs to two of these groups, B and C, thus only results not contained within this groups have specific growth rates statistically different from the original composition.

Figure 10 - Individual plot of the triplicates for the determined μ of PE-2 strain for 1SMol compositions.



Source: The author

The results for the conditions with increased and decreased concentrations of ammonium were statistically different from each other and from the original composition. The ammonium salt was added to the medium as an inorganic nitrogen source, playing a fundamental role in the biosynthesis of amino acids for biomass formation in *S. cerevisiae* (Albers et al., 1996). The different μ values obtained for the altered nitrogen concentrations in relation to the original composition (0.281 ± 0.001 h^{-1} and 0.371 ± 0.005 h^{-1} against 0.350 ± 0.003 h^{-1}) indicates that PE-2 strain growth rate is sensitive to the nitrogen concentration in the medium in the range tested. Nonetheless, it is important to highlight that in the condition with no inorganic nitrogen, no other sources of phosphate were added and the decreased μ might also be due to the absence of phosphate in the medium.

Weak acids in the non-dissociated form, in low pH values, are able to diffuse into the yeast cell's cytosol, where they dissociate accumulating protons in the intracellular environment. This might lead to decreased DNA and RNA synthesis rates, reduced metabolic activity and disrupted electrochemical proton gradients (Swinnen et al., 2014). To cope with the lowered pH in the cytoplasm, cells excrete the protons at the expense of ATP, which

depletion may decrease the biomass yield (Holyoak et al., 1996). As the medium pH was not low enough to maintain the weak acids in non-dissociated form, no growth significant inhibition was observed. The effect on μ was positive for the conditions without organic acids and doubled concentration ($0.359 \pm 0.003\ h^{-1}$ and $0.372 \pm 0.006\ h^{-1}$, respectively) in relation to the original composition ($0.350 \pm 0.003\ h^{-1}$).

The experiments with different concentrations of the trace elements and calcium had similar results. When compared to the original composition none of the conditions were significantly different and several effects might explain these results. Magnesium is able to alleviate toxic effects of metals contained in the trace elements group, such as cobalt, manganese, zinc and copper (Walker, 2004). Thus, as Mg^{2+} is the second most abundant cation in the medium, it is possible it is exerting a protective effect, alleviating the detrimental effects of increased concentration of Co, Mn, Zn, Cu and Ca, present in the trace element group. Additionally, resistance to toxic concentration of metals can be strain specific. For instance, Basso et al., 2011 have shown that the toxic effects of aluminium in cell viability, ethanol yield and aluminium cell accumulation were less pronounced industrial strain CAT-1 than in PE-2 strain and baker's yeast. Thus, it is possible that PE-2 strain is not sensitive to these concentrations of Co, Mn, Zn, Cu and Ca tested in this work. Nonetheless, this no conclusion can be drawn from this experiment alone.

Magnesium, potassium and sulphur are the most present elements in molasses, thus, to emulate conditions of a molasses containing higher and lower levels of these elements, the concentration of the Mg & K stock solution, was reduced 0.2 times and increased 2 times. It was expected that the composition with increased salt concentration would induce a stress response that impaired growth rate. The results (Table 12) for μ were statistically not different from the original composition ($0.350 \pm 0.003\ h^{-1}$) but different among the reduced and increased concentration conditions ($0.360 \pm 0.005\ h^{-1}$ and $0.347 \pm 0.001\ h^{-1}$). Thus, no notable effects from this nutritional group could be observed for the tested ranges in relation to the specific growth rate. It was expected that the higher condition would impose a stress factor that could result in impaired growth, nonetheless, other important parameters that indicate osmotic stress response, such as increased glycerol formation (Blomberg, 1997), were not measured in this stage.

The results discussed in section 5.1.3 indicated that the concentration of at least one of the seven vitamins added to the medium is deficient in molasses, but PE-2 strain is able to cope with such restriction. Thus, to assess this strain vitamin demands, this nutritional group

concentration was altered to contain 200 %, 50 %, 10 % and 0 % of the original composition. The μ obtained for the all version of the media containing alterations to the vitamin content did not present a significant difference for the confidence level used (Table 12), although the lowest vitamin concentration presented a remarkably lower value ($0.338 \pm 0.003\ h^{-1}$) in comparison to the other concentrations. These results indicate that PE-2 strain is adapted to media with low vitamin content, being less sensitive to their absence as already demonstrated by Stambuk et al. (2009), studying variations in the copy number of genes related to the biosynthesis of pyridoxine and thiamine.

Perli, Moonen, van den Broek, Pronk and Daran (2020) used adaptive laboratory evolution and reverse engineering to develop *S. cerevisiae* strains able to grow in chemical defined media. First, the authors evolved CEN.PK113-7D strain in serial transfer experiments employing chemically defined media with the absence of each vitamin. Genome sequencing of the resulting 6 strains, one for each vitamin other than biotin, revealed mutations that were linked to fast growth in media with the absence of its respective vitamin. The mutations were introduced in non-evolved CEN.PK113-7D strains, which were able grow in medium lacking a single vitamin with at least 87 % of the rates observed in media containing the vitamin (Perli, Moonen, et al., 2020). Considering this, PE-2 ability to grow on the media lacking vitamins indicates that the fermentation musts for ethanol production in Brazil, where it was isolated from (L. C. Basso et al., 2008) might exert this selective pressure, having low vitamin concentrations.

The results for OD_{nax}, an indirect parameter for how cells could grow, were also compared using Tukey test with 95% confidence. The results obtained (Table 13) showed that only the lowest nitrogen concentration evaluated affected this parameter, whereas all the remaining tested compositions had no significant difference in the OD_{600max}. Again, nitrogen is an essential nutrient for cell growth (Walker, 2014), and the absence of ammonium in the medium can significantly impact cell concentration. The observed biomass formation probably used nitrogen sources available from the “Maillard simulation” solution added to the medium, which contained glutamine, asparagine and aspartic acid all of which are regarded as being rapidly assimilated by S. *cerevisiae* (Stewart et al., 2013).

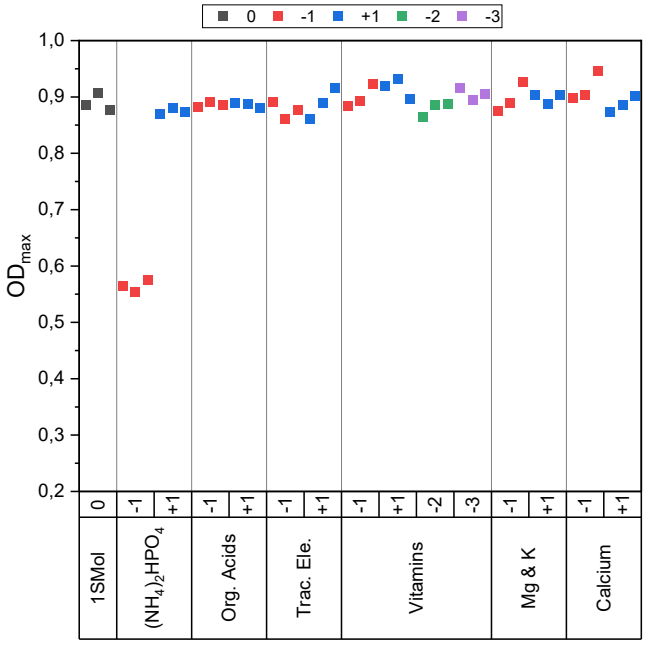
Table 13 - Maximum absorbance for PE-2 strain grown on original 1SMol composition and conditions with increased or decreased composition of each compound group

	OD_{600max}	Tukey Test groups
Original	0.890 ± 0.016	A
$(NH_4)_2HPO_4$ -1	0.565 ± 0.011	B
$(NH_4)_2HPO_4$ +1	0.874 ± 0.005	A
Org. Acids -1	0.887 ± 0.004	A
Org. Acids +1	0.886 ± 0.004	A
Trac Ele -1	0.876 ± 0.015	A
Trac Ele +1	0.889 ± 0.028	A
Vitamins +1	0.916 ± 0.018	A
Vitamins -1	0.900 ± 0.020	A
Vitamins -2	0.880 ± 0.012	A
Vitamins -3	0.906 ± 0.011	A
Mg & K Stock -1	0.897 ± 0.027	A
Mg & K Stock +1	0.898 ± 0.009	A
Calcium -1	0.916 ± 0.027	A
Calcium +1	0.887 ± 0.014	A

Source: The author

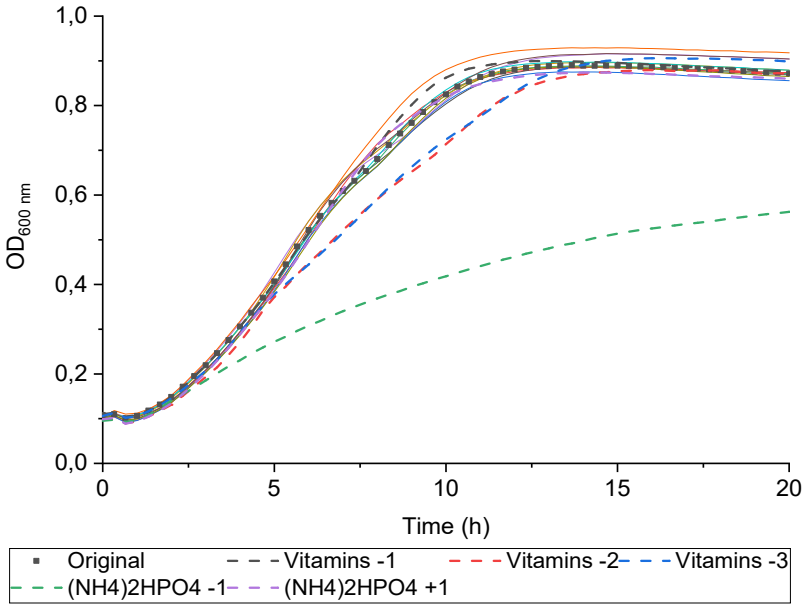
Comparison of Figure 10 and Figure 11 highlights the results, showing that PE-2 μ values are more sensitive to medium composition than OD_{max}. Overall, the results have showed several overlapping growth curves (Figure 12), illustrating that the industrial strain PE-2 seems to be not very sensitive to the conditions tested, besides the nitrogen deficient condition and the very low vitamin conditions, as reinforced by the statistical analysis.

Figure 11 - Individual value plot for absorbance after 20h of PE-2 strains on variations of 1SMol with modification of each class of component.



Source: The author

Figure 12 – Growth curves for PE-2 for all the conditions tested.



Source: The author

5.3.2. CEN.PK113-7D strain

The same physiological parameters were obtained for CEN.PK113-7D strain, a reference strain. Table 14 presents the mean values and standard deviations for calculated μ values as well as the Tukey test results, in which means that do not share a letter are significantly different (α=0,05). An individual plot of the triplicates was also constructed to facilitate data visualization (Figure 13).

To a certain extent, the results obtained for CEN.PK113-7D strain were similar to the results for PE-2. The inorganic nitrogen source concentration had the same effects observed on μ, with increasing concentrations leading to higher growth rates ($0.201 \pm 0.002\ h^{-1}$, $0.362 \pm 0.0104\ h^{-1}$, and $0.374 \pm 0.004\ h^{-1}$, respectively). The results for increased and decreased concentrations of Mg and K ($0.358 \pm 0.009\ h^{-1}$, $0.369 \pm 0.004\ h^{-1}$) were also not different from the original composition ($0.362 \pm 0.010\ h^{-1}$) at the confidence level used, as for PE-2.

Table 14 - Specific growth rate for CEN.PK113-7D strain grown on original 1SMol composition and conditions with increased or decreased composition of each compound group

	μ (h^{-1})	Tukey Test groups					
Original	0.362 ± 0.010	A	B	C			
$(NH_4)_2HPO_4$ -1	0.201 ± 0.002						F
$(NH_4)_2HPO_4$ +1	0.374 ± 0.004	A					
Org. Acids -1	0.371 ± 0.006	A	B	C			
Org. Acids +1	0.373 ± 0.009	A	B				
Trac Ele -1	0.374 ± 0.005	A					
Trac Ele +1	0.353 ± 0.003		B	C			
Vitamins +1	0.371 ± 0.003	A	B	C			
Vitamins -1	0.352 ± 0.005			C			
Vitamins -2	0.295 ± 0.001				D		
Vitamins -3	0.258 ± 0.003					E	
Mg & K Stock -1	0.358 ± 0.009	A	B	C			
Mg & K Stock +1	0.369 ± 0.004	A	B	C			
Calcium -1	0.371 ± 0.014	A	B	C			
Calcium +1	0.371 ± 0.007	A	B	C			

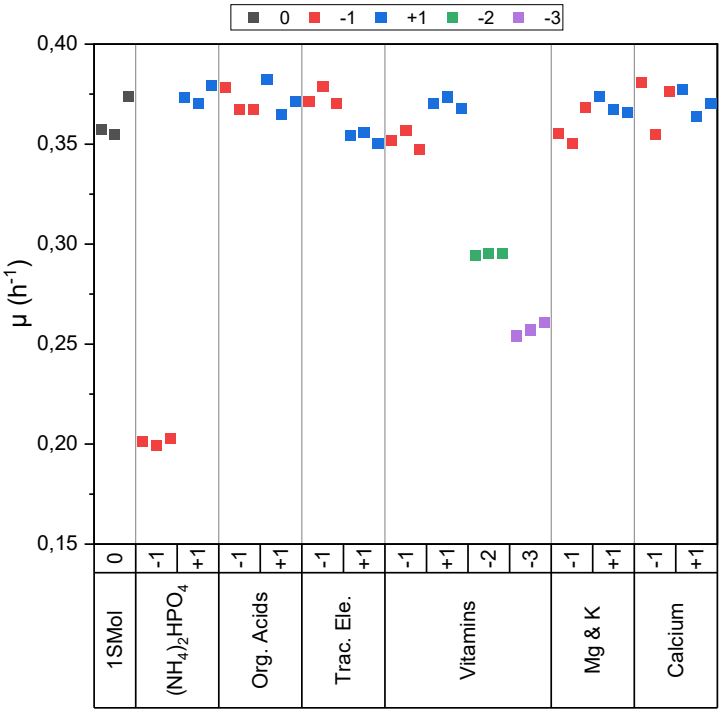
Source: The author

Results for altered concentrations of trace elements and calcium also followed a trend similar to the observed for PE-2 strain. Increased and decreased concentrations of both groups

yielding μ values statistically not different from the original composition. This reinforces the hypothesis that neither the concentrations of the trace elements and calcium have not been increased to toxic levels or that magnesium concentration is sufficiently high to promote the alleviating effects of toxic metals reported by Walker, 2004.

