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Ethanol production from deproteinized cheese whey fermentations by co-cultures of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*

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An application of the co-culture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* for ethanol production from deproteinized cheese whey was established. Among several co-cultures examined, the co-culture of *S. cerevisiae* UFLA KFG33 (ethanol over-producer) and *K. marxianus* (UFLA KF22) showed the highest value of ethanol production (16.02±0.11 g L^{-1}) and the highest yield of ethanol by fermentation time (0.22±0.05 g L h^{-1}), according to *Qp* (volumetric productivity). These yeasts also showed the highest value of cell mass concentration in final fermentation (1.02±0.01 g L^{-1}). The co-cultures were performed in 72 h fermentation at 28°C with shaking at 100 rpm. The results indicate that this methodology is a promising technique for the production of ethanol using deproteinized cheese whey.

Key words: Yeasts, Rep-PCR, lactose fermentation, whey.

INTRODUCTION

Cheese whey represents an important source of environmental pollution (Koutinas et al., 2009; Magalhães et al., 2010; Dragone et al., 2011). One of the most attractive options to limit the proliferation of whey pollution is the bioconversion of whey to ethanol employing yeasts, especially the *Kluyveromyces* species. The presence of lactose as the only fermentable carbohydrate in whey confines its use to selective fermentations involving microorganisms that are capable of breaking down lactose with the enzyme β-galactosidase (Zafar and Owais, 2006; Magalhães et al., 2010). In addition to lactose, whey also contains vitamins and minerals, Ethanol has tremendous applications in chemical, pharmaceutical and food industries in the form of raw materials, solvents and fuels (Plessas et al., 2008). It is which may improve the physiological activity of the cells (Zafar and Owais, 2006). very important to choose a yeast strain with suitable physiological characteristics to achieve optimal utilization of lactose from whey.

Not many yeast strains are capable of fermenting lactose to ethanol. The most commonly used distiller yeast *Saccharomyces cerevisiae* cannot ferment lactose since it lacks both β-galactosidase and a lactose permease system. This inability to ferment lactose prevents *S. cerevisiae* from using cheesewhey as fermentation substrate.

Alternative methods have been explored for utilization of cheese whey by *S. cerevisiae*. Champagne used one β-galactosidase-positive micro-organism to hydrolyze lactose first to provide suitable substrate for subsequent fermentation by *S. cerevisiae* (Guo et al., 2010). Most of

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the *Kluyveromyces* species are capable of using lactose in cheese whey for ethanol fermentation.

Despite their close phylogenetic relationship, there are still certain technological aspects which *Kluyveromyces* cannot industrially compete with *Saccharomyces*. However, fermentation strategies of mixed culture employed to overcome substrate limitations were considerably successful, which has been widely used in producing ethanol fermentation and single-cell protein, vitamin production, and disposing of waste water (Guo et al., 2010).

The main objective of this investigation was to study ethanol production from cheese whey by yeast strains, including *K. marxianus* in co-culture with *S. cerevisiae*, an ethanol over-producer that is unable to metabolize lactose. *K. marxianus* initiates the hydrolysis of lactose to fermentable sugars, and these released sugars can then be utilized by *S. cerevisiae*. This co-culture system might be able to generate an increase in ethanol production. Although several researchers have reported the growth of yeast strains such as *K. marxianus* (Zafar and Owais, 2006; Plessas et al., 2008; Oda et al., 2010) and *K. fragilis* (Dragone et al., 2011) on deproteinized cheese whey, the use of deproteinized cheese whey as a culture medium for ethanol production by co-culture yeasts has not been explored in a comprehensive manner.

MATERIALS AND METHODS

Cheese whey must preparation

Cheese whey from natural sources was obtained from the cheese produced by Cooperativa Agrícola Alto Rio Grande (Lavras-MG, Brazil). The cheese whey, containing a lactose concentration of 46 g L^{-1} and a pH of 4.5, was used as the fermentation medium. Cheese whey was deproteinized by heat treatment at 115°C for 15 min. The precipitates were removed by centrifugation at 5600 x g at 10°C for 15 min, and the supernatant was used as the fermentation medium (must).

Microorganisms

Microbial isolates were obtained from the culture collection of the microbiology laboratory, Federal University of Lavras, Brazil. *S. cerevisiae* (UFLA KFG33), which is an ethanol over-producer, and *K. marxianus* (UFLA KF01, UFLA KF54, UFLA KF22) strains were used in the experiments.

