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ORIGINAL ARTICLE

Rice bran extract as an alternative nutritional supplement for *Kluyveromyces marxianus*

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Abstract

The *Kluyveromyces marxianus* yeast has been arousing great interest as a biocatalyst for biorefineries due to its ability to assimilate different sugars, in addition to its rapid growth, thermotolerance, and GRAS status. In this research, different sources of nutrients, such as rice bran and extracts of malt, yeast, and peptone, were evaluated for the cultivation of *K. marxianus* ATCC 36,907 aiming at ethanol production in submerged fermentations conducted in a batch system. Mineral supplementation of the medium was also evaluated. The best results of process performance for production, yield, and productivity in ethanol were obtained in the medium supplemented with rice bran extract, calcium chloride, and ammonium sulfate (25.50 g/L; $Y_{(P/S)}$ 0.49 g/g; Q_P 2.13 g/Lh), and the medium supplemented only with yeast-malt extract peptone (YMP) (24.88 g/L; $Y_{(P/S)}$ 0.50 g/g; Q_P 2.06 g/Lh), after 12 h of cultivation. Supplementation of the medium with malt extract without adding mineral salts also contributed to similar values of production and yield in ethanol after 24 h of cultivation (25.59 g/L; $Y_{(P/S)}$ 0.50 g/g; Q_P 1.16 g/Lh^{-1}). The results revealed no need for mineral supplementation of the media added with YMP or malt extract for ethanol production by the yeast. On the other hand, rice bran extract, mainly associated with calcium chloride and ammonium sulfate, represents an excellent and inexpensive source of nutrients for this yeast and has the potential to replace traditional commercial supplements.

Keywords Bioprocesses · Ethanol · Fermentation · Non-conventional yeast · Nutritional requirements

1 Introduction

Rice is the second most consumed cereal in the world. According to data released by OECD-FAO [1], the average world production of rice between 2017 and 2019 was 515 million tons. For this, 164.9 million hectares were cropped, with an average yield of 3.12 t/ha. Over the next 10 years, global rice production is expected to increase by 66.8 million tons, reaching approximately 582 million tons in 2029 [1].

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Rice bran consists primarily of the outer layers of brown rice that are removed during the milling process to produce polished rice. Rice bran is a source of nutrients such as vitamin A and B, tocopherols with vitamin E activity, 11–12% of protein, 12–15% of lipids, and 2,750 kcal/kg of metabolizable energy. It has been used as a source of low-cost nutrients in animal feed, mainly pigs and poultry [2, 3], as well as in the human diet [4].

Rice bran extract (RBE) proved to be an alternative supplement for the proliferation and the production of mammalian cells [5] and the cultivation of probiotic microorganisms [6] and bacterial cellulose synthesis by *Acetobacter xylinum* [7]. RBE was able to effectively replace yeast extract in ethanol fermentation by *Saccharomyces cerevisiae*, contributing to the sustainability of the ethanol industry by reducing costs for large-scale fermentation [8].

The potential of RBE as a culture medium supplement was also demonstrated in second-generation ethanol studies from cellulosic and hemicellulosic fractions. RBE was nutritionally as effective as corn steep during

the simultaneous saccharification and fermentation (SSF) of rice straw with *S. cerevisiae* since it also increased the sugar concentration, thus improving the initial growth rate of the yeast and rapidly creating a stable SSF condition [9]. During the fermentation of sugarcane bagasse hemicellulosic hydrolysate, RBE was a promising alternative nitrogen source for yeast species such as *Scheffersomyces shehatae*, *Spathaspora arborariae* [10], and *Scheffersomyces (Pichia) stipitis* for ethanol production and *Candida guilliermondii* for xylitol production [11].

Kluyveromyces marxianus is a hemiascomycetous, homotallic yeast, widely known for its biotechnological potential due to traits such as thermotolerance, the fastest growth rate of any eukaryotic microbe, with a low doubling time of approximately 70 min [12]; wide intraspecific genetic diversity [13]; the capacity to assimilate a wide range of sugars such as sucrose, lactose, inulin, xylose, and xylose/glucose [14, 15]; and secretion of lytic enzymes, and the production of ethanol by fermentation from lactose [12, 16].

As a result of its long history of association with safe food and other products, it has achieved the status of GRAS (Generally Recognized As Safe) and QPS (qualified presumption of safety) in the USA and the European Union, which makes it a suitable yeast for the production of pharmaceutical and food-grade proteins [17].

Studies have shown that *K. marxianus* represents a novel candidate for second-generation ethanol production, mainly in SSF processes, due to its ability to grow and produce ethanol at temperatures above 40 °C [18, 19]. More recently, the genome sequencing of *K. marxianus* strains [20], in addition to transcriptomic analysis [20, 21] and evolutionary adaptation studies [22], have brought more profound knowledge about the potentialities of this non-conventional yeast, arousing interest in its use as a microbial cell factory for the production of substances of industrial interest. Considering that metabolomic data can more directly reflect the cellular metabolism than transcriptomic or proteomic data, a recent study has used untargeted metabolomic analysis to explore *K. marxianus*'s stress responses during high-temperature fermentation, thus providing rich information for further metabolic engineering towards improved stress tolerance and efficient bioethanol production [23]. To overcome the low ethanol tolerance in *K. marxianus*, a modified Monod model was designed, and it can contribute to the future application of this yeast in large-scale fermentations [24]. Moreover, advancements in synthetic biology, for example, the design of a set of promoters for controlling gene expression [25] as well as the CRISPR-Cas9 system (clustered regularly interspaced short palindromic repeats with Cas9)-mediated genome editing, will enable to develop an engineered yeast for the production of biochemicals and biopharmaceuticals having a myriad of industrial applications [26].

