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Keywords: cell growth, shake flask, cell growth quantifier, kluyveromyces marxianus.

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Parallelized Biomass Monitoring of Two Distinct *Kluyveromyces Marxianus* Strains in Shake Flask Cultivation

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ABSTRACT

Kluyveromyces marxianus, a non-conventional yeast, carries traits deemed suitable for industrial applications, such as ethanol production, exhibiting advantages over Saccharomyces cerevisiae in terms of growth rate and thermotolerance. Non-invasive parallel monitoring of biomass in shake flask cultures allows for efficient microorganism characterization, providing much-needed and accurate data on these strains through continuous sampling. Therefore, this study aimed to assess the behaviour of two K. marxianus strains during continuous shake flask cultivation. Strain IZ 1339 exhibited a constant, however, slower growth pattern when compared to strain FT 146L, which grew constantly up until the 12 h, after that the strain presented flocculation, affecting the quality of the readings. Strain IZ 1339 also had a higher ODmax value when compared to FT 146L, nevertheless, their growth rate was similar, showing that both strains had a satisfactory performance in both concentrations of molasses. Non-invasive monitoring makes it possible to accompany the growth pattern of the strains, indicating that both K. marxianus strains perform well when grown in a sugarcane molasses medium. This feature makes these K. marxianus strains an interesting non-conventional alternative to S. cerevisiae when it comes to industrial application.

Keywords: cell growth, shake flask, cell growth quantifier, kluyveromyces marxianus.

I. INTRODUCTION

Kluyveromyces marxianus is a homothallic, hemiascomycetous yeast observed to have potential and many beneficial traits for industrial applications (KARIM; GERLIANI; AÏDER, 2020), such as bioethanol production from both sugarcane and cheese whey, protein derived from biomass, enzyme production such as inulinase and β-galactosidase, pharmaceutical compounds (LANE; MORRISSEY, 2010), aromatic compounds and food-grade proteins, due to its Qualified Presumption of Safely (QPS) and GRAS status in European Union and United States, respectively (KARIM; GERLIANI; AÏDER, 2020).

Some of the traits that make this yeast a promising candidate for biotechnological application is thermotolerance, high growth rates, and a broad range of substrates (FONSECA et al., 2008). *K. marxianus*, like *S. cerevisiae*, is a respiro-fermentative yeast. Although *K. marxianus* is generally classified as Crabtree negative, it does carry the genes necessary for ethanol productions and will veer towards the fermentative lifestyle under certain conditions, questioning the Crabtree status of this species (LANE; MORRISSEY, 2010).

This diversity in measurements is not primarily based on manual error but on the physiological differences of strains used in different studies. Strain preservation, origin, and manipulation from stock to growth medium, all play a major role in the physiological diversity of this yeast known to present high levels of intraspecific polymorphism (BELLOCH et al., 1998; FONSECA et al., 2007).

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This divergence is also explained by the intraspecific variations and the fact that most studies utilize one single strain as the representative of the species. It can be concluded that *K. marxianus* is capable of carrying out simultaneous fermentation and respiration, and the shift between these pathways is strain-specific (LANE; MORRISSEY, 2010).

This metabolic shift responsible for the Crabtree effect results from multiple related factors, and these may not express themselves equally for all strains, creating a spectrum between Crabtree negative and Crabtree positive, which explains why some, but not all *K. marxianus* strains are effective ethanol producers (HONG et al., 2007; LANE; MORRISSEY, 2010; NONKLANG et al., 2008).

As for *K. marxianus*, there are conflicting data regarding the maximum specific growth rate, particularly due to differences in experimental conditions and the intraspecific variation displayed by this species (KARIM; GERLIANI; AÏDER, 2020). The untapped biotechnological potential of *K. marxianus* serves as a guide for future developments, such as genetics, evolutionary engineering and other physiological and molecular tools for *K. marxianus* (KARIM; GERLIANI; AÏDER, 2020).