Regarding the concentration of organic acids, similar effect was observed in the behaviour of CEN.PK113-7D and PE-2 strains, with conditions with decreased and increased organic acids concentration did not significantly changed μ values ($0.371 \pm 0.006\ h^{-1}$ and $0.373 \pm 0.009\ h^{-1}$) in relation to the intermediate concentration ($0.362 \pm 0.010\ h^{-1}$).

Figure 13 - Individual plot of the triplicates for the determined μ of CEN.PK113-7D strain for 1SMol compositions.

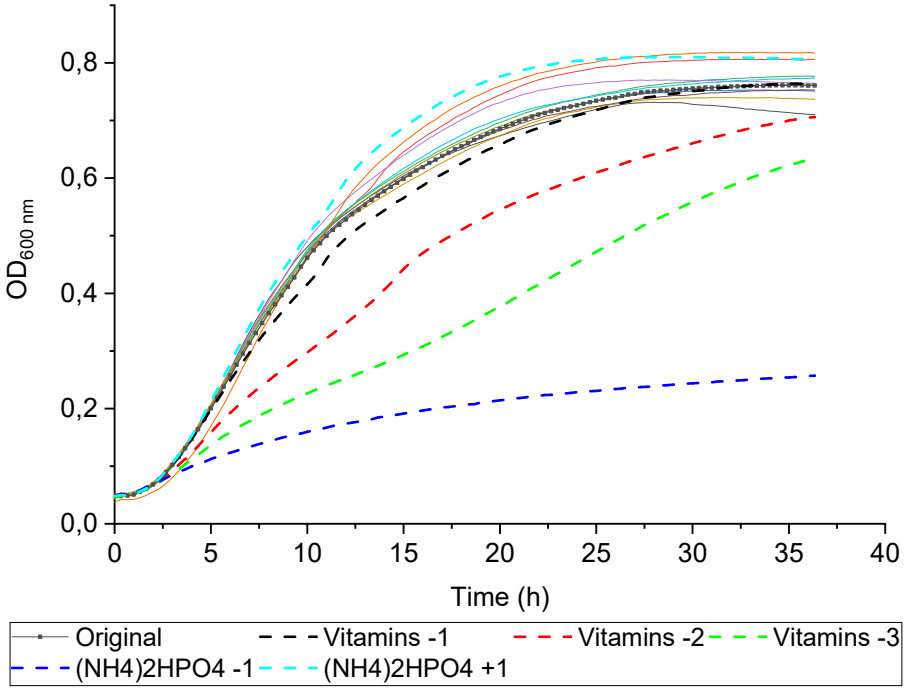


Source: The author

As expected, based on the results obtained in section 5.1.3, the effect vitamin concentration on the specific growth rate of the laboratory strain CEN.PK113-7D was more pronounced than on industrial PE-2. In this case, all conditions tested, with 200%, 100%, 50%, 10% and 0% of the vitamin content, had increasingly lower values for μ, although only the later two presented statistically different results from the previous conditions.

Consequently, for the lowest vitamin concentrations, fermentation times were significantly longer as highlighted on Figure 14.

Figure 14 - CEN.PK113-7D growth curves for original 1SMol composition and conditions with different nutrient concentrations.

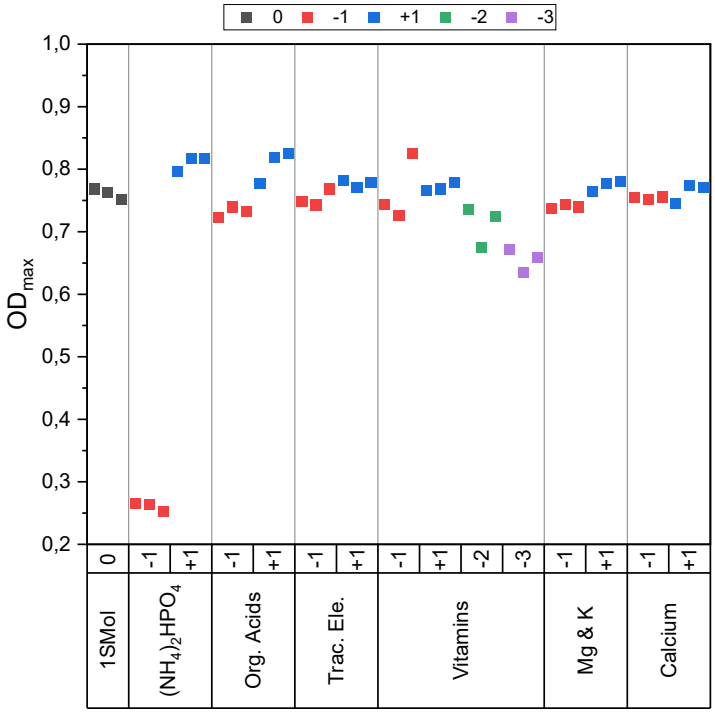


Source: The author

The effect of each composition on the OD_{max} for CEN.PK113-7D was also evaluated. As a result of the lower growth rates, fermentation times were bigger for the laboratory strain. After 20 h, time at which PE-2 had achieved its OD_{max} values, CEN.PK113-7D growth for some conditions was still on-going and the experiment was kept running for additional 16 h. Table 15 presents the absorbance values for both 36 h as well as grouping information using Tukey method with 95% confidence. Figure 15 present the individual plot values for OD_{max} at 36 h.

The data obtained shows that this laboratory strain is more sensitive to variations in the composition of the media. This is highlighted by the large number of groupings in comparison to PE-3. Although the time extension made the original composition statistically different from only 2 conditions (decreased ammonium and vitamins in the lowest concentration), this behaviour is largely different from the observed for PE-2, for which the OD_{max} were unsensitive to the tested conditions, except for ammonium concentration.

Figure 15 - Individual plot values for CEN.PK113-7D OD_{600} at 36 h.



Source: The author

5.4 Mixed effects of ammonium and vitamin concentration and comparison to industrial molasses

As reported previously (section 5.3), the largest effects observed on the growth kinetics of PE-2 and CEN.PK113-7D were due to variations in concentration of ammonium and vitamins in the 1SMol medium. Thus, media formulation containing variations in these nutritional groups were proposed (Table 16). Growth kinetics experiments in microplates were performed with these new formulations and compared to cultivations using 5 industrial molasses samples (USJ, RAIZEN, PEDRA, LUCELIA and IRACEMA). In the previous experiments, the ammonium biphosphate was the only ammonium source used and lower ammonium concentrations tested also resulted in lower phosphate concentration, thus mixed effects could have been observed. In this and any following experiment, ammonium concentration was increased by the addition of ammonium sulphate and the condition with decreased ammonium concentration had its phosphate content corrected by the addition of potassium phosphate. Similarly, phosphate concentration was increased by the addition of potassium phosphate and ammonium sulphate was used to correct the ammonium content in

the condition with decreased phosphate concentration. As previous results (section 5.3) showed that the effect of altered potassium and magnesium sulphates concentrations were not significant, it was assumed that correcting the concentrations with these salts would not interfere significantly in the physiological parameters investigated.

Table 15 - Absorbance readings for CEN.PK113-7D strain grown on original 1SMol composition and conditions with increased or decreased composition of each compound group.

Composition	OD_{600max}	Tukey Test groups				
Original	0.761 ± 0.008	A	B	C		
$(NH_4)_2HPO_4$ -1	0.261 ± 0.007					E
$(NH_4)_2HPO_4$ +1	0.811 ± 0.012	A				
Org. Acids -1	0.732 ± 0.009		B	C		
Org. Acids +1	0.807 ± 0.025	A				
Trac Ele -1	0.753 ± 0.013	A	B	C		
Trac Ele +1	0.778 ± 0.006	A	B			
Vitamins +1	0.771 ± 0.007	A	B			
Vitamins -1	0.765 ± 0.052	A	B	C		
Vitamins -2	0.712 ± 0.032			C	D	
Vitamins -3	0.655 ± 0.019				D	
Mg & K Stock -1	0.741 ± 0.003		B	C		
Mg & K Stock +1	0.775 ± 0.009	A	B			
Calcium -1	0.754 ± 0.002	A	B	C		
Calcium +1	0.763 ± 0.016	A	B	C		

Source: The author

The expected results from this set of experiments were that the different compositions would present a range of growth curves profiles similar to the different growth curves obtained for industrial molasses. However, in general, all compositions tested resulted in slower growth rates in comparison to the industrial molasses (Figure 16), which was considered unsuitable to the purpose of this work, as it was intended to obtain growth curves very similar to the ones observed for industrial molasses. In the previous section, the concentration of ammonium and vitamins in the medium were shown to effect both strains' growth, but even the conditions with the highest concentrations of these components could not match growth profiles obtained in industrial molasses. In addition, previous experiments did not evaluate variations in the amino nitrogen concentration in the medium. The source of amino nitrogen in 1SMol is the "sugar stock" solution which proportion was maintained constant, to avoid fluctuations in the sucrose and glucose/fructose ratios. Moreover, it is very likely that due to formation of Maillard degradation products, as well as thermal degradation

during the autoclaving step of this solution, amino acid concentration in the synthetic medium would be lower than expected (Table 10). This might affect the availability of nitrogen to yeast cells. Therefore, to avoid such uncertainties, another version of the media was formulated, named 2SMol, with the complete removal of the "sugar stock" proposed by Lino et al (2018), substituted for a mixture of sucrose, glucose and fructose, and filter sterilized amino acids, to avoid thermal degradation.

Table 16 - Compositions tested varying ammonium content and vitamin concentration.

Conditions	Ratio in relation to 1SMol Composition	
	Ammonium	Vitamins
1SMol 1	1	0,25
1SMol 2	0	0,25
1SMol 3	2	0,25
1SMol 4	1	0,5
1SMol 5	0	0,5
1SMol 6	2	0,5
1SMol 7	1	0
1SMol 8	0	0
1SMol 9	2	0

Source: The author

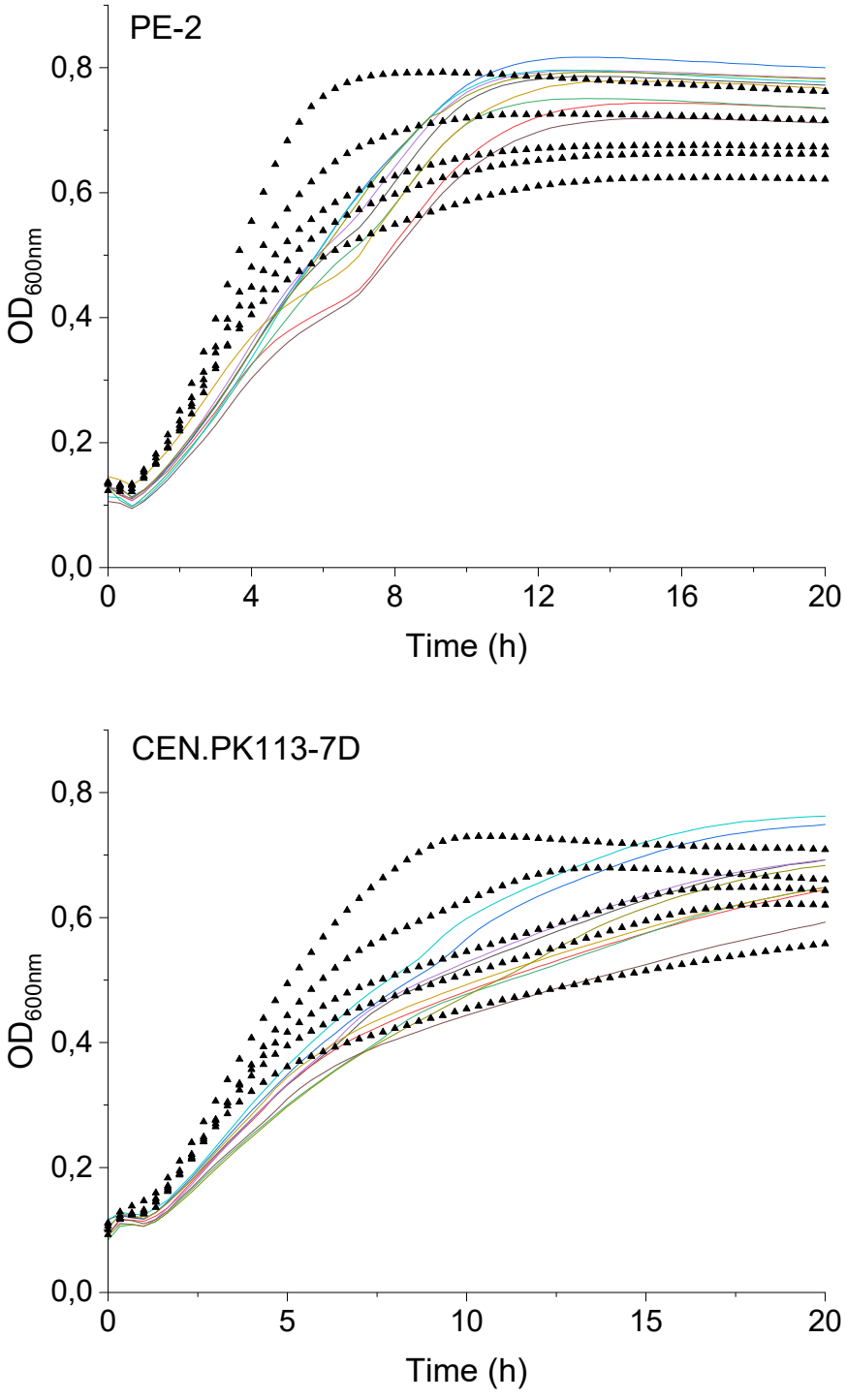
The synthetic composition 2SMol is identical to the 1SMol composition except for the amino acid content. Initially, the filter sterilized amino acid would be added the concentrations listed on Table 10, however the low solubility of aspartic acid in water made the medium formulation difficult and was partially substituted for glutamine to reach the same final nitrogen concentration, resulting in the concentrations presented on Table 17.