Inoculum preparation and fermentation process

The inoculum was prepared by cultivating each yeast strain separately in MYGP (Merck, Whitehouse Station, USA) culture medium, containing 100 mg L^{-1} chloramphenicol (Sigma, St. Louis, USA) and 50 mg L^{-1} chlortetracycline (Sigma, St. Louis, USA) to inhibit bacterial growth, until the population reached a density of 10^5 cells m L^{-1}. Yeasts cells were harvested by centrifugation for 10 min at 5600 x g. Subsequently, the yeasts were resuspended in cheese whey, inoculated into 250 mL of cheese whey must be incubated at 28°C for 72 h with shaking at 100 rpm. The inoculation was performed in pairs for each of the co-culture combinations of *K. marxianus* and *S. cerevisiae* (UFLA KF01 and *S. cerevisiae*, KF54 UFLA and *S. cerevisiae*, and KF22 UFLA and *S. cerevisiae*). The fermentation processes were performed in triplicate. Samples of the fermented products were aseptically removed at the beginning and end of each fermentation run for chemical and microbiological analyses.

Microbiological analysis

The enumeration of yeasts was carried out using MYGP agar (Merck, Whitehouse Station, USA) culture medium. Plates were incubated at 28°C for 120 h, and colony forming units (cfu mL^{-1}) were quantified. Colonies for identification were taken at random from each plate containing isolated colonies. The number of colonies used for identification was equal to the square root of the total number of each morphotype in plate.

The identification of all yeast isolates was determined using repetitive extragenic palindromic sequences (rep-PCR) using the primer $(GTG)_5$ (5′-GTG GTG GTG GTG GTG-3′). The DNA was extracted from the pure cultures. Yeasts colonies were picked from agar surfaces, suspended in a PCR buffer (Invitrogen, Foster City, USA), and heated to 95°C for 15 min. The extracts were used for PCR without further processing. Rep-PCR (GTG_5-primer) was carried out as described by Pereira et al. (2012). Amplification products were separated by electrophoresis on a 1.8% (w/v) agarose gel at 70 V for 4 h and stained with SYBR Green (Invitrogen, Foster City, CA, USA). DNA fragments were visualized by UV transillumination, and images were captured using a Polaroid camera. A ladder marker (GeneRuler 100 bp DNA Ladder Plus) was used as a size reference. The rep-PCR-profiles were normalized, and a cluster analysis was performed using Bionumerics V6.5 software package (Applied Maths, Sint-Martens-Latem, Belgium).

The rep-PCR profiles for known yeast strains were compared to the population estimate rep-PCR profile of each morphotype isolated in the plates.

Analytical methods

Chemicals

The pH of fermented products was measured at room temperature using a digital pH meter (Micronal, B474 model, Germany). The soluble solids were determined using a digital refractometer (ATAGO, PR-1000, Brazil ATAGO, LTDA), and the results were expressed in ºB.

HPLC analysis

Ethanol and organic acids (lactic and acetic) were quantified by high-performance liquid chromatography (HPLC). Analyses were carried out using a Shimadzu chromatograph, model LC-10 Ai (Shimadzu Corp., Japan), equipped with a dual detection system consisting of an Ultra Violet detector (UV) and a Refractive Index Detector (RI — 10A). A Shimadzu cation-exchange column (Shim-pack SCR-101H, 7.9 mm × 30 cm), was operated at 30°C to ethanol and 50°C to organic acids, using 100 mM perchloric acid as the eluent at a flow rate of 0.6 mL min^{-1}. The acids were detected via UV absorbance (210 nm), while the ethanol were detected via RI. Individual acids and alcohols were identified by comparison of their retention times with the retention times of certified standards. The quantification of alcohols and acids were performed using calibration curves obtained from standard compounds (Duarte et

Table 1. Yeast cell counts in fermentations.

Yeast strains	Initial count (cfu mL^{-1})	Final count (72 h) (cfu mL^{-1})
UFLA KF01	$2.9x10^5±0.1^a$	$1.6x10^8±0.1^c$
UFLA KF01 / UFLA KFG33	$1.5x10^5±0.1^b$ / $1.2x10^5±0.1^b$	$1.6x10^8±0.1^e$ / $1.1x10^7±0.1^e$
UFLA KF54	$3.1x10^5±0.1^a$	$1.7x10^8±0.1^c$
UFLA KF54 / UFLA KFG33	$1.4x10^5±0.1^b$ / $1.1x10^5±0.1^b$	$1.8x10^8±0.1^e$ / $1.3x10^7±0.1^e$
UFLA KF22	$3.1x10^5±0.1^a$	$2.9x10^8±0.1^d$
UFLA KF22 / UFLA KFG33	$1.5x10^5±0.1^b$ / $1.3x10^5±0.1^b$	$1.1x10^9±0.1^f$ / $2.1x10^8±0.1^f$

Data represent the mean values of duplicates ± standard deviation; Different letters indicate significant differences ($p < 0.05$); Yeast strains: UFLA KF01, UFLA KF54 and UFLA KF22 = *Kluyveromyces marxianus*, UFLA KFG33 = *Saccharomyces cerevisiae.*

al., 2011). All samples were examined in triplicate.