It is worth noting that malt extract (ME), yeast extract (YE), and peptone have been used to propagate yeasts and molds. Such materials are nutritionally rich supplements for the formulation of culture media, but they are relatively expensive. Malt extract contains a high concentration of carbohydrates, particularly maltose, and a complex mixture of peptides. Yeast extract provides carbonaceous compounds, sulfur, trace nutrients, and a complex mixture of peptides. Peptone provides nitrogenous and carbonaceous compounds, long-chain amino acids, and vitamins. However, there is no exact composition for these hydrolysates. The commercial products themselves do vary from one manufacturer to the other. Recently, the peptide content of a commercial YE was investigated by setting up a complete analytical workflow based on mass spectrometry (peptidomics), which allowed for the identification of around 4,600 different oligopeptides ranging from 6 to more than 30 amino acids in length [27].

Ammonium sulfate (AS) and di-ammonium phosphate (DAP) are mineral salts relevant to *K. marxianus* growth. AS and DAP act as supplementary nitrogen in protein anabolism and the gene expression of glycolytic and fermentative pathway components, favoring sugar conversion into ethanol by yeasts such as *S. cerevisiae* [28]. Calcium acts as a key cofactor in enzymatic reactions and a second messenger in several signal transduction pathways in eukaryotic cells [29].

Despite all the features mentioned above and the growing number of studies that promote *K. marxianus* as a promising industrial host for the biosynthesis of biofuels and other valuable chemicals, separately or in a biorefinery context, so far, there are no studies regarding the use of rice brain extract for second-generation ethanol production by *K. marxianus*. The few reports found in the literature reveal the use of rice bran solution (10% w/v) by *K. marxianus* to produce higher alcohols and acetate esters [30]. Thus, the present study proposed to evaluate the use of RBE as an economical and alternative nutrient source for the formulation of a medium to improve its fermentative performance.

2 Material and methods

2.1 Microorganism and inoculum cultivation

The experiments were conducted with the thermotolerant yeast *Kluyveromyces marxianus* ATCC 36,907, obtained from the Tropical Culture Collection (CCT) from the André Tosello Foundation. The culture was maintained in malt extract agar slants (30 g/L malt extract, 5.0 g/L mycological peptone, and 15 g/L agar), at 4 °C. The inoculum was grown in Erlenmeyer flasks of 250 mL, containing 100 mL of the YMP medium (10 g/L glucose, 3.0 g/L malt extract, 3.0 g/L yeast extract, and 5.0 g/L peptone). Cultivation was performed in a rotary incubator shaker (Marconi MA-420) at

200 rpm, temperature of 35 °C, for 16 h [31]. Then, the cells were centrifuged (Fanen 206 MP) at 2000 rpm for 20 min, washed with sterile distilled water, and after further centrifugation, resuspended in sterile distilled water to be used as inoculum at an initial cellular concentration of 1.0 g/L.

2.2 Rice bran extract (RBE) preparation and fermentation conditions

Rice bran was supplied by the food company Itasa Alimentos Ltda from Santa Tereza do Oeste, Paraná, Brazil. The concentrated water-soluble rice bran extract was prepared by mixing 200 g of rice bran with 1.0 L of 50 mmol/L sodium citrate buffer solution. The mixture was autoclaved at 110 °C (0.5 atm) for 15 min. Then, the solids were separated by centrifugation (Fanen 206 MP) at 2000 × g, 20 min, under sterile conditions, and the supernatant was stored at 4 °C before being used as a culture medium supplement.

To evaluate the effect of different nutrients on the metabolism of *K. marxianus*, the following media were prepared: non-supplemented medium (T1: 90 g/L glucose); medium supplemented with RBE (T2: 90 g/L glucose and 20 g/L RBE); medium supplemented with RBE plus mineral nutrients (T3: 90 g/L glucose, 20 g/L RBE, 0.1 g/L $CaCl_2$ and 2.0 g/L $(NH_4)_2SO_4$); medium supplemented with malt extract (T4: 90 g/L glucose and 3.0 g/L malt extract); medium supplemented with yeast extract (T5: 90 g/L glucose and 3.0 g/L yeast extract); medium supplemented with peptone (T6: 90 g/L glucose and 5.0 g/L peptone); and control-YMP medium (T7: 90 g/L glucose, 3.0 g/L malt extract, 3.0 g/L yeast extract, and 5.0 g/L peptone). All the media were prepared with 50 mmol/L sodium citrate buffer, pH 5.5, to avoid pH decrease due to the production of acetic acid by the yeast. The glucose concentration of 90 g/L was defined by Tavares et al. [32] as the optimal substrate concentration for *K. marxianus* ATCC 36,907.