However, in order to better explore the biotechnological potential of a yeast strain, it is essential to understand its metabolism and response to growth medium and other factors, such as temperature, pH, sugar consumption and biomass concentration, even more so in the case of production of compounds whose titer are linked to biomass production (FONSECA et al., 2007).

Monitoring the growth of cultures in shake flasks has been traditionally carried out by manual sampling and offline biomass analysis, however, this process is insufficient for modern bioprocess monitoring, due to low data density, invasive sampling and lack of parallelization. Non-invasive parallelized biomass monitoring of cultures in a shake flask under agitation allows the characterization of microorganisms in a precise and efficient way, providing high data density and accuracy (BRUDER et al., 2016).

In order to characterize the growth profile of two *K. marxianus* strains, this study evaluated growth in shake flasks under continuous agitation through an online, automated biomass monitoring system, aiming to better understand the differences in metabolism of two strains cultivated under the same conditions.

II. MATERIAL AND METHODS

2.1 Microorganisms and Substrate

Two *Kluyveromyces marxianus* strains were utilized: strain IZ 1339 (native strain isolated from Drosophila) (GOMES et al., 2003; LEAL et al., 2008), kindly provided by Prof. Dr. Luiz Humberto Gomes (ESALQ/USP), and strain FT 146L (isolated from ethanol production), kindly provided by Fermentec Ltda (Piracicaba, SP, Brazil).

Strains were inoculated on Petri dishes containing YPDA medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ glucose; 18 $g.L^{-1}$ agar), and, subsequently transferred to cryotubes containing skim milk as a cryoprotectant for maintenance at -80°C.

Sugarcane molasses, a by-product of the sugar industry, utilized in this study was provided by Sugar and Ethanol Industries from the region of Piracicaba, São Paulo, Brazil. The molasses was diluted to the desired concentrations and sterilized at 121° C, 1 atm, for 15 min. Aliquots were stored at -20°C.

2.2 Study of Growth Profile of K. marxianus

Cultivations was carried out utilizing sterile sugarcane molasses (SCM), diluted to 8 and 15 °Brix (M8 and M15, respectively). Both strains were previously cultivated in YPD medium, and the cell suspension was adjusted to O.D.600 1,6. Subsequently, 1 mL of the cell suspension was inoculated in 50 mL of M8 and M15 in an Erlenmeyer flask (250 mL).

Biomass growth was monitored online and non-invasively by the CGQ dispositive ("Cell

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Growth Quantifier", Aquila Biolabs), readings were performed at approximately every 4 sec. The experiments were carried out in duplicates, at 30°C for 24 h. Both yeast strains were also cultivated in YPD medium, which was utilized as a reference.

The CGQ (Cell Growth Quantifier) method has the advantage of high data density and non-invasive sampling, thus, eliminating possible manual errors, sampling biases, sedimentation and equipment calibration. For both strains, graphs were obtained, detailing backscatter and maximum growth rate (h^{-1}).

The measurement of cell density by backscattering takes place through light radiated by an LED located at the base of the equipment, which interacts with the cells and is then reflected back by a photodiode, which converts the light into an electrical signal. This method allows the reading of higher cell densities, in the range of 0.1 to 150 O.D.600, without the need for any dilution (BRUDER et al., 2016).

2.3 Parameters Analysis

After the 24-hour period of growth, the samples cultivated in M8 and M15 were centrifuged at 2046 g for 3 min (NT-815, Novatecnica), and the supernatant was collected for analysis. Parameters were determined at 0 and 24 h of cultivation.

The pH was determined through a digital pH meter (LUCA-210, Lucadema). Total Acidity (acetic acid g/L) was determined by the titratable total acidity method (BRASIL, 1986).

Residual sugars were determined through DNS method (MILLER, 1959) in order to determine sugar consumption.

IV. RESULTS AND DISCUSSION

In order to evaluate the biomass data and strain-specific characteristics, strain IZ 1339 and FT 146L were grown on diluted sugarcane molasses (M8 and M15).