Volumetric productivity (Qp)

For *QP* determination, the following equation was used according to the method of Duarte et al. (2011): [$Qp = (Pf − Pi)/t f$].

Where, *Pi* is the initial concentration of ethanol; *Pf* is the ethanol concentration at the end of fermentation and *tf* is the total time of fermentation

Biomass yeasts

Measuring dry weight of the biomass yeast cells were harvested by centrifugation for 10 min at 10.000 rpm. The pellets were washed twice with distilled water and weighed every 24 h of drying at 100°C until the weight stabilized.

Statistical analysis

The fermentation processes were carried out in triplicate, and the mean values ± standard deviations are reported in Tables 1 and 2. Tukey's test was performed using the Statgraphics Plus software program to evaluate statistical significance (level of $p < 0.05$) of differences between the fermentation sets and to compare the means among the samples.

RESULTS AND DISCUSSION

The focus of this study was to evaluate the possible synergistic effects of a mixed fermentation of *K. marxianus* and *S. cerevisiae* yeasts on ethanol production. The strategy was designed to promote improvement in ethanol production from deproteinized cheese whey. The cheese whey fermentations were carried out using *K. marxianus* monocultures or co-cultures containing both *K. marxianus* and *S. cerevisiae*. According to the study of Zafar and Owais (2006) and Oda et al. (2010) shaking the fermentation container during the fermentation time results in higher alcohol content.

All strains of *K. marxianus* and *S. cerevisiae* used in this study were able to grow in deproteinized cheese whey. Table 1 shows the identities of the yeast strains, as well as the microbial enumeration values. The enumeration values (cfu mL^{-1}) of the isolated viable yeasts ranged from minimum values of $1.6x10^8±0.1$ cfu mL^{-1} and $1.1x10^7±0.1$ cfu mL^{-1} in a co-culture of *K. marxianus* (UFLA KF01) and *S. cerevisiae* (UFLA KFG33) to maximum values of $1.1x10^9±0.1$ cfu mL^{-1} and $2.1x10^8±0.1$ cfu mL^{-1} in a co-culture of *K. marxianus* (UFLA KF22) and *S. cerevisiae* (UFLA KFG33). The three monocultures of *K. marxianus* resulted in lower cell counts than the co-culture fermentations of *K. marxianus* and *S. cerevisiae*.

A total of 125 isolates were obtained and molecularly characterized using the rep-PCR technique (Figure 1). The profile analysis using the $(GTG)_5$-PCR method resulted in identification of the isolates by comparison of the rep-PCR profiles to known yeast strains (Figure 2). This technique verified the microbial count of each morphotype in agar plates. These results confirmed the presence of the *S. cerevisiae* and *K. marxianus* yeasts in the final fermentation.

The fermentations were monitored in 72 h periods at 28°C by determining the acidity and °B value. Table 2 lists the pH values of the fermentations. The pH values decreased at the end of the process, with the lowest value for the *S. cerevisiae* and *K. marxianus* (UFLA KF22) co-culture (3.02±0.05). The end of fermentation was indicated by a value of 0 °B (total consumption of the lactose substrate) at 72 h of fermentation, and the °B value was evaluated every 8 h.

High performance liquid chromatography was used to analyze organic acids and ethanol (Table 2). The lactic acid content reached a maximum value of 4.02 g L^{-1} for the *S. cerevisiae* and *K marxianus* (UFLA KF22) co-culture. This may have been due to a larger population of *K. marxianus* (UFLA KF22), which produced an increased amount of lactic acid (Plessas et al., 2008).

Acetic acid was also formed during the fermentation process, reaching a maximum value of 2.07 g L^{-1} in 72 h

Table 2. Fermentation end products and pH values for fermentations.