The assays were performed in triplicate in Erlenmeyer flasks of 250 mL containing 100 mL of the culture media. The flasks were incubated in a rotary incubator shaker (Marconi MA-420) at 200 rpm, 40º C for 96 h [32]. Every 12 h, aliquots were taken to evaluate cell growth, glucose consumption, ethanol production, and by-products such as acetic acid and glycerol.

2.3 Analytic methods

The rice bran was characterized by determining moisture, crude protein, and lipid contents. Moisture was determined by weighing 5.0 g of rice bran (triplicate) with subsequent drying in an oven at 105 °C for 24 h. The samples were cooled in a desiccator for 30 min, re-weighed, and the moisture was determined by the difference of the weighing values recorded and expressed as a percentage [33].

Crude protein content was determined using the micro-Kjeldahl method (nitrogen-protein conversion factor of 6.25) and the lipids by the Soxhlet method [33].

Glucose, ethanol, acetic acid, and glycerol were measured by high-performance liquid chromatography (HPLC) on a Shimadzu LC-20A system equipped with a refractive index detector and column Phenomenex Rezex ROA-Organic Acid H + (8%) 150 × 7.8 mm, using H_2SO_4 0.005 mol/L as mobile phase, a flow rate 0.6 mL/min, 10 µL injection volume, and oven temperature of 65 °C [31]. The samples were filtered through polytetrafluoroethylene (PTFE) syringe filters (pore 0.45 µm, diameter 13 mm), and the mobile phase was vacuum filtered through hydrophilic polyvinylidene fluoride (PVDF) membrane (0.45 µm). The concentrations of the compounds were determined from curves obtained with high purity standards (98–99%).

The cellular concentration of the inoculum and during the fermentations were determined by spectrophotometry (Femto 700 Plus) by measuring the optical density (OD) at 600 nm and by converting values using a standard curve that correlated the $OD_{600\ nm}$ values to the dry weight of cells grown in YMP medium (1 g cells/L 1:10 corresponded to $OD_{600\ nm}$ 0.6).

2.4 Statistical analysis

Statistical analysis was performed using parametric analysis of variance tests (ANOVA) after checking the results' normal distribution and the variance's homoscedasticity. The Tukey post-test compared the groups, and the value set for statistical significance was $p < 0.05$.

3 Results and discussion

3.1 Chemical characterization of rice bran

The raw rice bran, as expected, presented a low moisture content (9.95%) and showed to be rich in minerals (9.87% mineral residue), lipids (17.24%), and crude protein (15.80%). These values corroborate those found by other authors concerning the moisture content (5.59–9.7%), lipids (13.43–20.79%), proteins (14–19.30%), and ashes (8.47–18%) [34, 35].

3.2 Effect of the nutritional conditions on glucose consumption, ethanol production, and cell growth

The profiles of glucose consumption, ethanol production, and cell growth observed in fermentations with the different nutritional media studied are shown in Figs. 1a, b, and c. Glucose (Fig. 1a) was fully consumed within 24 h in the

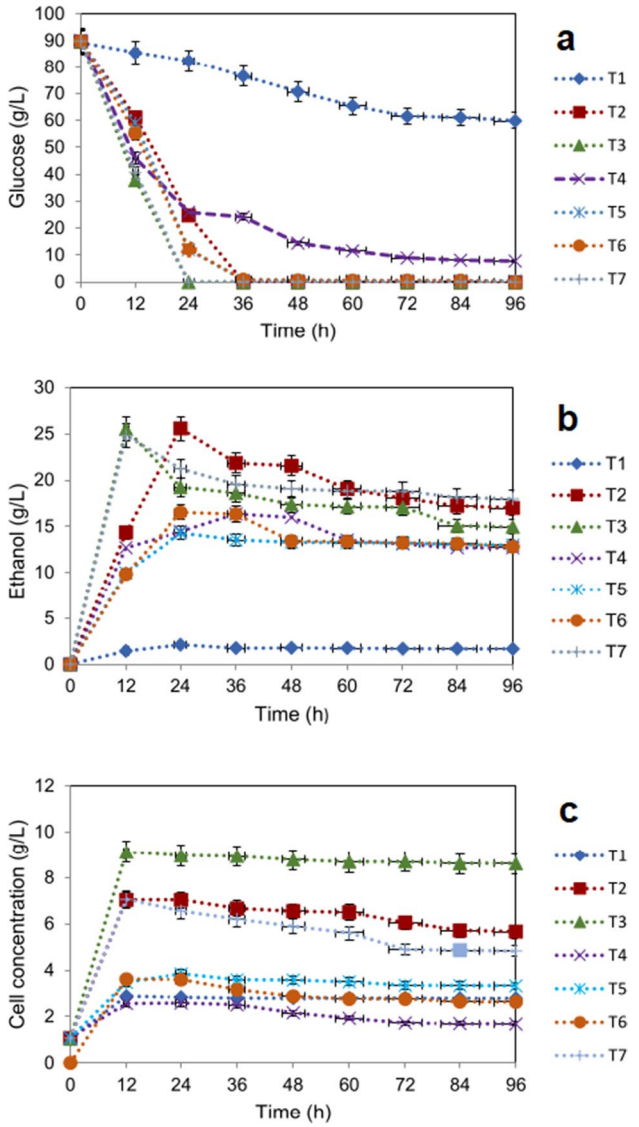

Fig. 1 Glucose consumption, ethanol production and cell concentration during cultivation of *K. marxianus* ATCC 36,907 in the non-supplemented medium, T1 (♦); medium supplemented with RBE, T2 (■); medium supplemented with RBE, calcium chloride and ammonium sulfate, T3 (▲); medium supplemented with malt extract, T4 (x); medium supplemented with yeast extract, T5 (x); medium supplemented with peptone, T6 (●); and control-YMP medium, T7 (+)