Table 17 - Amino acid concentrations in 1SMol and 2SMol

	1SMol	2SMol	1SMol	2SMol
Amino acid	$g.L^{-1}$		Nitrogen in $g.L^{-1}$	
Glutamine	3.8	4.15	0.73	0.80
Aspartic Acid	2.2	1.5	0.23	0.16
Asparagine	1.42	1.42	0.3	0.3
Total	**7.42**	**7.07**	**1.26**	**1.25**

Source: The author

Figure 16 - Growth curves for 1SMol with variations in ammonium and vitamins content (lines) and industrial molasses samples (▲)



Source: The author

5.5 2SMol composition initial amino acid concentration evaluation and group effects

5.5.1 2SMol amino acid concentration

Prior to the assessment of each nutritional group effect in this version of the medium, as done for 1SMol in section 5.3, the amino acid concentration effect on yeast grown was assessed in microplate assays and compared to results obtained for industrial molasses. Initially, five concentrations were tested with 100%, 75%, 50%, 25% and 0% of the amino nitrogen concentrations for 2SMol listed on Table 17. Additionally, industrial molasses from Pedra, Iracema and São José mills were used to benchmark the results. The maximum specific growth rate (μ), OD_{max} and deceleration time were used as parameters to compare the results, Table 18.

Overall, μ calculated values for PE-2 were larger than those for CEN.PK113-7D but within each strain its value remained constant for all synthetic compositions containing amino acids, with statistically different values found only for the conditions without its addition. The μ values found for the industrial molasses were within the range found for the synthetic compositions containing 100% to 25% of the amino acids content from 2SMol, as demonstrated by the multiple comparison test.

The results for OD_{max} were similar between the tested strains, although these results were not compared in pairwise tests, showing larger values as the amino acid concentration increased, nonetheless the two largest values gave the similar results (0,92 ± 0,01 and 0,91 ± 0,02 for PE-2 and 0,91 ± 0,01 and 0,90 ± 0,01 for CEN.PK113-7D), indicating that the strains might be insensitive to the amino acid concentration in this range. The different industrial molasses presented statistically different OD_{max} values, within the range found for synthetic compositions containing 100 – 75% to 0% of the amino acids content from 2SMol.

Table 18 – Maximum specific growth, maximum optical density, and deceleration time for 2SMol compositions with different amino acids concentrations and industrial molasses.

Condition	μ (h^{-1})		ODmax		Tdecel (h)	
	PE-2*	CEN.PK113-7D†	PE-2†	CEN.PK113-7D†	PE-2*	CEN.PK113-7D*
AA 100%	0,420 ± 0,001 B	0,363 ± 0,004 AB	0,919 ± 0,012 A	0,912 ± 0,010 A	8,67 ± 0,00 D	11,67 ± 0,58 D
AA 75%	0,429 ± 0,00 B	0,36 ± 0,00 B	0,910 ± 0,018 A	0,900 ± 0,014 A	8,89 ± 0,19 D	14,34 ± 0,33 D
AA 50%	0,427 ± 0,00 B	0,36 ± 0,00 AB	0,859 ± 0,019 AB	0,877 ± 0,021 A	8,67 ± 0,00 D	16,34 ± 2,60 BCD
AA 25%	0,404 ± 0,01 B	0,36 ± 0,01 B	0,813 ± 0,018 BC	0,801 ± 0,017 B	11,78 ± 0,19 C	19,78 ± 0,39 C
AA 0%	0,349 ± 0,00 C	0,31 ± 0,00 C	0,682 ± 0,017 D	0,667 ± 0,007 D	24,89 ± 1,50 A	33,12 ± 1,26 A
IRA	0,496 ± 0,01 A	0,39 ± 0,02 A	0,902 ± 0,015 A	0,827 ± 0,016 B	5,78 ± 0,19 E	10,78 ± 0,19 D
PEDRA	0,426 ± 0,011 B	0,37 ± 0,01 AB	0,682 ± 0,057 D	0,666 ± 0,019 D	8,56 ± 0,19 D	14,56 ± 0,51 D
USJ	0,417 ± 0,010 B	0,38 ± 0,01 AB	0,746 ± 0,009 CD	0,716 ± 0,017 C	17,56 ± 0,77 B	26,00 ± 0,88 B

*Pairwise comparisons using Games-Howell test α=0,05.

†Pairwise comparisons using Tukey test with α=0,05.

Source: The author

The deceleration time was also more sensitive to the amino acid concentrations, with progressively lower amino acid concentration leading to increased deceleration times, growth duration. The industrial strain PE-2 has lower values as compared to CEN.PK113-7D strain for the same amino acid concentration, for both industrial and synthetic molasses, indicating an overall better fitness to consume carbon sources and grow in these conditions. For this parameter, the tested industrial molasses presented values within the obtained for the synthetic compositions except for IRA, which presented the smallest result presenting a statistically different value.

Thus, as the condition containing 25% of the amino acid content of the 2SMol medium presented intermediate values in relation to the industrial molasses, it was chosen as a base concentration for a following experiment, to evaluate the effect of each component of the media in yeast growth. This condition will be regard as 2SMolAA25, in which AA25 represents 25% of the amino acid content of original 2SMol composition.

5.5.2 Evaluation of each component group effects in 2SMolAA25

To assess the effect of each component group in the growth of PE-2 and CEN.PK113-7D strains, similar to that discussed in section 5.3, 17 conditions were tested, with alterations in 2SMolAA25, as listed on Table 19.

Table 19 – Compositional variations in relation to 2SMolAA25 used in microplate assays to assess yeast sensitivity.

Condition	Concentration ratio in relation to 2SMolAA100	
	Lower	Higher
2SMolAA25	-	
Ammonium	0 (-1)	2 (+1)
Organic Acids	0 (-1)	2 (+1)
Trace elements	0.1 (-1)	10 (+1)
Vitamins	0.1 (-1)	2 (+1)
Mg & K	0.2 (-1)	2 (+1)
Calcium	0 (-1)	2 (+1)
Amino acids	0 (-1)	0.5 (+1)
Phosphate	0.5 (-1)	2 (+1)

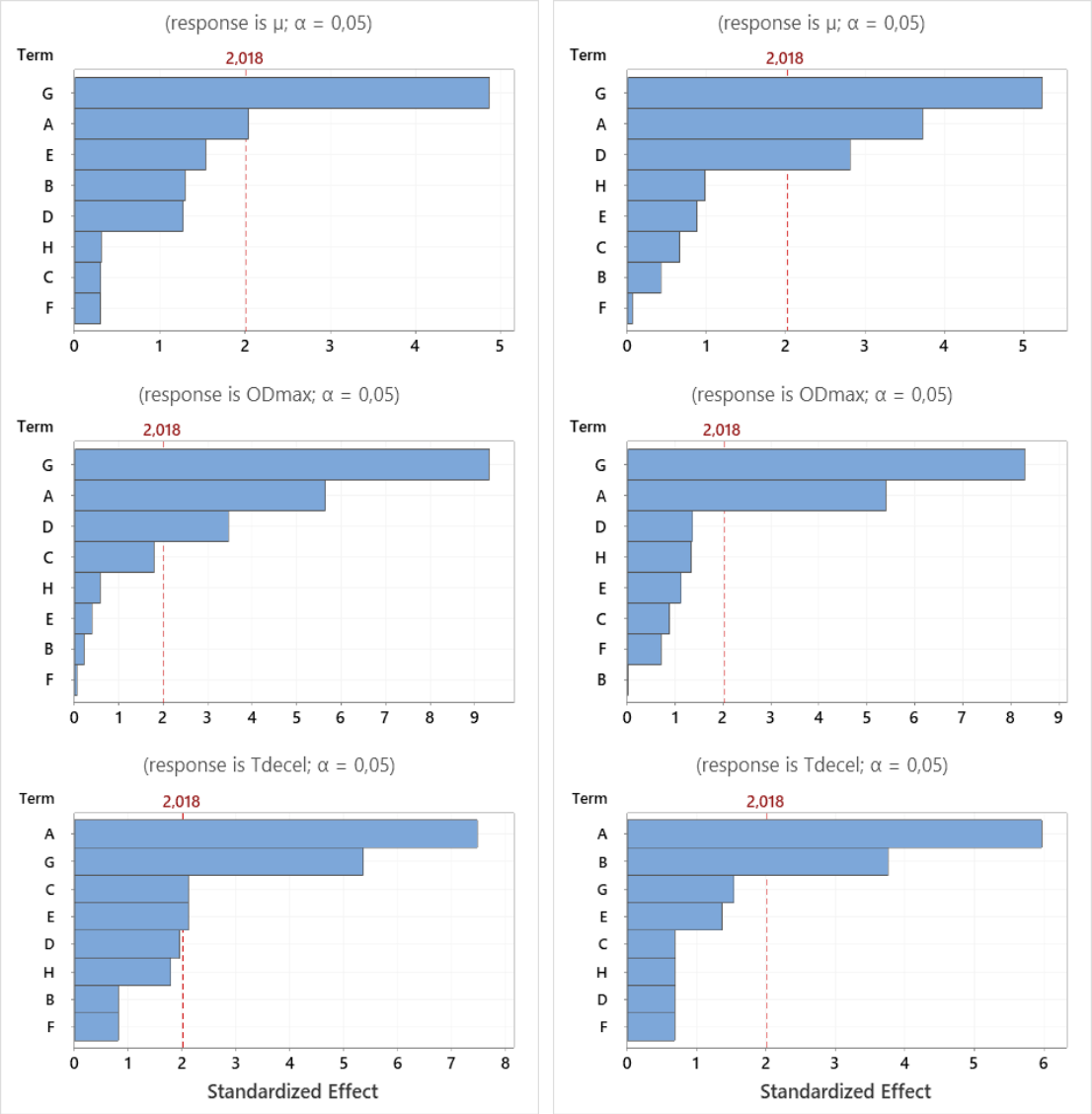
Source: The author

In this case, instead of using pairwise comparison test to determine which media components had the largest effects on each parameter, Pareto charts of normalized effects were constructed for each parameter and strain, using Minitab. This tool displays the absolute value of the effect for each variable, allowing the visual identification of potentially important effects, Figure 17 Additionally, in this section the results for PE-2 and CEN.PK113-7D will be discussed together. The obtained mean values and standard deviations from triplicates are presented on Table 20 for PE-2 strain and Table 21 for CEN.PK113-7D strain. Individual triplicate results were plotted in a grouped scatter graph in APPENDIX A.

For the specific growth rate, the most important variables for both strains were the amino acids and ammonium concentration, respectively. This result is consistent with the experiments described previously in this section, for the effects of amino acid concentration on μ for both strains, as well as in the results discussed in section 5.3 for the effect of ammonium concentration. While the vitamin concentration had the 3rd largest effect on CEN.PK113-7D against 5th for PE-2 strain, evidencing the capability of PE-2 strains to overcome low growth factor concentrations.

Figure 17 - Pareto chart of the standardized effects for PE-2 and CEN.PK113-7D in 2SMolAA25 with variations in concentration of nutritional groups. A=Ammonium, B=Organic acids, C=Trace elements, D=Vitamins, E=Mg & K, F=Calcium, G=Amino acids and H=Phosphate.