Yeast strains / Fermentation time	Ethanol (g L^{-1})	Lactic acid (g L^{-1})	Acetic acid (g L^{-1})	pH
UFLA KF01/0 h	n.d.	0.03±0.01^{e}	0.02±0.01^{e}	4.51 ±0.02^{l}
UFLA KF01/72 h	6.19±0.01^{a}	1.38±0.01^{f}	0.79±0.01^{i}	3.52±0.50^{m}
UFLA KF01 and UFLA KFG33/0 h	n.d.	0.02±0.01^{e}	0.02±0.01^{e}	4.52±0.10^{l}
UFLA KF01 and UFLA KFG33/72 h	9.03±0.11^{c}	1.39±0.01^{f}	0.81±0.01^{i}	3.61 ±0.02^{m}
UFLA KF54/0 h	n.d.	0.03±0.01^{e}	0.02±0.01^{e}	4.51 ±0.02^{l}
UFLA KF54/72 h	6.39±0.01^{a}	1.39±0.01^{f}	0.79±0.01^{i}	3.51 ±0.02^{m}
UFLA KF54 and UFLA KFG33/0 h	n.d.	0.04±0.01^{e}	0.02±0.01^{e}	4.52±0.05^{l}
UFLA KF54 and UFLA KFG33/72 h	10.01±0.11^{b}	1.39±0.01^{f}	0.79±0.01^{i}	3.51±0.01^{m}
UFLA KF22/0 h	n.d.	0.03±0.01^{e}	0.02±0.01^{e}	4.51 ±0.02^{l}
UFLA KF22/72 h	10.79±0.01^{b}	2.19±0.01^{g}	1.69±0.01^{j}	3.31 ±0.02^{n}
UFLA KF22 and UFLA KFG33/0 h	n.d.	0.03±0.01^{e}	0.02±0.01^{e}	4.51 ±0.02^{l}
UFLA KF22 and UFLA KFG33/72 h	16.02±0.11^{d}	4.02±0.01^{h}	2.07±0.01^{k}	3.02±0.05^{o}

Data represent the mean values of duplicates ± standard deviation; Different letters indicate significant differences ($p < 0.05$); n.d. = not detected; Yeast strains: UFLA KF01, UFLA KF54 and UFLA KF22 = *Kluyveromyces marxianus*, UFLA KFG33 = *Saccharomyces cerevisiae.*