The growth profile of the strains (Figure 1) shows that strain IZ 1339 exhibited a distinct growth pattern in the Reference (cultivated in YPD medium). Adaptation took around 6.5 h, followed by the initial rapid growth phase, which then shifted to a much slower growth. This behavior is similar to that observed by Bruder et al. (2016) in *Saccharomyces cerevisiae*, where the authors attribute this growth pattern to the positive Crabtree-effect, the rapid growth phase is associated with ethanol formation, followed by the typical metabolic shift to respiratory ethanol metabolization. Similar behaviour can be observed for the Reference in Figure 1, for both repetitions, in strains cultivated in glucose (superior left and right).

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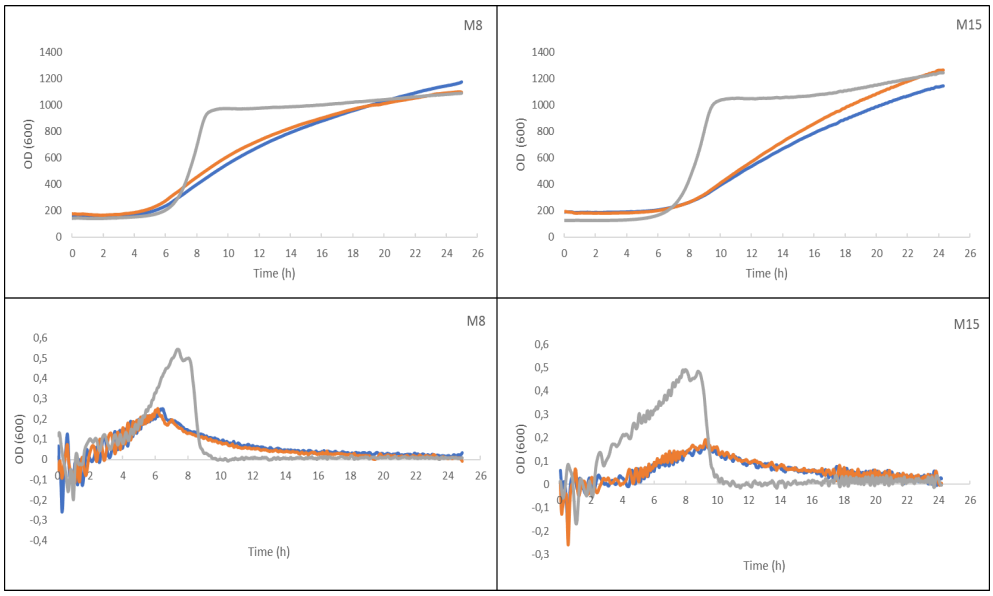

Figure 1: Growth of strain IZ 1339 in sterile molasses 8º Brix (M8) (left), 15º Brix (M15) (right) and PD 2% (Reference), at 30°C during 24 h. Experiments performed in duplicates. Sup. Growth rate by backscattering, Inf. Maximum specific growth rate

As for the growth in molasses, there was not an evident rapid growth phase. The adaptation period was similar to the Reference, around 6 to 7 h, followed by a slower growth curve. In terms of cell density, as determined by backscattering, both molasses concentrations (M8 and M15) and the Reference were fairly similar.

The maximum specific growth rate for strain IZ 1339 in M8 (Figure 1, bottom row) presented a peak, related to the maximum growth rate recorded, around 6 h of cultivation (0.24 h^{-1}). The Reference displayed a higher growth rate, probably due to the exponential growth phase (0.54 h^{-1}). Fonseca et al. (2013) observed growth rates of 0.39 and 0.49 h^{-1} utilizing 10g/L of supplemented carbon source.

When grown in M15, strain IZ 1339 also presented smaller growth rates when compared to growth in M8, reaching higher values around 9 h of cultivation at 0.17 h^{-1}, as opposed to 0.49 h^{-1} observed in the Reference (Figure 1, left).