PE-2 CEN.PK113-7D

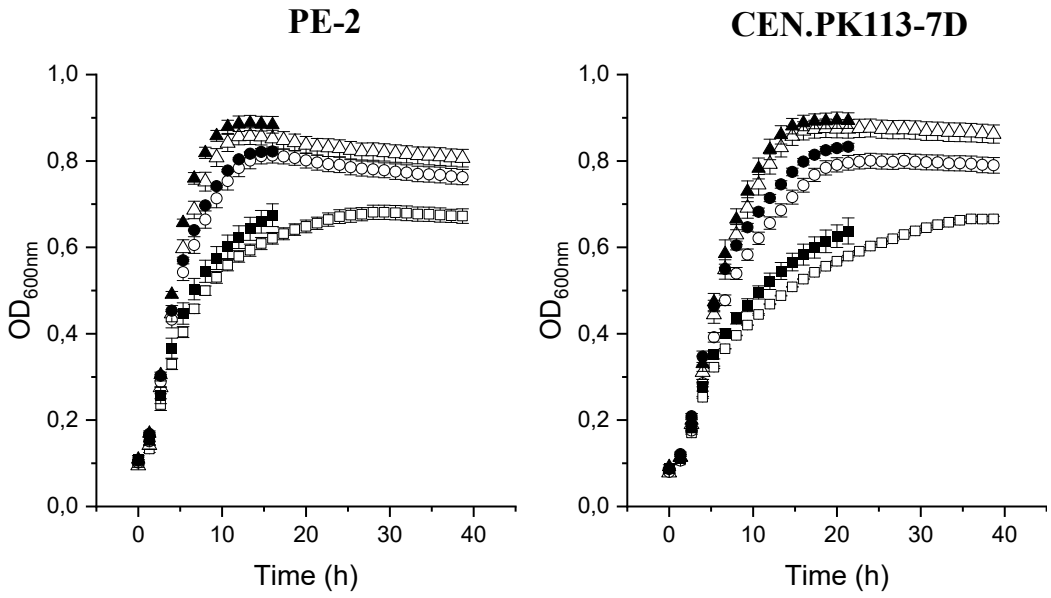


Source: The author

Regarding OD_{max}, amino acid and ammonium concentration had the largest observed effects for both strains, which is again consistent with data previously discussed. Unexpectedly, vitamin concentration was found to have significant effect on PE-2 OD_{max} results, differently from what was observed in section 5.3.1, in which OD_{max} did not differ significantly between the tested conditions for vitamin concentration.

The results for T_{decel} indicate that for both strains, ammonium concentration present the largest effect for this parameter; however, amino acids concentration showed significantly smaller value for CEN.PK113-7D in comparison to PE-2. This can be explained as T_{decel} could not be accurately estimated in this set of experiment as measurements ceased before growth could completely stabilize, particularly for the condition with lower amino acids concentration. This can be evidenced by the comparison of results from Table 18 and from Table 20 and Table 21 for 2SMolAA0, 2SMolAA25 and 2SMolAA50, in which the values obtained for μ and OD_{max} in the different experiments presented small variations between the two datasets whereas T_{decel} presented significantly different values, particularly for the lowest amino acids concentration. The growth curves obtained for both set of experiments, presented on Figure 18, may explain the difference in T_{decel} results. While for the first set of experiments (data with empty symbols) readings were done for approximately 40 hours, the latter lasted approximately 20 hours (data with filled symbols). It was assumed by that time that the growth curves were sufficiently monitored to allow differentiation between the tested conditions. Thus, the estimation of the time difference between the end of the exponential phase and the point of maximum absorbance could not be estimated appropriately. Nonetheless, the growth curves presented on Figure 18 and the results from section 5.5.1., demonstrate higher amino acids concentration in the medium significantly decrease T_{decel}.

Figure 18 - Growth curves for PE-2 and CEN.PK113-7D strains in 2SMolAA0 (■□), 2SMolAA25 (●○), 2SMolAA50 (▲△), for two independent experiments. Filled symbols refer to data which parameters are listed on Table 18, empty symbols refer to data which parameters are listed on Table 20 and Table 21



Source: The author

Three of these nutritional groups were chosen for a sequential study: ammonium, amino acids and vitamin. The first two presented significant effects for all the parameters measured, whereas the vitamin content presented a more pronounced effect on CEN.PK113-7D maximum specific growth rate. It is expected that the different growth behaviour between molasses is most likely the result of concentration variation in these components, thus a factorial design with three levels will be used to evaluate interactions between these variables and to establish a statistical model to evaluate these parameters to predict or emulate different molasses with these parameters.

Table 20 – Growth parameters for PE-2 using 2SmolAA25 and variations in nutritional groups concentrations.

Condition	μ (h^{-1})		OD_{Max}		T_{decel} (h)	
	Lower level	Higher level	Lower level	Higher level	Lower level	Higher level
2SMolAA25	0,416 ± 0,003		0,823 ± 0,006		12,22 ± 0,77	
Ammonium	0,428 ± 0,011	0,400 ± 0,008	0,812 ± 0,013	0,688 ± 0,025	8,22 ± 0,19	13,33 ± 0,00
Organic Acids	0,432 ± 0,014	0,414 ± 0,012	0,796 ± 0,017	0,800 ± 0,005	10,67 ± 0,00	10,11 ± 0,19
Trace elements	0,407 ± 0,007	0,403 ± 0,017	0,778 ± 0,021	0,817 ± 0,014	11,78 ± 0,51	10,34 ± 0,58
Vitamins	0,393 ± 0,003	0,411 ± 0,021	0,736 ± 0,029	0,812 ± 0,025	13,00 ± 0,33	11,67 ± 0,88
Mg & K	0,433 ± 0,005	0,412 ± 0,015	0,789 ± 0,008	0,798 ± 0,020	12,00 ± 0,58	10,56 ± 0,51
Calcium	0,425 ± 0,010	0,421 ± 0,016	0,810 ± 0,025	0,809 ± 0,024	12,22 ± 0,84	11,67 ± 0,33
Amino acids	0,357 ± 0,016	0,424 ± 0,010	0,682 ± 0,026	0,889 ± 0,017	13,33 ± 0,00	9,67 ± 0,33
Phosphate	0,419 ± 0,023	0,415 ± 0,016	0,787 ± 0,024	0,800 ± 0,006	11,56 ± 0,39	12,78 ± 0,39

Source: The author

Table 21 – Growth parameter for CEN.PK113-7D using 2SmolAA25 and variations in nutritional groups concentrations.

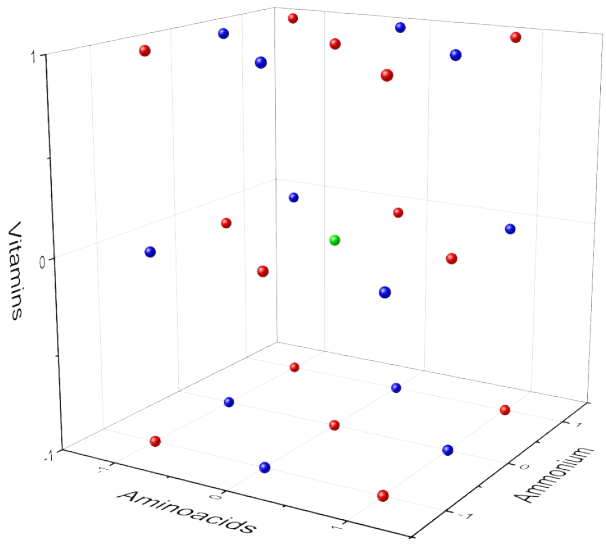
Condition	μ (h^{-1})		OD_{Max}		T_{decel} (h)	
	Lower level	Higher level	Lower level	Higher level	Lower level	Higher level
2SMolAA25	0,387 ± 0,004		0,833 ± 0,008		17,00 ± 0,00	
Ammonium	0,404 ± 0,007	0,367 ± 0,004	0,857 ± 0,018	0,689 ± 0,001	13,11 ± 1,02	17,00 ± 0,00
Organic Acids	0,382 ± 0,002	0,387 ± 0,009	0,813 ± 0,016	0,812 ± 0,025	14,22 ± 0,51	16,67 ± 0,33
Trace elements	0,367 ± 0,006	0,373 ± 0,007	0,852 ± 0,012	0,825 ± 0,043	16,11 ± 1,02	16,56 ± 0,19
Vitamins	0,348 ± 0,006	0,377 ± 0,004	0,836 ± 0,028	0,878 ± 0,020	16,89 ± 0,39	16,45 ± 0,77
Mg & K	0,388 ± 0,006	0,379 ± 0,006	0,840 ± 0,008	0,806 ± 0,038	16,89 ± 0,19	16,00 ± 0,67
Calcium	0,384 ± 0,012	0,385 ± 0,003	0,818 ± 0,045	0,840 ± 0,021	16,89 ± 0,19	16,45 ± 0,39
Amino acids	0,335 ± 0,004	0,388 ± 0,010	0,638 ± 0,030	0,896 ± 0,019	17,00 ± 0,33	16,00 ± 0,67
Phosphate	0,365 ± 0,011	0,375 ± 0,007	0,797 ± 0,011	0,838 ± 0,014	16,22 ± 0,19	16,67 ± 0,58

Source: The author

5.6 3^3 factorial design

A 3^3 full factorial design, yielding 27 conditions represented visually on Figure 19, was used to study the interactions between the concentration of amino acids, ammonium and vitamins on specific growth rate, maximum absorbance and deceleration time for PE-2 and CEN.PK113-7D strain. This methodology allowed the creation of a statistical model, using Minitab®, for μ, OD_{max} and $T_{decel,}$ given in Equation 2 for the coded variables in which the response is represented by y and the linear and interaction terms are represented b_i and b_{ij}, respectively. Additionally, two industrial molasses samples were also tested in the same experimental runs, allowing their results comparison to the model obtained from 2SMol variations.

Figure 19 – Visual representation of a 3^3 Full factorial design



Source: The author

$$\hat{y} = b_0 + b_1\, Amino\ acids + b_2 Ammonium + b_3\, Vitamins + b_{11}\, Amino\ acids.Amino\ acids + b_{22}\, Ammonium.Ammonium + b_{33}\, Vitamins.Vitamins + b_{12}\, Amino\ acids.Ammonium + b_{13}\, Amino\ acids.Vitamins + b_{23}\, Ammonium.Vitamin \quad (2)$$

Each factor level is defined on Table 22, as a percentual of the original content of 2SMol, these ranges were the same tested for ammonium in section 5.5.2; however, amino acids and vitamin ranges were increased to cover a wider dimensional space. Each condition,

including the central point was run in triplicate and the results in terms of means and standard deviations are presented on APPENDIX B, as well as the predicted values obtained by the adjusted model.

Table 22 – Investigated factors and their levels with coded values in brackets.

Investigated factors	Factor levels in % of 2SMol content		
Amino acids	0 (-1)	37,5 (0)	75 (+1)
Ammonium	0 (-1)	100 (0)	200 (+1)
Vitamin	0 (-1)	100 (0)	200 (+1)

Source: The author

The obtained model coefficients for both strains are presented on μ, OD_{max} and T_{decel} are presented on Table 23, Table 24 and Table 25, respectively.

For the maximum specific growth rat, the adjusted quadratic model R^2 values were close to 89% for both strains indicating a good adjustment of the model to the data, while values for R^2 adjusted and R^2 predicted close to R^2, indicate that the model was not overfit. Model coefficients for maximum specific growth rate for both PE-2 and CEN.PK113-7D strain (Table 23) show that all factors linear and quadratic terms for amino acid, ammonium and vitamin concentration are significant to the model ($p < 0,05$). The amino acid and ammonium interaction concentrations is significant for both strains, ammonium and vitamin interaction are not significant for PE-2 strain and ammonium and vitamin are not significant for both strains. Additionally, linear and interaction model coefficient terms for CEN.PK113-7D were larger in comparison to PE-2 values, indicating a larger sensitivity of the maximum specific growth rate to amino acid, ammonium, and vitamin concentration.

Results for OD_{max} show significant effect for all linear factors; however, quadratic effect for vitamin concentration was not significant for both strains, as well as the interaction effect between ammonium and vitamin concentrations. As opposed to the results for maximum specific growth rate, the coefficient terms for CEN.PK113-7D were not consistently higher than PE-2, indicating the response to changes in the concentration of amino acids, ammonium and vitamin are not for both strains. R^2 values for CEN.PK113-7D were slightly lower than for PE-2, nonetheless the values for R^2, adjusted R^2, and predicted R^2 were within close ranges, suggesting the model adequacy to the data.

Table 23 – Model coefficients for maximum specific growth rate obtained from the 3^3 factorial design for PE-2 and CEN.PK113-7D strain. 1=Amino acids, 2=Ammonium, 3=Vitamins.

Term	PE-2				CEN.PK113-7D			
	Coefficient	Standard Error	t-Value	p-Value	Coefficient	Standard Error	t-Value	p-Value
b_0	0,424	0,006	68,820	0,000	0,401	0,008	53,100	0,000
b_1	0,044	0,003	15,540	0,000	0,046	0,004	13,030	0,000
b_2	0,026	0,003	9,130	0,000	0,032	0,004	9,130	0,000
b_3	0,023	0,003	8,230	0,000	0,035	0,004	9,890	0,000
b_{11}	-0,037	0,005	-7,550	0,000	-0,048	0,006	-7,860	0,000
b_{22}	-0,023	0,005	-4,680	0,000	-0,033	0,006	-5,460	0,000
b_{33}	-0,024	0,005	-4,810	0,000	-0,031	0,006	-5,170	0,000
b_{12}	-0,030	0,003	-8,510	0,000	-0,048	0,004	-11,140	0,000
b_{13}	0,006	0,003	1,730	0,089	0,008	0,004	1,870	0,065
b_{23}	0,011	0,003	3,010	0,004	0,006	0,004	1,330	0,188
R^2		89,08%				89,40%		
R^2 adjusted		87,69%				88,05%		
R^2 predicted		85,57%				86,34%		

Source: The author

Table 24 – Model coefficients for maximum absorbance obtained from the 3^3 factorial design for PE-2 and CEN.PK113-7D strain. A=Amino acids, B=Ammonium, C=Vitamins.