treatment T3 (medium supplied with RBE and calcium chloride and ammonium sulfate), as well as in the treatment T7 (YMP medium). In treatments T2, T5, and T6, glucose was consumed within 36 h, whereas in treatment T4, this consumption was slower and remained around 10 g/L of residual glucose in the medium at the end of fermentation. As expected, in fermentations containing glucose (T1), the consumption was much slower, with a residual glucose content of 59.86 g/L after 96 h.

Nitrogen is a necessary macroelement for synthesizing amino acids, the backbone of proteins, and therefore of enzymes vital for maintaining metabolism. Nitrogen availability also influences the kinetics of glucose assimilation due to the combined effect of different nitrogen and glucose concentrations on the expression of HXT transcripts in hexose uptake systems (HXT). The activity of hexose uptake systems has been considered an essential factor in limiting the activity of alcoholic fermentation in yeasts [36].

As illustrated in Fig. 1b, ethanol production occurred under all the cultivation conditions, being the maximum production (25.50 g/L) observed in the treatments T3 (glucose associated with RBE and minerals) and T7 (control-YMP medium: 24.88 g/L), both after 12 h. Similar ethanol production (25.59 g/L) was observed in the T2 treatment (medium based on glucose supplemented with RBE), however, in a longer fermentation time (24 h).

The medium containing only glucose (T1) presented the lowest ethanol production, on average 2.11 g/L. Likewise, lower ethanol concentrations were observed in the media based on glucose supplemented with malt extract (T4: 16.34 g/L), yeast extract (T5: 14.29 g/L), and peptone (T6: 16.51 g/L) compared to T3 and T7 fermentation conditions. These results indicate that RBE favors glucose consumption and ethanol production by *K. marxianus* ATCC 36,907, especially when supplemented with calcium chloride and ammonium sulfate, with results similar to those obtained in the YMP medium (T7).

In addition to the role of calcium as a cofactor in enzymatic reactions [29], mineral nutrients such as calcium have a protective effect at appropriate concentrations, contributing to the maintenance of the structural stability of the plasma membrane, acting in the maintenance of the permeability barrier in adverse conditions, as in the presence of ethanol [37]. Salts such as KCl/KOH are suggested to increase potassium uptake allowing an efficient proton efflux, increased membrane integrity, and strengthened membrane electrochemical potential. In *K. marxianus* Kmx24, supplementation of the culture medium with KCl/KOH (40/10 mM) resulted in more significant cell viability and greater tolerance to acetic acid, an inhibitor present in lignocellulosic biomass hydrolysates, with improved production of second-generation ethanol [38]. Ammonium sulfate, besides providing sulfur, is an inorganic nitrogen source easily assimilated by yeasts [39]. This nutrient is part of the structure of amino acids such as cysteine and methionine, essential for protein synthesis [40].

It is important to note that except in the T1 fermentation condition (Fig. 1b), ethanol concentration gradually decreased in the fermented media. It can be attributed to the consumption of ethanol as a carbon source by the yeast, presumably due to the depletion of glucose in the fermentation medium. Other authors, such as Tavares et al. [32], also reported ethanol consumption by this same *K. marxianus* strain during fermentation in semi-defined media. Similarly,

Leonel et al. [41] verified the same phenomenon in fermentations with *K. marxianus* and *C. guilliermondi* FTI 20,037 grown in a medium based on apple pomace hemicellulosic hydrolysate after 72 h of fermentation. According to Mo et al. [22], two routes may exist for directly consuming ethanol in *K. marxianus*. One way is via cytoplasmic ADH6, which catalyzes ethanol to acetaldehyde, facilitated by $NADP^+$. The other is mitochondrial ATF1, which promotes ethanol esterification with the aid of acetyl-CoA.

The effect of RBE as a nitrogen source was also evaluated with *S. stipitis* NRRL Y-7124 in sugarcane bagasse hemicellulosic hydrolysate. The medium supplemented with RBE plus ammonium sulfate and calcium chloride presented better results than the medium containing yeast extract and peptone, as well as the medium containing yeast extract, peptone, and malt extract, with maximum ethanol production of 8.6 g/L, 8.1 g/L, and 7.4 g/L, respectively [42].

Martiniano et al. [10] evaluated the potential of RBE for ethanol production by yeast strains isolated from Brazilian forests (*Scheffersomyces shehatae* CG8-8BY and *Spathaspora arborariae* UFMG-HM19.1A) and concluded that it is a promising nitrogen source to replace peptone and yeast extract. *S. shehatae* and *S. arborariae*, both in a fermentation medium consisting of sugarcane bagasse hemicellulosic hydrolysate supplemented with RBE, produced 17.0 g/L and 5.4 g/L of ethanol, respectively. In this context, when rice bran was evaluated as a cheap nutritional supplement for ethanol production by *S. cerevisiae* in fed-batch fermentation with simultaneous saccharification of rice straw, a high ethanol production (69.3 g/L) was found by Mochidzuki et al. [9].