As for strain FT 146L, growth in the Reference medium (2% glucose) presented a similar pattern to strain IZ 1339, with a rapid growth phase followed by a slower growth, much as described by Bruder et al. (2016). The one notable difference for strain FT 146L was that M15 yielded a higher biomass concentration, over 1200 (Figure 2, top row).

Growth in M8 presented a constant growth curve up until 15 h, starting to decline shortly after, unlike the Reference, which remained stable. The maximum growth rate graph (Figure 2, bottom row) showed abnormal peaks at the beginning of cultivation, after 18 h. This probably occurred because the strain presented flocculation, which makes it difficult to accurately read the cell density.

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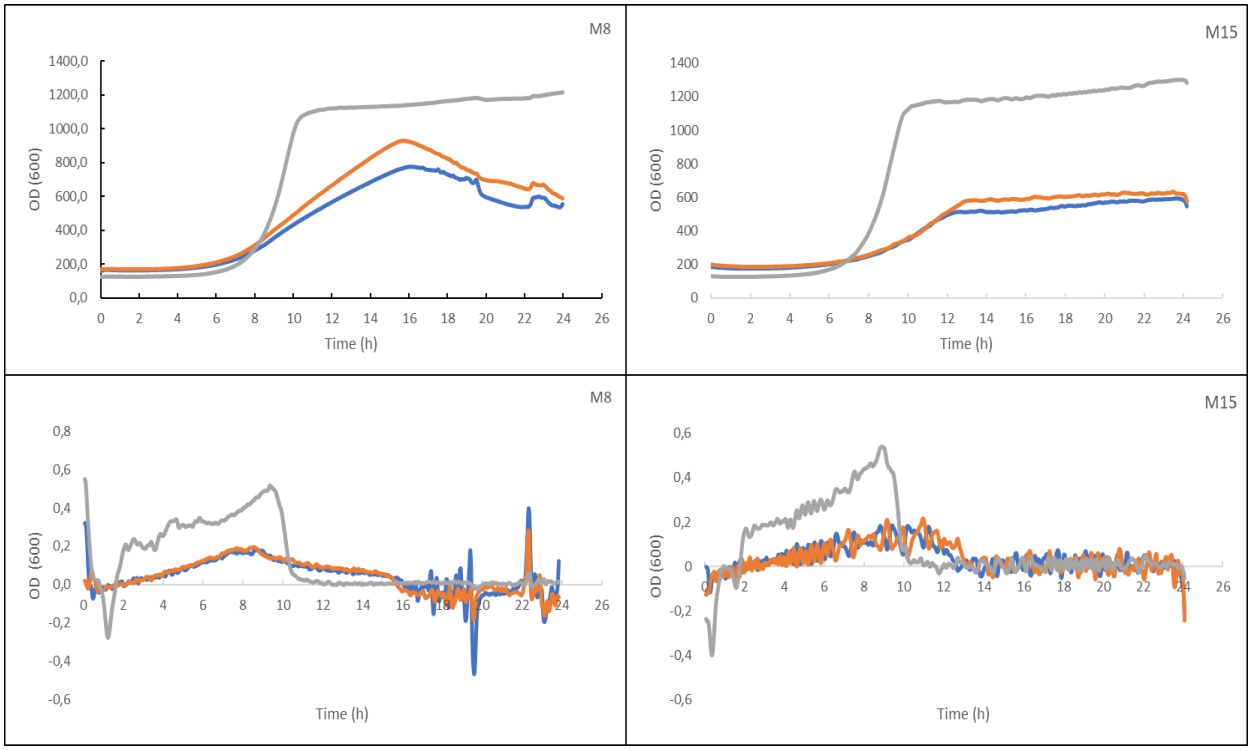

Figure 2: Growth of strain FT 146L in sterile molasses 8º Brix (M8) (left), 15º Brix (M15) (right) and YPD 2% (Reference), at 30°C during 24 h. Experiments performed in duplicates. Sup. Growth rate by backscattering, Inf. Maximum specific growth rate.