Term	PE-2				CEN.PK113-7D			
	Coefficient	Standard Error	t-Value	p-Value	Coefficient	Standard Error	t-Value	p-Value
b_0	0,914	0,017	55,310	0,000	0,903	0,021	44,040	0,000
b_1	0,159	0,008	20,840	0,000	0,167	0,009	17,580	0,000
b_2	0,076	0,008	9,890	0,000	0,074	0,009	7,840	0,000
b_3	0,032	0,008	4,230	0,000	0,044	0,009	4,650	0,000
b_{11}	-0,081	0,013	-6,100	0,000	-0,056	0,016	-3,400	0,001
b_{22}	-0,042	0,013	-3,160	0,002	-0,055	0,016	-3,370	0,001
b_{33}	-0,017	0,013	-1,280	0,205	-0,006	0,016	-0,360	0,722
b_{12}	-0,066	0,009	-7,060	0,000	-0,103	0,012	-8,840	0,000
b_{13}	0,034	0,009	3,640	0,001	0,029	0,012	2,480	0,016
b_{23}	0,014	0,009	1,450	0,150	0,016	0,012	1,370	0,176
R^2		90.34%				82,35%		
R^2 adjusted		89,11%				82,37%		
R^2 predicted		87,49%				79,69%		

Source: The author

Deceleration time for PE-2 was significantly lower for PE-2 strain in comparison to CEN.PK113-7D strain, as demonstrated by the large difference in the constant b_0 value (Table 25). For these parameters, the quadratic interaction for ammonium and amino acids concentration were not significant (p-value>0,05) for both strains, amino acid and ammonium interaction was not significant for PE-2, and interaction between vitamin and ammonium concentration was not significant for CEN.PK113-7D. As observed for μ and maximum absorbance, R^2, R^2 adjusted, and R^2 predicted were within similar ranges, indicating an overall fitness of the model to the data.

Table 25 – Model coefficients for deceleration time obtained from the 3^3 factorial design for PE-2 and CEN.PK113-7D strain. A=Amino acids, B=Ammonium, C=Vitamins.

Term	PE-2				CEN.PK113-7D			
	Coefficient	Standard Error	t-Value	p-Value	Coefficient	Standard Error	t-Value	p-Value
b_0	11,137	0,378	29,480	0,000	18,274	0,949	19,260	0,000
b_1	-4,315	0,175	-24,670	0,000	-4,889	0,439	-11,130	0,000
b_2	-2,297	0,175	-13,130	0,000	-3,704	0,439	-8,430	0,000
b_3	-0,766	0,175	-4,380	0,000	-4,463	0,439	-10,160	0,000
b_{11}	0,352	0,303	1,160	0,249	1,148	0,761	1,510	0,136
b_{22}	0,037	0,303	0,120	0,903	-0,444	0,761	-0,580	0,561
b_{33}	0,704	0,303	2,320	0,023	2,871	0,761	3,770	0,000
b_{12}	0,111	0,214	0,520	0,606	1,482	0,538	2,750	0,007
b_{13}	-0,620	0,214	-2,900	0,005	-4,084	0,538	-7,590	0,000
b_{23}	-0,796	0,214	-3,720	0,000	-0,806	0,538	-1,500	0,139
R^2		92,13%				87,59%		
R^2 adjusted		91,12%				86,02%		
R^2 predicted		89,60%				84,04%		

Source: The author

The obtained equations were then used to estimate the concentration ranges that could, theoretically reproduce the results obtained by the two molasses samples tested, from Iracema (IRA) and São José (USJ) sugarcane mills. These two samples were chosen for their distinct growth patterns. Both presented similar values for maximum specific growth rate and maximum optical density and different T_{decel}. The calculated parameters for PE-2 and CEN.PK113-7D in each of these molasses are presented on Table 26. As observed for the synthetic compositions tested, PE-2 strain had larger μ and ODmax, and smaller t_{decel} values. Finally, the results obtained were between the ranges found for the synthetic compositions

tested, indicating that the synthetic medium presents flexibility and sensitivity, allowing the obtention of different results, as different industrial molasses usually present.

Table 26 - Values obtained for maximum specific growth rate, maximum optical density and deceleration time for PE-2 and CEN.PK113-7D strains.

Strain	PE-2			CEN.PK113-7D		
Param.	μ (h^{-1})	OD_{max}	t_{decel} (h)	μ (h^{-1})	OD_{max}	t_{decel} (h)
IRA	0,47 ± 0,00	0,93 ± 0,03	5,22 ± 0,19	0,37 ± 0,00	0,83 ± 0,02	10,11 ± 1,02
USJ	0,43 ± 0,01	0,86 ± 0,03	17,89 ± 0,19	0,35 ± 0,01	0,80 ± 0,04	29,78 ± 1,54

Source: The author

Nonetheless, the exact results obtained three parameters of the industrial molasses samples could not be matched with a single synthetic molasses composition, which might be due to the presence of other components present in industrial sample that can affect yeast growth behaviour.

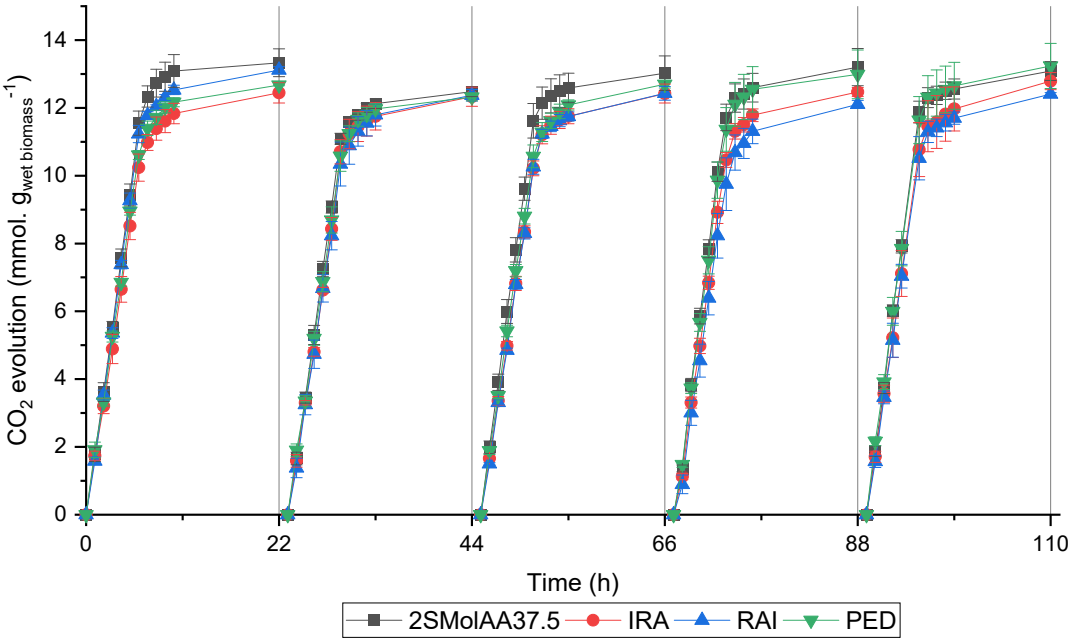
5.7 Validation in small scale fed-batch fermentations mimicking the Brazilian process

Although microplate assays are useful for assessing a high number of conditions, the proposed synthetic medium must be validated in assays more representative of the ethanol production from sugarcane-based substrates, such as the methodology described by Raghavendran et al. (2017). Thus, 2SMolAA37,5 composition, presented in Table 27, was used as substrate in small scale fed-batch fermentations with cell recycle and its results were compared to fermentations using industrial molasses from Iracema, Pedra and Raízen Univalem mills. Industrial molasses samples were diluted to contain 180 $g.L^{-1}$ TRS, to assess whether it would reproduce the obtained by the fermentation of industrial substrates over at least five fermentation cycles. The yeast PE-2 was propagated as described in materials and methods section, using molasses from Iracemápolis mill collected in September 2020.

Sugar consumption profiles for the synthetic composition, monitored by tube weight loss, presented similar results to the industrial molasses, without pronounced and or consistent differences over cycles (Figure 20). Results are represented in terms of mmol $CO_2.g_{wet\ biomass}^{-1}$ to normalize possible deviations in consumption speed due to yeast mass gain or loss over cycles (Raghavendran et al., 2017). On the first cycle, the synthetic composition was apparently more favourable to sugar consumption than the natural media, while on the second cycle, every medium presented indistinguishable results. Again, on the third cycle, the

synthetic medium fermentation ended slightly sooner than for the other media, however, on the following cycles, no sharp differences could be observed.

Figure 20 – CO_2 profiles for 2SMolAA37.5 and industrial molasses from Iracema, Raízen and Pedra mills, normalized by wet biomass. All experiments were carried out in triplicates and results are represented as mean ± standard error.



Source: The author

Synthetic must pH was 5, lower than the industrial molasses samples used, with pH around 5.5 (Figure 21). Nonetheless, for all the conditions a similar pattern was observed, for the first two cycles wine pH drops in relation to the must, then for the following cycles the pH remains constant, with minimal variation between the second and fifth cycles even to match the exact pH of a specific sample. Additionally, yeast cell viability remained above 90% (Figure 22) for synthetic and industrial molasses samples, suggesting that the pH difference found in this experiment was not critical (Figure 21). The pH is a critical parameter to ensure proper fermentation efficiency and maintain cell viability and although pH values observed for synthetic molasses lower than the industrial molasses, they were within a non-stressful range for *S. cerevisiae* industrial strains. Nonetheless, this is a parameter that can be easily adjusted during formulation of the medium even to match the exact pH of a specific sample.

Table 27 – 2SmolAA37,5 composition

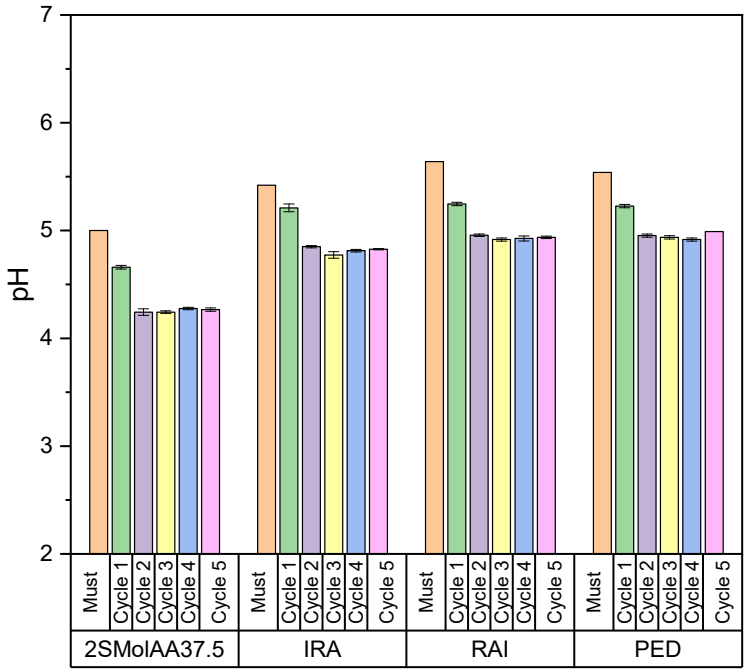
Class	Compound	Concentration	Stock solution for medium preparation
Carbon sources ($g.L^{-1}$)	Sucrose	160	Sugar stock - 2.5x Concentrated
	Glucose	20	
	Fructose	20	
Maillard reaction products simulation ($g.L^{-1}$)	Glutamine	1.56	Amino acids Stock – 2.5x Concentrated
	Aspartic Acid	0.56	
	Asparagine	0.53	
Inorganic nitrogen source ($g.L^{-1}$)	$(NH_4)_2HPO_4$	1.00	Inorganic Nitrogen Stock - 100x Concentrated
Organic acids ($g.L^{-1}$)	Trans-aconitic acid	2.00	Organic Acids Stock Solution - 20x Concentrated
	l-malic acid	1.00	
	Citric acid	0.01	
	KOH	1.96	
Trace elements[1] ($mg.L^{-1}$)	$EDTA.Na_2$	150.00	Trace Elements Stock Solution - 100x Concentrated
	$CaCl_2.2H_2O$	45.00	
	$MnCl_2.4H_2O$	10.00	
	$ZnSO_4.7H_2O$	45.00	
	$CuSO_4 \cdot 5H_2O$	3.00	
	KI	1.00	
	$CoCl_2.6H_2O$	3.00	
	H_3BO_3	10.00	
	$FeSO_4.7H_20$	30.00	
	$Na_2MoO_4.2H_20$	4.00	
Vitamins[2] ($mg.L^{-1}$)	Inositol	25.00	Vitamin Stock Solution - 1000x Concentrated
	Nicotinic acid	1.00	
	Calcium pantothenate	1.00	
	Biotin	0.05	
	Pyridoxine hydrochloride	1.00	
	Thiamine hydrochloride	1.00	
	Para-aminobenzoic acid	0.20	
Magnesium and potassium ($g.L^{-1}$)	K_2SO_4	5	Mg & K Stock Solution - 50x Concentrated
	$MgSO_4.7H_2O$	10	
Calcium ($g.L^{-1}$)	$CaCl_2.2H_2O$	0.5	Calcium Stock - 500x Concentrated

Source: The author

[1] As described by Verduyn et al. (1992), 10-fold increase in each component concentration

[2] As described by Verduyn et al. (1992)

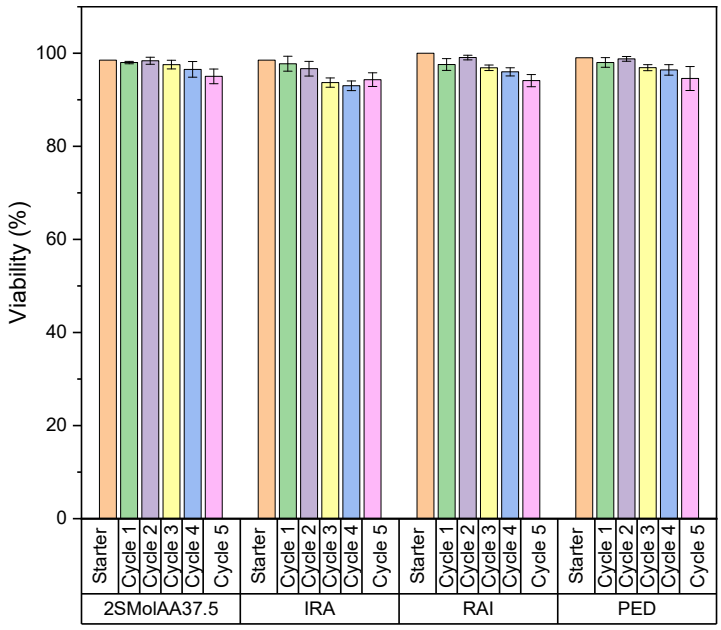
Figure 21 - Must and wine pH for five cycles of fed batch fermentations with cell recycle using 2SMolAA37.5 and industrial molasses from Iracema, Raízen and Pedra mills.