Besides the carbon source, nitrogen is one of the essential nutrients for ethanol production, in addition to vitamins and trace elements necessary for yeast growth and metabolites production. Rice bran is rich in vitamins such as vitamin E, niacin, thiamin, and minerals such as calcium, phosphorus, potassium, and magnesium, among others [43], which may have contributed to better fermentation performance in fermentations containing RBE. As already mentioned, RBE, yeast and malt extracts, and peptone have a complex nutritional composition (yet chemically undefined) that varies according to the source and the manufacturing process. Identifying substances in complex media, especially the peptides diversity, is still a technically challenging step.

The growth profiles of *K. marxianus* ATCC 36,907 in the different media evaluated (T1 to T7) are shown in Fig. 1c. All treatments showed the same trend, with maximum growth observed between 12 and 24 h, coincident with the period of maximum ethanol production (Fig. 1b). A correlation between cell growth and ethanol production was already expected since ethanol is a primary metabolite.

Similar to that observed for glucose consumption and ethanol production, the highest cell concentration (9.11 g cell/L) was verified in the treatment T3 (supplemented with RBE, calcium chloride, and ammonium sulfate), followed by the treatment T7 (control-YMP medium, 7.05 g cell/L), and treatment T2 (supplemented with RBE, 7.03 g cell/L). On the other hand, a slight decrease in the growth of *K. marxianus* was observed between 48 and 72 h in the treatments T2, T4, and T7. Oda et al. [44] correlated the cell viability of *K. marxianus* with the amount of ethanol produced and found that cell viability decreased dramatically with ethanol accumulation in the medium.

The effect of RBE supplementation on yeast growth has been reported in several studies. *S. shehatae* CG8-8BY and *S. arborariae* UFMG-HM19 showed the highest cell growth (2.27 and 5.38 g/L, respectively, from the initial 0.5 g/L) when grown in sugarcane bagasse hemicellulosic hydrolysate supplemented with 20 g/L of RBE, 2.0 g/L of ammonia sulfate, and 0.1 g/L of calcium chloride [10]. In the same study, the authors reported that in a medium supplemented with 3.0 g/L of yeast extract, the growth of both yeasts was lower. In fermentations performed by Milessi et al. [42], the supplementation of sugarcane bagasse hydrolysate with 10% (v/v) of RBE, 0.1 g/L of calcium chloride, and 2.0 g/L of ammonium sulfate supported the growth of *S. stipitis* NRRL Y-7124 (12.8 g/L in 96 h from the initial 1.0 g/L). However, the highest cell concentration was obtained from the fermentation of the medium supplemented with yeast extract and/or peptone. Although microorganisms can assimilate different nitrogen sources, inorganic and organic, some may have preferences for one or the other, depending on their nutritional needs [45], which can vary between the species and strains [46]. For example, in fermentation using *Lactobacillus plantarum*, peptides with molecular weights in the range of 200–1,400 Da present in the oat extract and 100–700 Da in the malt extract were preferentially absorbed and utilized by the microorganism [47].

3.3 Effect of the nutritional conditions on by-products formation

The formation of by-products, such as acetic acid and glycerol, by *K. marxianus* in the different cultivation media was quantified and is depicted in Fig. 2a and b. The highest acetic acid production (9.54 g/L), which accounted for approximately 1/3 of ethanol production, occurred at 12 h in T3 treatment, coinciding with the highest cell growth and ethanol production rates. Similar performance was observed in the control treatment T7 (7.68 g/L), while in the other treatments, the maximum production occurred between 24 and 36 h, not undergoing subsequent variations.

The production of high levels of acetic acid is a common feature of *K. marxianus* species, occurring in the exponential growth phase [48]. This behavior has also been reported with the ATCC 36,907 strain, which produced 1.0–1.5 g/L

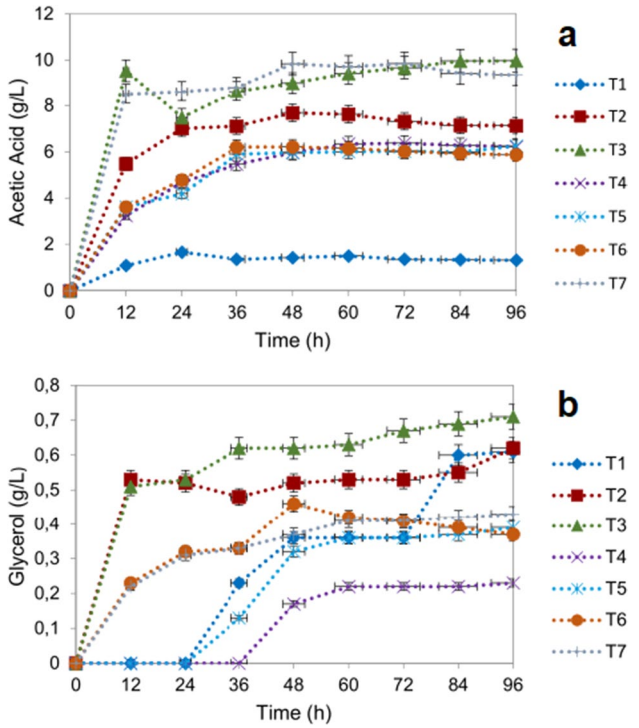

Fig. 2 Acetic acid and glycerol production during cultivation of *K. marxianus* ATCC 36,907 in the non-supplemented medium, T1 (♦); medium supplemented with RBE, T2 (■); medium supplemented with RBE, calcium chloride and ammonium sulfate, T3 (▲); medium supplemented with malt extract, T4 (x); medium supplemented with yeast extract, T5 (✱); medium supplemented with peptone, T6 (●); and control-YMP medium, T7 (+)