For industrial applications, such as the production of enzymes, flocculation is a desirable trait in *K. marxianus*, as a means to obtain higher cell density, therefore increasing productivity in bioreactor operations. Flocculation is a mechanism that occurs in some yeast strains as a result of non-sexual aggregation of single cells into a multicellular mass, which then sediments at the bottom of the medium. The mechanism behind flocculation is correlated by cell wall proteins (ALMEIDA et al., 2003; VERSTREPEN et al., 2003).

Growth in M15 presented a notably small growth rate and cell concentration when compared to M8 and the Reference, even though the growth curve was more stable throughout the 24 h of cultivation. Cell density for both media was smaller than strain IZ 1339 in both concentrations assayed.

Growth rates for M15 also presented abnormal peaks in the reading, due to the flocculent behaviour, even though the peaks have a more uniform pattern, which indicates a more constant maximum growth rate throughout the 24 h period, averaging 0.18 h^{-1}. It is worth noting that the Reference was grown in YPD, a complex medium that provides all nutrients necessary for yeast growth, the sugar, vitamins, minerals and amino acids present in the medium act as carbon and nitrogen sources.

The *K. marxianus* strains were also cultivated on molasses, a raw byproduct of the production of sugar and ethanol, consisting of 75–85% total solids, 30–36% sucrose, 10– 17% fructose + glucose, 10–16% ash, and minor varying compositions of oligosaccharides, polysaccharides, organic acids, proteins, and nitrogen compounds (CARIOCA; LEAL, 2019). Therefore, there are notable differences in the composition, and mainly, available sugars to stimulate growth.

It is worth noting that while the Reference was grown in YPD medium containing glucose, the duplicates for M8 and M15 had to hydrolyze the sucrose present in the medium, which would explain the slower growth curves when compared to the Reference.

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Overall, strain IZ 1339 presented a more constant growth pattern, and higher growth rate/biomass concentration compared to strain FT 146L. Table 1 shows the average maximum specific growth rate for each strain and media concentration, as well as the Reference.

Table 1: Average values of maximum specific growth rates μmax (h^{-1}) presented by strains IZ 1339 and FT 146L in M8, M15 and reference YPD 2%

Medium	Strain	
	IZ 1339	FT 146L
YPD 2%	0.54	0.54
M8	0.25	0.18
M15	0.18	0.19

M8 – sterile molasses 80 g/L; M15 – sterile molasses150 g/L; study was conducted in duplicates

From the average growth rate (h^{-1}) values, it is possible to infer that the Reference provided better conditions for both strains to grow, as for the molasses in both concentrations; there were not significant variations in growth rate values. Strain IZ 1339 had a higher biomass concentration and average growth rate for M8, however, strain FT 146L had a higher average growth rate for M15, despite having a lower concentration of biomass.

From a biotechnological standpoint, strain IZ 1339 seems to be more adapted for biomass production in this particular condition, while strain FT 146L grows at a faster rate, adapting more easily to the growth medium.

Maximum specific growth rate (μmax h^{-1}) of 0.56 was obtained during batch cultivations by Fonseca et al. (2007), utilizing glucose as the sole carbon source at 10 g/L, in a complex mineral medium, supplemented for growth optimization. However, there are sparse and conflicting data regarding the maximum specific growth rate for *K. marxianus*, due to the intraspecific variation and the distinct conditions assayed (KARIM; GERLIANI; AÏDER, 2020). Fonseca et al. (2007) highlights the diversity of measurements is not based on measurement errors, but on the physiological differences of strains used in different studies. It is possible to speculate that strain preservation, origin, and manipulation play a major role in this physiological diversity. *K. marxianus* is known to present high levels of intraspecific polymorphism, and may be prone to high mutation rates that result in rapid and unexpected evolution during the propagation process (BELLOCH et al., 1998).