Source: The author

Regarding yeast cell biomass variation, the synthetic medium also presented similar results as the ones observed for the industrial molasses, showing an increase between cycles 1 and 2, and remaining constant throughout the remaining cycles. This is another evidence that the synthetic composition reproduces the results obtained by the fermentation of industrial molasses samples. Similar results were previously reported in fermentations using the same conditions, employing different molasses samples and strains (Prado et al., 2020; Raghavendran et al., 2017).

Figure 22 – Yeast cell viability at propagation step and after each fermentation cycle for the synthetic composition and industrial molasses samples.

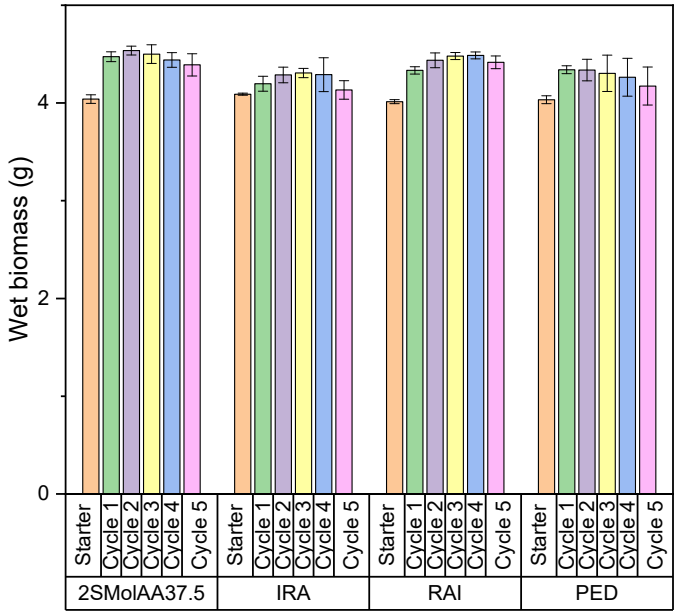


Source: The author

Ethanol yield, calculated as percentage of the theoretical maximum conversion yield (0.511 g of ethanol per g of hexose-equivalent) was also compared among media, and the synthetic composition also presented similar values as the ones obtained by fermentation of the industrial molasses samples (Figure 24). Although we observed high values for this parameter, close to 100%, the different media presented similar results and no particular trend was observed for a single sample, a similar behaviour observed for this parameter by Lino et al., (2018).

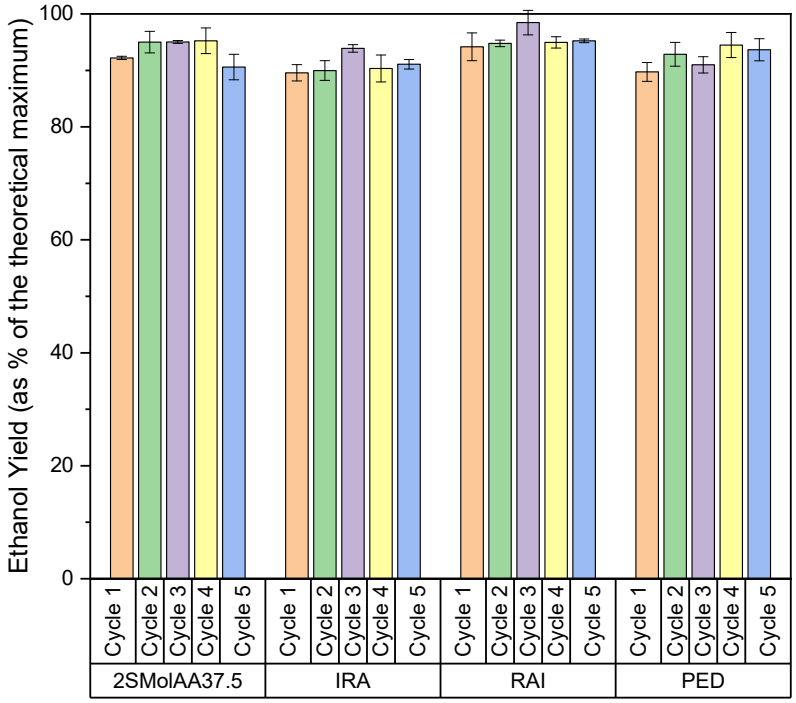
Finally, glycerol concentration in wine was the only measured parameter for which the synthetic medium did not presented similar results as the ones observed for industrial molasses. While glycerol in wine samples from industrial molasses remained around 3 $g.L^{-1}$, for the synthetic wine glycerol concentration was significantly higher, around 4 $g.L^{-1}$, especially after the second cycle. This set of experiments was not designed to identify possible causes for this difference, and other experiments should be conducted to investigate glycerol production by yeasts in the proposed medium, as glycerol is not a desired product in this process.

Figure 23 – Wet biomass at propagation step and after each fermentation cycle for the synthetic composition and industrial molasses samples.



Source: The author

Figure 24 - Ethanol yield of wine as percentage of the maximum theorical value.



Source: The author

Figure 25 – Glycerol concentration in $g.L^{-1}$ in wine for five cycles of fed batch fermentations with cell recycle

	2SMolAA37.5	Iracema	Raízen	Pedra
Cycle 1	3,03 ± 0,03	2,81 ± 0,09	2,77 ± 0,11	2,70 ± 0,06
Cycle 2	3,73 ± 0,49	2,89 ± 0,17	2,81 ± 0,12	2,71 ± 0,08
Cycle 3	4,13 ± 0,75	2,92 ± 0,05	2,91 ± 0,03	2,66 ± 0,09
Cycle 4	3,98 ± 0,08	3,00 ± 0,15	2,85 ± 0,19	3,17 ± 0,08
Cycle 5	4,02 ± 0,13	3,18 ± 0,19	3,25 ± 0,33	3,19 ± 0,20

Source: The author

Overall, the results obtained for the fermentation 2SmolAA37.5 were comparable to results obtained by the fermentation of industrial molasses samples in terms of pH, biomass, cell viability and ethanol yield. Glycerol concentrations in the wines were higher for the synthetic medium, which deserves further investigation. Nonetheless, these results demonstrated that the proposed synthetic medium composition can mimic molasses in fed-batch fermentations with cell recycle.

5 CONCLUSION

Different molasses samples yielded different results (in terms of growth profiles and physiological parameters – growth rates and final OD) in batch fermentations conducted on microplates with both laboratory and industrial yeast strains, illustrating that even industrial strains, considered as more adapted to harsh conditions than lab ones, can struggle to grow on molasses. This also highlights the need for a standardized medium that effectively simulates molasses-based media. Specifically, on the differences between laboratory and industrial strains, CEN.PK family strains showed to be more sensitive to vitamin content than PE-2 and SA-1 strains. When vitamin concentrations were low, the growth curves showed a linear pattern and when vitamin concentration was increased, laboratory and industrial strains showed the same growth patterns.

The composition proposed by Lino et al (2018) was evaluated and modification were proposed this work. In general, the proportion of sucrose and glucose/fructose content was adjusted to match that of molasses. The mineral composition was extensively increased to match recent literature data. And vitamin and trace element stock solutions were also changed

to incorporate solutions already used by research groups working with yeasts. The initial proposed composition, 1SMol, was formulated in 7 groups to assess the effect of each nutrient group on the growth profile of yeasts cells. Among all groups tested, nitrogen source (as ammonia) and vitamins affected significantly both industrial and laboratory strain's growth profiles and physiological parameters.

In view of different growth profiles between industrial molasses and 1SMol cultivations, another version of the medium was formulated, 2SMol, substituting the solution containing Maillard reaction products, present in 1SMol, to pure sugar and amino acids solution. This formulation promoted growth profiles similar to industrial molasses. Additionally, it was possible to identify that amino acid, ammonium and vitamin concentrations had the largest impact in the maximum specific growth rate, maximum absorbance and deceleration time and a response surface methodology was used to study the interactions between the three nutrients and these parameters.

Finally, benchmarking the synthetic molasses medium against 3 industrial molasses samples in a small-scale fed-batch fermentation experiment that mimicked the industrial conditions employed in Brazil, showed that CO_2 production profile, pH, yeast cell viability, yeast wet biomass and ethanol yield were similar among all media, indicating that the proposed medium is an adequate medium to reproduce the results obtained by the fermentation of industrial molasses.

Therefore, we propose that the formulation 2SMol can be considered a defined medium to be employed in laboratory studies interested in fermentation of molasses by yeast cells, allowing standardization in molasses-based investigations. We encourage that experiments could and should be done to take the most of this medium, challenging what we know about sugarcane first generation ethanol fermentation. The following questions remain to be answered yet: Is low nitrogen concentration in molasses decisive to the high ethanol yield observed in sugarcane substrates, if they had more nitrogen we would necessarily obtain more biomass and less ethanol? What is the effect of high salt concentration in ethanol fermentation, are "spent" molasses bad musts? What are the effects of low vitamin concentration on fermentation? Although this work did not address any of these questions objectively, it provided a tool for them to be answered.

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[1] According to APA (American Psychological Association)

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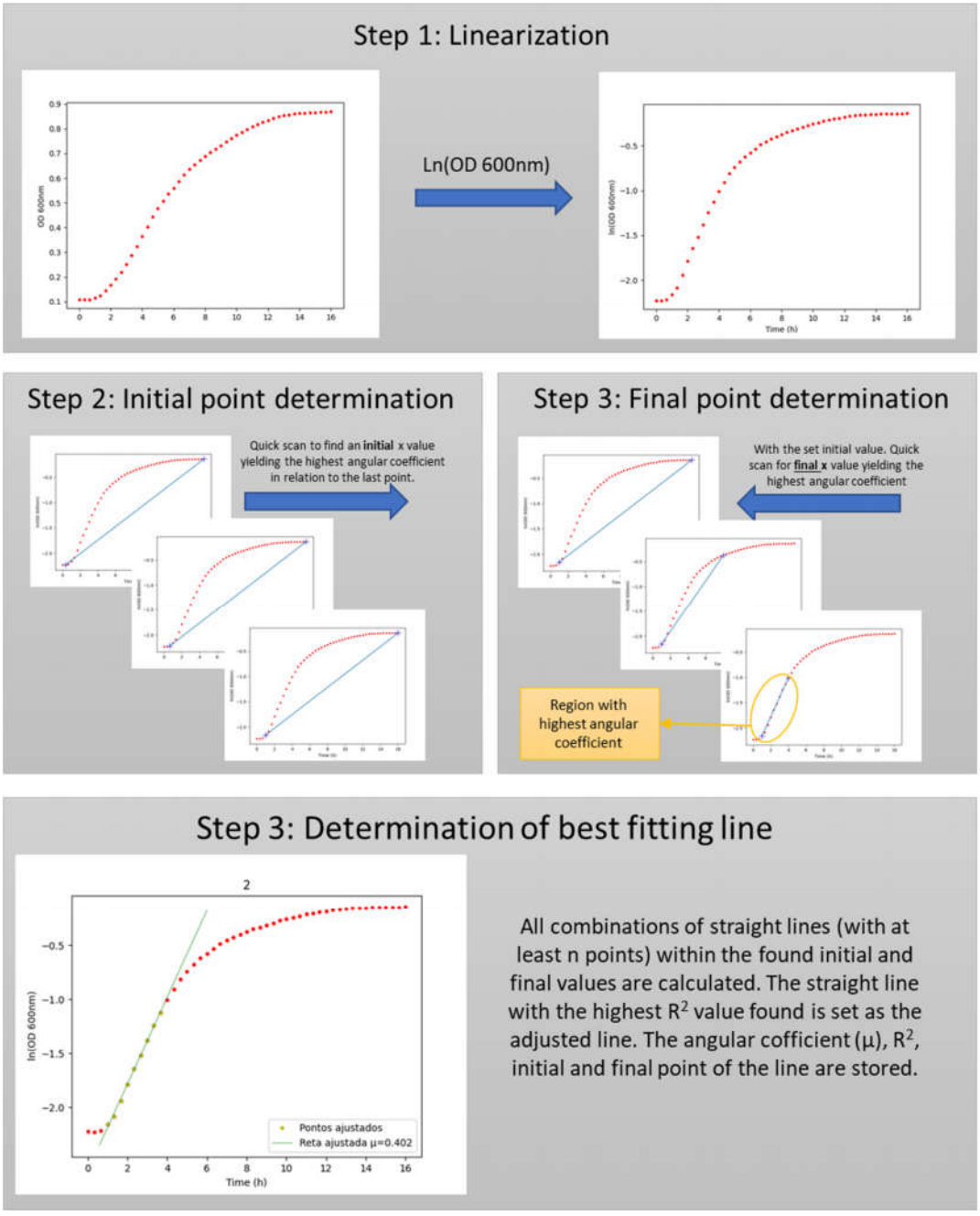
APPENDIX A – Script and orientations used to determine maximum specific growth rate, maximum absorbance, and deceleration time in batch cultivations in microplates

The algorithm used follows the logic described in Figure A.1.