of acetic acid when grown in a synthetic medium (90 g/L glucose) at 40 °C [32]; NBRC 1777 strain, with a maximum acetic acid concentration of 7.0 g/L when grown in YPD broth (500 g/L glucose continuously fed) under an anaerobic condition at 30 °C [49]; and IMB3 strain, with a maximum acetic acid concentration of 2.6 g/L in submerged fermentation with sugarcane bagasse 15% (w/v) at 45 °C [50].

A possible explanation for the high levels of acetic acid in *K. marxianus* is that the enzyme pyruvate decarboxylase has low activity. In contrast, acetaldehyde dehydrogenase and acetyl CoA synthetase have relatively high activity [51], which are enzymes involved in the production of acetate in situations of cytoplasmic redox imbalance since it is produced along with NADH and also when there is a need for cytoplasmic acetyl-CoA for biomass production [48].

Regarding glycerol production (Fig. 2b), the fermentations supplemented with RBE, T2, and T3, promoted the highest glycerol concentrations, 0.53 and 0.52 g/L at 12 h and 0.71 and 0.62 g/L at 96 h, respectively. Different from the observed for acetic acid, in some treatments, glycerol production did not coincide with the ethanol formation. Glycerol production was only observed after 36 h in the treatments T1 and T5 and after 48 h in T4.

Relatively higher concentrations of glycerol have been observed for *K. marxianus*. Camargo, Gomes, and Sene [31] verified the formation of up to 2.62 g/L of glycerol by this same strain, *K. marxianus* ATCC 36,907, in the SSF using sunflower bran, while Pessani et al. [52], using the IMB3 strain, observed glycerol concentrations of 2.4 and 3.2 g/L, with an increase in temperature from 37 to 45 °C, during the SSF of switchgrass.

As the acetic acid, glycerol is a by-product commonly formed by yeast species to maintain redox balance in response to stress conditions such as high temperature, presence of ethanol, and oxygen-limiting conditions [53]. In addition, its accumulation can occur in osmotic stress

Table 1 Fermentative parameters and maximum ethanol concentrations during the cultivation of *K. marxianus* ATCC 36,907 in different nutritional fermentation conditions

Treatment	Ethanol [a]	$Y_{P/S}$	$Y_{X/S}$	Q_X	Q_P
	(g/L)	(g/g)	(g/g)	($g/L.h^{-1}$)	($g/L.h^{-1}$)
T1 (24 h)	2.11 ± 0.08^{g}	0.38 ± 0.01^{c}	0.12 ± 0.004^{f}	0.15 ± 0.001^{d}	0.09 ± 0.003^{g}
T2 (24 h)	25.59 ± 0.77^{a}	0.50 ± 0.02^{a}	1.16 ± 0.03^{c}	0.50 ± 0.02^{b}	$1.16 \pm 0.03^{c,b}$
T3 (12 h)	25.50 ± 0.80^{b}	0.49 ± 0.01^{a}	2.13 ± 0.06^{a}	0.67 ± 0.02^{a}	2.13 ± 0.06^{a}
T4 (36 h)	16.34 ± 0.51^{e}	$0.29 \pm 0.007^{d,f,g}$	1.06 ± 0.03^{d}	0.12 ± 0.003^{e}	0.45 ± 0.01^{f}
T5 (24 h)	14.28 ± 0.41^{f}	$0.32 \pm 0.009^{b,f}$	0.83 ± 0.03^{e}	0.20 ± 0.006^{c}	0.59 ± 0.018^{e}
T6 (24 h)	16.51 ± 0.49^{d}	$0.28 \pm 0.008^{e,g}$	0.82 ± 0.025^{e}	0.21 ± 0.011^{c}	0.69 ± 0.02^{d}
T7 (12 h)	24.88 ± 0.75^{c}	0.50 ± 0.02^{a}	2.06 ± 0.05^{b}	0.50 ± 0.01^{b}	2.06 ± 0.06^{b}

Fermentation media: T1 (non-supplemented medium), T2 (medium supplemented with RBE), T3 (medium supplemented with RBE, calcium chloride, and ammonium sulfate), T4 (medium supplemented with malt extract), T5 (medium supplemented with yeast extract), T6 (medium supplemented with peptone); T7 (control-YMP medium)

[a]Maximum ethanol concentration, $Y_{P/S}$: yield of glucose to ethanol (g/g), $Y_{X/S}$: yield of glucose to cells (g/g), Q_P: volumetric ethanol productivity ($g/L.h^{-1}$), Q_X: volumetric cell productivity ($g/L.h^{-1}$)

Averages followed by distinct lowercase letters differ statistically in the column by the Tukey test at 5% significance ($p < 0.05$)