4.1 Experimental Variables

After 24 h of cultivation, the supernatant was obtained by centrifugation. The parameters of the supernatant were evaluated for pH, total titrated acidity and residual sugar concentration (Table 2).

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Table 2: Post-Cultivation Parameters Analysis Values for Strains IZ 1339 and FT 146L in both Media

Samples	T (h)	RS (g/L)	TRS (g/L)	Consumed sugar (%)	Acidity (g/L)	pH
M8	0	7.33	80.62	*	0,62	5.61
IZ1339 M8	24	24.35	46.70	42.1	3.62	3.86
FT146L M8	24	18.32	39.81	50.6	2.25	4.19
M15	0	14.95	135.77	*	1.17	5.52
IZ1339 M15	24	89.98	74.12	45.4	4.27	4.20
FT146L M15	12	13.65	82.44	39.3	2.49	4.92
FT146L M15	24	111.35	132.23	2.61	3.03	4.03

M8 – sterile molasses 80 g/L; M15 – sterile molasses150 g/L; Acidity = concentration of acetic acid (g/L); T = time of sampling; RS = reducing sugars; TRS = total reducing sugars

It is possible to observe that neither of the strain was able to consume all of the sugar content in the medium, whether to produce biomass or, likely, to produce ethanol. Strain FT 146L was able to consume half of the sugar present in M8, while strain IZ 1339 consumed 42% (33.9 g/L out of 80 g/L). As for M15, there were even more residual sugars left at the end of the growth period, with strain IZ 1339 consuming 45.4% (74.1 g/L), as opposed to strain FT 146L, which consumed 39.3 % (53.33 g/L) at the 12 h of cultivation.

On average, both *K. marxianus* strains consume around 45% of the total sugars present in the growth medium. It is worth noting that strain FT 146L presented flocculation midway through the cultivation period, around the 12h mark, which is why the readings around 24h are not as accurate. Because growth in CGQ cannot be interrupted for external sampling, an experiment was done again in order to sample total soluble sugars and other parameters described in Table 2.

The behaviour herein observed for strain FT 146L could be triggered due to fructose or the total sugars inhibiting growth and causing flocculation. This could also occur due to this strain being suffering mutations throughout the generations, causing unstable behaviour (KARIM; GERLIANI; AÏDER, 2020; LANE; MORRISSEY, 2010).

Korkoutas et al. (2002) produced wine utilizing *K. marxianus* strain IMB3 and noticed that, while the final product had good quality and reached the desired ethanol concentration, there was a relatively high content of residual sugars, presumably due to a combination of cell density and temperature, which require further exploration. Plessas et al. (2008) utilized *K. marxianus* strain IFO 288 to produce lactic acid from cheese whey, with an initial sugar concentration of 36 g/L, and observed 0.4 g/L of residual sugar concentration of 36 g/L, and observed 0.4 g/L of residual sugars after the fermentation. The outcome of sugar consumption can vary depending on the employed conditions, which is why it is essential to understand a particular strain behaviour and metabolism.

As for total acidity, no condition demonstrated a significant increase, being the highest concentration M15 for strain IZ 1339, at 4.27 g/L, a regular byproduct of fermentation. Acetic acid production under fermentative conditions is linked to glycerol formation via redox balancing (EGLINTON et al., 2002), also, aeration and sugar content are also responsible for the increase of organic acids during fermentation, such as acetic acid, produced by yeast metabolic activity (LEE et al., 1999).

V. CONCLUSION

Characterizing a strain via growth-based methods provides essential data to understand sugar consumption and biomass production. The CGQ method for online biomass monitoring proved to be a valuable tool regarding growth rates and

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biomass data with high-resolution and non-invasive sampling. It was possible to infer that both *K. marxianus* strains had distinct behaviour and diverging growth patterns when cultivated under the same conditions.

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