Figure A.1 – Algorithm used to calculate the steepest region in the ln(OD) vs time curves

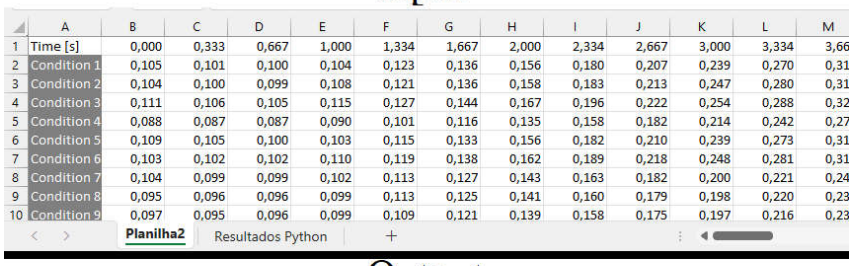


Source The author

The following script was used, developed in Python and ran in Microsoft Visual Studio Code.

Figure A.2 – Data input in .xlsx file and the output generated in a new sheet in the same .xlsx file

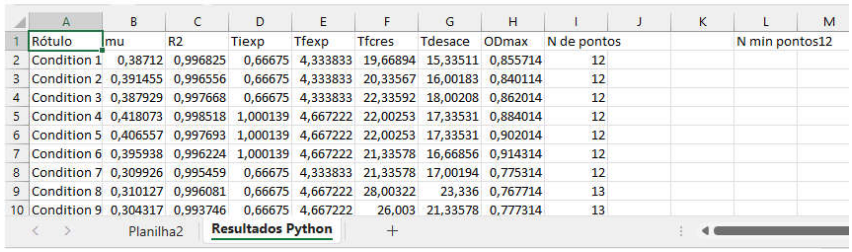
Input



	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Time [s]	0,000	0,333	0,667	1,000	1,334	1,667	2,000	2,334	2,667	3,000	3,334	3,66
2	Condition 1	0,105	0,101	0,100	0,104	0,123	0,136	0,156	0,180	0,207	0,239	0,270	0,31
3	Condition 2	0,104	0,100	0,099	0,108	0,121	0,136	0,158	0,183	0,213	0,247	0,280	0,31
4	Condition 3	0,111	0,106	0,105	0,115	0,127	0,144	0,167	0,196	0,222	0,254	0,288	0,32
5	Condition 4	0,088	0,087	0,087	0,090	0,101	0,116	0,135	0,158	0,182	0,214	0,242	0,27
6	Condition 5	0,109	0,105	0,100	0,103	0,115	0,133	0,156	0,182	0,210	0,239	0,273	0,31
7	Condition 6	0,103	0,102	0,102	0,110	0,119	0,138	0,162	0,189	0,218	0,248	0,281	0,31
8	Condition 7	0,104	0,099	0,099	0,102	0,113	0,127	0,143	0,163	0,182	0,200	0,221	0,24
9	Condition 8	0,095	0,096	0,096	0,099	0,113	0,125	0,141	0,160	0,179	0,198	0,220	0,23
10	Condition 9	0,097	0,095	0,096	0,099	0,109	0,121	0,139	0,158	0,175	0,197	0,216	0,23

Planilha2 | Resultados Python | +

Output



	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Rótulo	mu	R2	Tiexp	Tfexp	Tfcres	Tdesace	ODmax	N de pontos			N min pontos12	
2	Condition 1	0,38712	0,996825	0,66675	4,333833	19,66894	15,33511	0,855714	12				
3	Condition 2	0,391455	0,996556	0,66675	4,333833	20,33567	16,00183	0,840114	12				
4	Condition 3	0,387929	0,997668	0,66675	4,333833	22,33592	18,00208	0,862014	12				
5	Condition 4	0,418073	0,998518	1,000139	4,667222	22,00253	17,33531	0,884014	12				
6	Condition 5	0,406557	0,997693	1,000139	4,667222	22,00253	17,33531	0,902014	12				
7	Condition 6	0,395938	0,996224	1,000139	4,667222	21,33578	16,66856	0,914314	12				
8	Condition 7	0,309926	0,995459	0,66675	4,333833	21,33578	17,00194	0,775314	12				
9	Condition 8	0,310127	0,996081	0,66675	4,667222	28,00322	23,336	0,767714	13				
10	Condition 9	0,304317	0,993746	0,66675	4,667222	26,003	21,33578	0,777314	13				

Planilha2 | Resultados Python | +

Source The author

```
from openpyxl import load_workbook
import matplotlib
import matplotlib.pyplot as plt
import numpy as np
from sklearn.linear_model import LinearRegression
################################################################

def main():

    Dados = []
    file = "ExemploDados.xlsx"
    book = 0
    workbook = load_workbook(filename=file)
    workbook.active = book
    sheet = workbook.active
    col_max= sheet.max_column #limits at which column data will be read
    intervalo = [2,col_max] #Data range for time (final time indicated by col_max)
    time_row = 1 #Line at which the time points are (1 by default)
    row_max = sheet.max_row #limits at which row data will be read
    data_row=[2,row_max] # Data range for which microplate wells will be evaluated
    graficos=str("s") #set “s” to generate graphs in separate files
    nmin=12 #number of points in adjusted
    for i in range(data_row[0],data_row[1]+1): #Iteration to calculate each row
        print("row:",i)
        dados = Analise(file,book,intervalo,time_row,i,nmin,graficos) #Calls function
“Análise”, using the parameters defined above
        Dados.append(dados)
    DadosPlanilha(file,Dados,nmin,data_row) #Function to print results in a new sheet
    return()

################################################################
```

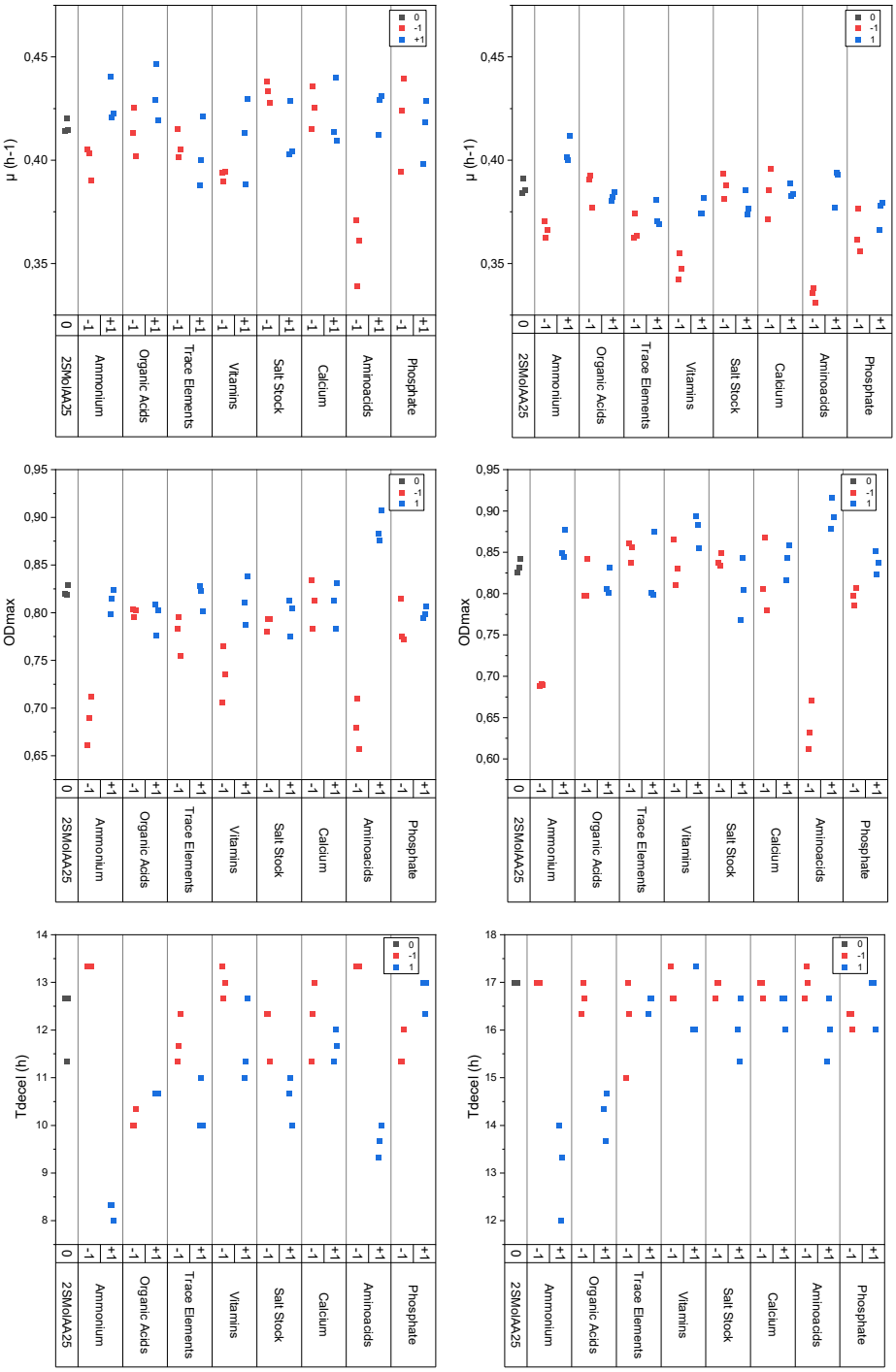
```
def DadosPlanilha(file,dados,nmin,data_row): # Function to print results in a new
sheet
    workbook =  load_workbook(filename=file)
    workbook.active = 0
    sheet = workbook.active
    for value in sheet.iter_cols(min_row=data_row[0], max_row=data_row[1], max_col=1,
values_only=True):
        title=value
    title=np.array(title)
    workbook.create_sheet("Resultados Python", 1)
    workbook.active = 1
    sheet = workbook.active
    for i in range(1,len(title)+1):
        sheet.cell(row=i+1, column=1).value = title[i-1]
    sheet.cell(row=1, column = 1).value = 'Rótulo' #Name in column1 of your data
    sheet.cell(row=1, column = 2).value = 'mu' #Mu in h^-1
    sheet.cell(row=1, column = 3).value = 'R2' #Adjusted line R^2
    sheet.cell(row=1, column = 4).value = 'Tiexp' #Inicial exponential time point
    sheet.cell(row=1, column = 5).value = 'Tfexp' # Final exponential time point
    sheet.cell(row=1, column = 6).value = 'Tfcres' #Time at which the growth curve
reachs ODmax
    sheet.cell(row=1, column = 7).value = 'Tdesace' #Tfcres-Tfexp
    sheet.cell(row=1, column = 8).value = 'ODmax' #OD max
    sheet.cell(row=1, column = 9).value = 'N de pontos' #Number of points in the
adjusted line
    numpontos='N min pontos' + str(nmin)
    sheet.cell(row=1, column = 12).value = str(numpontos)
    for i in range(2,len(dados)+2): #Prints data in the correct order
        for j in range(2,10) :
            sheet.cell(row=i, column=j).value = dados[i-2][j-2]
    workbook.save(filename=file)
    return()


#################################################################

def Analise(file,book,intervalo,time_row,data_row,nmin,graficos):
    workbook = load_workbook(filename=file)
    workbook.active = book
    sheet = workbook.active
    start = False
    end = False
    y1 = []
    y2 = []
    for value in sheet.iter_rows(min_row=time_row, max_row=time_row,
min_col=intervalo[0],max_col= intervalo[1], values_only=True):
        x=value
    for value in sheet.iter_rows(min_row=data_row, max_row=data_row,
min_col=intervalo[0],max_col= intervalo[1], values_only=True):
        y=value
    y=np.log(np.array(y))
    x=np.array(x) #Divide by /3600 if time is in seconds-> x=np.array(x)/3600
    grofin=np.max(y)
    groratio=0

    for i in range(0,intervalo[1]-1):
        groratio=grofin/y[i]
        if groratio>=1:
            #print(i)
            #print(x[i])
            fincres=x[i]
            break
```

```
    n = AcharStart(x,y) #Calls function to determine exponential phase start
    m = AcharEnd(x,y,n) # Calls function to determine exponential phase end
    original = Coeficientes(x,y)
    cortada = Coeficientes(x[n:m],y[n:m])
    a,b,c,mu,d = MelhorAjuste(x,y,m,n,nmin)
    ##print(x[a],x[b-1],a,b-1)
    print("velocidade específica:", mu)
    print("R2 =", c)
    mu = mu.tolist()
    mu = mu[0]

    if graficos[0]=='s':
        plt.plot(x,y,'r''.')
        plt.plot(x[i],y[i],'m''D',label='ODmax')
        plt.plot(x[a:b],y[a:b],'y''.',label='Pontos ajustados')
        xa=np.linspace(0,8,8)
        label="Reta ajustada μ="+str(round(mu, 3))
        plt.plot(xa,mu*xa+d,'g',linewidth=0.5,label=str(label))
        plt.xlabel("Time (h)")
        plt.ylabel("ln(OD 600nm)")
        plt.title(str(data_row-1)+' '+str((file[:-5])))
        plt.legend(loc='upper right')
        plt.savefig(str(data_row-1)+' '+str((file[:-5]))+' '+str(nmin)+' pontos.png')
        plt.clf()
    return(mu,c,x[a],x[b-1],fincres,fincres-x[b-1],np.exp(grofin),b-a)

################################################################

def AcharStart(x,y): #Defines beginning of exponential phase
    PosiçãoInicial = 1
    m1 = (y[-1] - y[0]) /(x[-1] - x[0])
    start = False
    while start == False:
        m2 = (y[-1] - y[PosiçãoInicial]) /(x[-1] - x[PosiçãoInicial])

        if m1 > m2:
            start = True
        else:
            m1 = m2
            PosiçãoInicial += len(x)//25 #Change values if mu determination is
incorrect. Larger values yield smaller steps and increase computation time.
    return(PosiçãoInicial)
################################################################

def AcharEnd(x,y,n):
    PosiçãoFinal = len(x)-1
    m1 = (y[-1] - y[n]) /(x[-1] - x[n])
    start = False

    while start == False:

        m2 = (y[PosiçãoFinal] - y[n]) /(x[PosiçãoFinal] - x[n])
        if (PosiçãoFinal-n)<len(x)//3: #Change values if mu determination is
incorrect. Larger values yield smaller steps and increase computation time.
            start = True
        else:
            if m1 > m2:
                start = True
            else:
                    m1 = m2
                    PosiçãoFinal -= len(x)//4 #Serves as correction if the step is too
large. Cant be larger than the previous value.
```

```
    return(PosiçãoFinal)

#################################################################

def Coeficientes(x,y):
    xr = x.reshape((-1,1))
    model = LinearRegression().fit(xr,y)
    a = model.coef_
    b = model.intercept_
    r = model.score(xr,y)
    coef = [a,b,r]
    return(coef)

#################################################################

def MelhorAjuste(x,y,m,n,nmin): #Using values defined by AcharStart and AcharEnd,
finds the line with at least nmin points with larger R^2 value

    model1 = LinearRegression().fit(x[n:m].reshape((-1,1)),y[n:m])
    r1 = model1.score(x[n:m].reshape((-1,1)),y[n:m])
    rmax = r1
    imax = n
    jmax = m
    amax = model1.coef_
    bmax = model1.intercept_
    BestFit = False

    for i in range(len(x[n:m])-nmin):
        for j in range(nmin+i,len(x[n:m])):
            model = LinearRegression().fit(x[i:j].reshape((-1,1)),y[i:j])
            r = model.score(x[i:j].reshape((-1,1)),y[i:j])

            if r > rmax:
                imax = i
                jmax = j
                rmax = r
                amax = model.coef_
                bmax = model.intercept_
    return(imax,jmax,rmax,amax,bmax)

#################################################################
main()
```