Table 2 Ethanol production from glucose under different nutritional and environmental conditions using various strains of *K. marxianus*

Strain	Process	Initial glucose (g/L)	Medium/nutrients	Ethanol yield		Reference
				Conc.(g/L)	$Y_{P/S}$ (g/g)	
K. marxianus ATCC 36,907	Shaking flasks, 40 °C, 200 rpm, 96 h	90	Semi-defined (20 g/L RBE, 0.1 g/L $CaCl_2$ and 2.0 g/L $(NH_4)_2SO_4$)	25.50	0.49	This study
			YMP broth (3.0 g/L malt extract, 3.0 g/L yeast extract and 5.0 g/L peptone)	24.88	0.50	
K. marxianus ATCC 36,907	5.0 L bioreactor with 1.2 L working volume, vacuum for product removal, 40 °C, 200 rpm, 36 h, K_{La} 0.2 h^{-1}	90	YMP broth (3.0 g/L malt extract, 3.0 g/L yeast extract and 5.0 g/L peptone)	34.13	0.38	[32]
K marxianus DMKU3-1042	Shaking flasks, 45 °C, 160 rpm, 18 h	20	YPD broth (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose)	10	0.48 ± 0.03 (12 h)	[55]
K. marxianus NCYC 3396	Shaking flasks, low oxygen, 48 °C, 100 rpm, 12 h	10	Semi-defined (2 M KOH, vitamins, and urea 2.3 g/L initial)	up to 10%	0.38 ± 0.01	[56]
K. marxianus UFV-3				up to 10%	0.40 ± 0.01	
K. marxianus UFV3	Shaking flasks, 37 and 42 °C, 180 rpm, 12 h	40	Minimal medium (2.5 g/L yeast extract, 2.5 g/L peptone, 2.0 g/L NH_4Cl, 1.0 g/L KH_2PO_4, 0.3 g/L MgSO4.7H_2O)	18.53–7.24	0.41–0.23	[57]
K. marxianus ATCC 8554				19.66–12.11	0.42–0.26	
K. marxianus CCT 4086				18.10–12.06	0.39–0.26	
K. marxianus NIRE-K3	Bioreactor with 5.0 L working volume, 45 °C	40	Semi-defined (yeast extract and minerals (K_2HPO_4, NaH_2PO_4, $MgSO_4$ and $(NH_4)_2SO_4$), optimized by Face-centered Central Composite Design (FCCD)	19.01	0.48	[58]
K. marxianus NIRE-K1				17.73	0.44	
K. marxianus Kmx24	Shaking flasks, 30 °C or 42 °C, 150 rpm, 48 h	50	YPD broth (5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, with or without KCl/KOH supplementation to increase acetic acid tolerance	17.00 – 24.18	0.38–0.46	[38]

situations as an osmolyte, protecting cells against cell lysis [54].

3.4 Effect of the nutritional conditions on the fermentative parameters

The fermentative parameters maximum ethanol concentration, conversion factor of glucose to ethanol ($Y_{P/S}$, ethanol yield), conversion factor of glucose to cells ($Y_{X/S}$), volumetric ethanol productivity (Q_P), and volumetric cell productivity (Q_X) are shown in Table 1. The ethanol yield ranged from 0.28 to 0.50 g/g, and as expected, according to the ethanol production trend (Fig. 1b), the treatments T2 (medium supplemented with RBE), T3 (RBE plus mineral salts), and T7 (YMP medium) presented the highest values. There were no significant differences between them ($p < 0.05$), and these yields are very close to the theoretical yield (0.511 g/g) of ethanol from glucose. In these fermentation conditions, ethanol yield values ($Y_{P/S}$) of 0.50 g/g (T2, 24 h), 0.49 g/g (T3, 12 h), and 0.50 g/g (T7, 12 h) were found, respectively.

Similar to that observed with the ethanol yield, the highest ethanol volumetric productivity (Q_P) also occurred in the treatments T2 (1.16 g/L.h^{-1}), T7 (2.06 g/L.h^{-1}), and T3 (2.13 g/L.h^{-1}). Treatment T3 showed a better result of volumetric productivity in ethanol in 12 h of cultivation, statistically differing ($p < 0.05$) from the other cultivation conditions. As expected, as it reflects the behavior of biomass production shown in Fig. 1c, the highest cell yield ($Y_{X/S}$) was observed in the treatment T3

Table 3 Ethanol production during cultivation of *K. marxianus* ATCC 36,907 in the different fermentation media at the fermentation times of 12, 24, and 36 h

Treatments	12 h	24 h	36 h
T1	1.42 ± 0.04^{cB}	2.63 ± 0.07^{cA}	2.79 ± 0.08^{aC}
T2	14.34 ± 0.43^{bB}	25.59 ± 0.75^{cA}	21.83 ± 0.62^{aA}
T3	25.50 ± 0.75^{aA}	19.24 ± 0.55^{bAB}	18.59 ± 0.51^{bAB}
T4	12.69 ± 0.35^{bA}	14.44 ± 0.41^{aB}	16.34 ± 0.49^{aAB}
T5	9.97 ± 0.28^{bB}	14.02 ± 0.40^{aB}	13.28 ± 0.49^{aB}
T6	9.88 ± 0.28^{bB}	16.51 ± 0.49^{aB}	16.32 ± 0.47^{aAB}
T7	24.81 ± 0.72^{aA}	21.22 ± 0.60^{bAB}	19.51 ± 0.52^{bA}