APPENDIX B – Grouped scatter graph for μ, ODmax and Tdecel for PE-2 and CEN.PK113-7D strain for 2SMolAA25 variations in nutritional groups.

PE-2 CEN.PK113-7D



APPENDIX C - Maximum specific growth rate, maximum absorbance and deceleration time for PE-2 and CEN.PK113-7D strains in 2SMol medium with different concentrations of amino acids, ammonium and vitamins, and in industrial molasses samples.

Table C-1 – CEN.PK113-7D

Condition	AA	AM	VT	μ (h^{-1})	μ (h^{-1}) pred	% Difference	OD_{max}	OD_{max} pred	% Difference	T_{decel} (h)	T_{decel} (h) pred	% Difference
1	0	**0**	**0**	0,39 ± 0,00	0,401	-3,25%	0,85 ± 0,01	0,903	-5,85%	16,45 ± 1,39	18,274	-11,11%
2	0	0	**1**	0,41 ± 0,01	0,405	0,53%	0,90 ± 0,02	0,941	-4,51%	17,11 ± 0,38	16,682	2,52%
3	0	0	-1	0,31 ± 0,00	0,336	-8,89%	0,77 ± 0,01	0,852	-10,22%	20,56 ± 3,24	25,608	-24,57%
4	0	1	**0**	0,39 ± 0,00	0,400	-2,86%	0,88 ± 0,01	0,922	-5,09%	13,67 ± 2,60	14,126	-3,35%
5	0	1	**1**	0,39 ± 0,01	0,409	-5,26%	0,94 ± 0,04	0,976	-3,58%	14,22 ± 2,12	11,728	17,55%
6	0	1	**-1**	0,30 ± 0,01	0,329	-9,94%	0,81 ± 0,02	0,856	-5,09%	24,45 ± 0,19	22,266	8,92%
7	0	-1	**0**	0,37 ± 0,00	0,336	10,03%	0,83 ± 0,01	0,773	7,43%	20,89 ± 0,51	21,534	-3,08%
8	0	-1	**1**	0,38 ± 0,01	0,334	11,15%	0,89 ± 0,01	0,795	10,52%	22,89 ± 0,19	20,748	9,36%
9	0	-1	-1	0,30 ± 0,02	0,276	6,67%	0,87 ± 0,07	0,739	15,37%	28,78 ± 1,90	28,062	2,50%
10	**1**	**0**	**0**	0,38 ± 0,00	0,399	-4,55%	1,01 ± 0,01	1,013	-0,72%	11,67 ± 4,04	14,533	-24,55%
11	1	0	**1**	0,39 ± 0,01	0,411	-6,08%	1,05 ± 0,02	1,080	-3,09%	6,78 ± 0,77	8,857	-30,66%
12	1	0	-1	0,31 ± 0,00	0,325	-4,21%	0,88 ± 0,01	0,935	-6,42%	27,00 ± 1,76	25,951	3,90%
13	1	1	**0**	0,38 ± 0,00	0,351	7,00%	1,02 ± 0,02	0,930	8,44%	10,11 ± 3,69	11,867	-17,35%
14	1	1	**1**	0,38 ± 0,00	0,368	2,57%	1,03 ± 0,00	1,013	1,65%	7,33 ± 0,33	5,385	26,58%
15	1	1	**-1**	0,30 ± 0,01	0,271	10,28%	0,88 ± 0,01	0,835	5,40%	25,34 ± 0,33	24,091	4,91%
16	1	-1	**0**	0,37 ± 0,01	0,382	-2,36%	0,96 ± 0,02	0,986	-2,23%	20,22 ± 4,35	16,311	19,35%
17	1	-1	**1**	0,39 ± 0,01	0,388	-0,39%	1,02 ± 0,04	1,037	-1,42%	10,11 ± 7,17	11,441	-13,14%
18	1	-1	-1	0,31 ± 0,00	0,314	-0,88%	0,91 ± 0,03	0,923	-2,03%	26,78 ± 1,35	26,923	-0,53%
19	-1	**0**	**0**	0,33 ± 0,00	0,308	7,75%	0,76 ± 0,02	0,680	10,90%	29,34 ± 0,33	24,311	17,13%
20	-1	0	**1**	0,33 ± 0,00	0,303	9,20%	0,79 ± 0,02	0,689	13,15%	29,56 ± 0,69	26,803	9,32%
21	-1	0	-1	0,29 ± 0,00	0,250	12,36%	0,74 ± 0,03	0,659	10,59%	30,11 ± 0,19	27,561	8,48%
22	-1	1	**0**	0,36 ± 0,00	0,355	0,52%	0,77 ± 0,01	0,802	-3,86%	16,78 ± 1,02	18,681	-11,33%
23	-1	1	**1**	0,35 ± 0,01	0,356	-0,41%	0,82 ± 0,01	0,827	-1,36%	17,34 ± 1,20	20,367	-17,49%
24	-1	1	**-1**	0,29 ± 0,00	0,291	-2,17%	0,77 ± 0,03	0,765	1,35%	22,00 ± 1,45	22,737	-3,34%
25	-1	-1	**0**	0,15 ± 0,01	0,195	-27,09%	0,37 ± 0,04	0,447	-21,12%	29,56 ± 0,77	29,053	1,71%
26	-1	-1	**1**	0,15 ± 0,01	0,185	-25,48%	0,36 ± 0,01	0,441	-22,93%	29,00 ± 1,20	32,351	-11,54%
27	-1	-1	-1	0,14 ± 0,00	0,143	-5,02%	0,37 ± 0,01	0,442	-20,38%	29,67 ± 0,33	31,497	-6,16%

Table C-2 – PE-2

Condition	AA	AM	VT	μ (h^{-1})	μ (h^{-1}) model	Error (%)	OD_{max}	OD_{max} ,model	Error (%)	T_{decel} (h)	T_{decel} (h) mode	Error (%)
1	0	**0**	**0**	0,42 ± 0,01	0,424	-0,74%	0,89 ± 0,03	0,914	-2,67%	11,33 ± 1,76	11,137	1,74%
2	0	0	**1**	0,43 ± 0,00	0,423	1,66%	0,88 ± 0,02	0,929	-5,14%	11,11 ± 0,96	11,075	0,34%
3	0	0	-1	0,38 ± 0,00	0,376	1,77%	0,81 ± 0,03	0,864	-6,58%	11,56 ± 0,19	12,607	-9,09%
4	0	1	**0**	0,40 ± 0,01	0,427	-6,00%	0,87 ± 0,03	0,947	-8,44%	8,00 ± 0,67	8,877	-10,95%
5	0	1	**1**	0,41 ± 0,00	0,437	-5,36%	0,97 ± 0,03	0,976	-0,56%	7,78 ± 0,51	8,019	-3,09%
6	0	1	**-1**	0,37 ± 0,01	0,369	0,16%	0,89 ± 0,02	0,885	0,99%	9,78 ± 0,19	11,143	-13,95%
7	0	-1	**0**	0,39 ± 0,01	0,374	3,70%	0,86 ± 0,02	0,796	6,88%	13,45 ± 0,77	13,471	-0,19%
8	0	-1	**1**	0,36 ± 0,02	0,364	-0,01%	0,84 ± 0,03	0,798	5,40%	15,89 ± 1,26	14,205	10,61%
9	0	-1	-1	0,36 ± 0,00	0,338	5,75%	0,85 ± 0,01	0,761	10,36%	15,78 ± 0,69	14,145	10,36%
10	**1**	**0**	**0**	0,42 ± 0,00	0,431	-3,35%	0,99 ± 0,01	0,992	0,16%	6,78 ± 0,96	7,174	-5,83%
11	1	0	**1**	0,42 ± 0,00	0,436	-3,25%	1,02 ± 0,01	1,042	-1,64%	4,67 ± 0,67	6,492	-39,10%
12	1	0	-1	0,34 ± 0,01	0,377	-9,65%	0,85 ± 0,02	0,909	-7,02%	9,78 ± 1,50	9,264	5,27%
13	1	1	**0**	0,42 ± 0,01	0,404	4,87%	1,01 ± 0,03	0,960	5,29%	4,78 ± 1,17	5,025	-5,16%
14	1	1	**1**	0,43 ± 0,00	0,420	1,22%	1,03 ± 0,04	1,023	0,72%	4,44 ± 1,35	3,547	20,20%
15	1	1	**-1**	0,36 ± 0,01	0,340	6,78%	0,90 ± 0,05	0,863	4,60%	9,56 ± 0,19	7,911	17,22%
16	1	-1	**0**	0,41 ± 0,02	0,411	-1,08%	0,93 ± 0,05	0,941	-1,70%	9,78 ± 0,84	9,397	3,91%
17	1	-1	**1**	0,42 ± 0,01	0,406	4,27%	0,98 ± 0,04	0,977	0,15%	10,00 ± 1,53	9,511	4,90%
18	1	-1	-1	0,37 ± 0,00	0,368	-0,94%	0,86 ± 0,04	0,871	-1,67%	9,22 ± 0,19	10,691	-15,91%
19	-1	**0**	**0**	0,36 ± 0,00	0,342	5,30%	0,73 ± 0,02	0,674	7,92%	16,89 ± 1,07	15,804	6,43%
20	-1	0	**1**	0,33 ± 0,01	0,336	-0,31%	0,72 ± 0,05	0,655	9,16%	16,89 ± 0,51	16,362	3,13%
21	-1	0	-1	0,33 ± 0,01	0,301	9,60%	0,73 ± 0,04	0,658	9,95%	17,56 ± 0,38	16,654	5,15%
22	-1	1	**0**	0,39 ± 0,00	0,375	4,33%	0,77 ± 0,02	0,773	-0,07%	13,56 ± 0,69	13,433	0,92%
23	-1	1	**1**	0,39 ± 0,00	0,379	3,44%	0,77 ± 0,02	0,768	-0,40%	13,45 ± 0,51	13,195	1,87%
24	-1	1	**-1**	0,29 ± 0,03	0,323	-12,30%	0,72 ± 0,07	0,745	-4,13%	14,89 ± 0,84	15,079	-1,27%
25	-1	-1	**0**	0,24 ± 0,01	0,263	-10,78%	0,43 ± 0,02	0,490	-13,62%	18,00 ± 0,00	18,249	-1,37%
	-1	-1	**1**	0,24 ± 0,00	0,246	-2,91%	0,41 ± 0,01	0,458	-12,41%	17,78 ± 0,19	19,603	-10,25%
27	-1	-1	-1	0,22 ± 0,01	0,233	-5,74%	0,43 ± 0,02	0,489	-12,73%	17,67 ± 0,33	18,303	-3,59%