Averages followed by the same lowercase letter in the row and capital in the column do not differ by the Tukey test at 5% significance ($p < 0.05$)

Table 4 Cell concentration during cultivation of *K. marxianus* ATCC 36,907 in different fermentation media at fermentation times of 12, 24, and 36 h

Treatments	12 h	24 h	36 h
T1	1.83 ± 0.05^{bD}	2.82 ± 0.07^{aC}	2.79 ± 0.08^{aD}
T2	7.03 ± 0.20^{aB}	7.06 ± 0.18^{aB}	6.66 ± 0.19^{aAB}
T3	9.11 ± 0.27^{aA}	8.96 ± 0.25^{aA}	8.93 ± 0.24^{aA}
T4	2.56 ± 0.07^{aC}	2.58 ± 0.06^{aD}	2.59 ± 0.05^{aD}
T5	3.47 ± 0.10^{bBC}	3.84 ± 0.12^{aC}	3.58 ± 0.10^{cB}
T6	3.61 ± 0.10^{aBC}	3.62 ± 0.11^{aC}	3.16 ± 0.09^{cB}
T7	7.06 ± 0.20^{aB}	6.55 ± 0.18^{bAB}	6.19 ± 0.15^{bC}

Means followed by the same lowercase letter in the row and uppercase in the column do not differ from each other by the 5% significance of the Tukey test ($p < 0.05$)

(2.13 g/g at 12 h, $p < 0.05$), corresponding to a growth rate of 0.67 g/L.h^{-1}. These values were followed by T7 (2.06 g/g) and T2 (1.16 g/g) at 12 h and 24 h of growth, respectively.

YMP medium at an initial glucose concentration of 90 g/L was also studied by Tavares et al. [32], which evaluated the effect of vacuum removal of ethanol on the fermentative performance of the same yeast strain in a bench bioreactor, using two 36 h sequential batches system. These authors reported lower values of yield and volumetric productivity in ethanol. Thus, our results indicate that RBE supplied the nutritional requirements of the strain ATCC 36,907, resulting in a better fermentative performance. The rice bran extract (RBE), mainly associated with calcium chloride and ammonium sulfate, is a viable and economical source of nutrients capable of favoring ethanol production by *K. marxianus* ATCC 36,907.

Table 2 presents an overview of the fermentative performance of several strains of *K. marxianus* cultivated in glucose media added with different nutritional supplements.

Table S1 (see Supplementary Table S1) shows the analysis of variance data (ANOVA) of the factors media and fermentation times, indicating that significant interaction occurred among the factors under evaluation ($F = 6.187$).

As can be seen from Table 3, T3 (RBE plus calcium chloride and ammonium sulfate) and T7 (YMP medium), which showed the highest ethanol productions (25.50 and 24.81 g/L, respectively) after 12 h of fermentation, did not differ from each other ($p > 0.05$), but differed among the other treatments ($p < 0.05$), except for T2, which showed the highest ethanol production, 25.59 g/L, after 24 h of fermentation.

The YMP broth has been long-established for the isolation and cultivation of yeasts and molds and presents in its composition carbon, nitrogen, vitamin B complex, nutrients, and other growth factors. Therefore, the use of RBE as a supplement, especially when in conjunction with calcium chloride and ammonium sulfate, as used in this study for the formulation of T3 medium, proved to be nutritionally equivalent and as efficient as YMP broth for ethanol production by *K. marxianus* ATCC 36,907.

Table S2 shows (see Supplementary Table S2) the ANOVA analysis of cell concentration considering the seven different treatments and the fermentation times since there was interaction among them ($F = 4.17$).

As shown in Table 4, the T3 treatment presented the highest cell concentration (9.11 g/L) at 12 h of fermentation, followed by treatments T2 and T7, which showed similar values without statistical difference ($p < 0.05$). The values of all treatments remained constant, with no significant statistical difference ($p > 0.05$) after 12 h of fermentation due to total sugar consumption in the first 36 h. This directly reflected in bioethanol production, with consequent reduction of productivity, as already observed.

4 Conclusions

By comparing water-soluble rice bran extract (RBE) with other nutrient sources for the cultivation of *K. marxianus* ATCC 36,907, it was demonstrated that RBE successfully supplied the nutritional requirements of this yeast, supporting growth, and ethanol production. When RBE was supplemented with calcium chloride and ammonium sulfate, the yeast showed the same fermentative performance as observed in the YMP medium. The medium based on RBE supplemented with minerals also favored the cell growth with maximum cell concentration and productivity at 12 h. Thus, RBE, mainly if supplemented with calcium chloride and ammonium sulfate, represents a suitable and low cost substitute for commercially available supplements for *K. marxianus* ATCC 36,907. Given the metabolic traits of *K. marxianus* and its ability to assimilate a variety of carbon sources, it would be worthwhile to evaluate RBE

supplementation for this yeast in lignocellulosic hydrolysates, together with an economic analysis, aiming at large-scale, sustainable ethanol production.

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Data availability The datasets generated during the current study are available in the manuscript itself.

Declarations

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