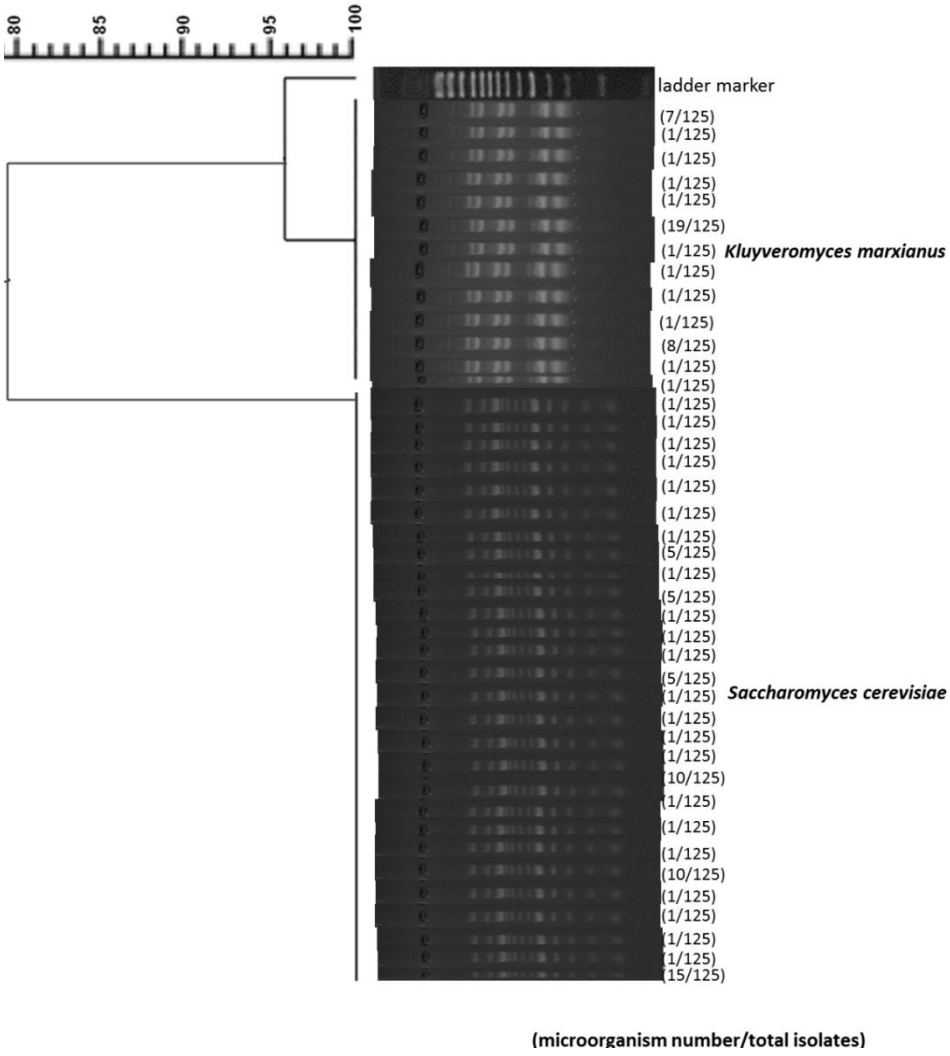

Figure 1. Cluster analysis of the 125 profiles obtained by rep-PCR of the yeasts.

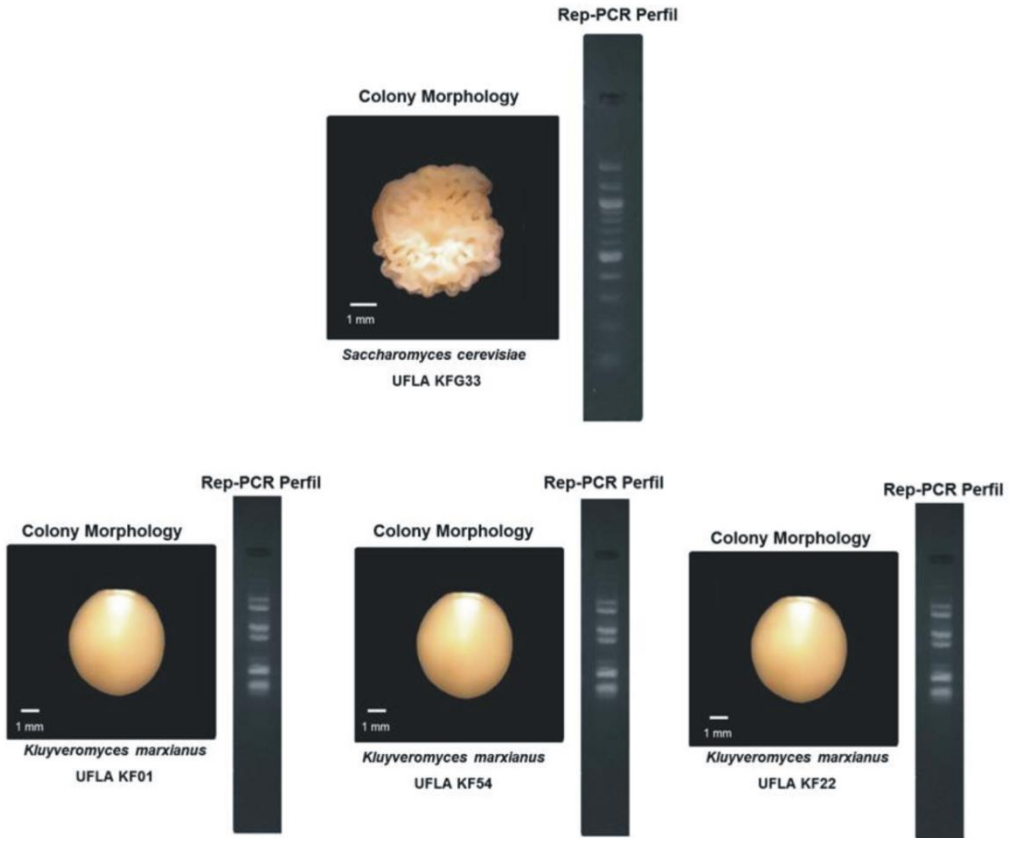

Figure 2. Microbial morphotypes and Rep-PCR profiles of yeasts.

of fermentation by the *S. cerevisiae* and *K. marxianus* (UFLA KF22) co-culture (Table 2). The acetic acid was likely formed by heterolactic metabolic pathways present in these yeasts. These results are of importance because lactic acid and acetic acid are also industrially relevant products. Thus, this methodology may also be applied to production of lactic acid and acetic.

The co-culture of *S. cerevisiae* and *K. marxianus* (UFLA KF22) also showed the highest rate of ethanol (16.02±0.11 g L^{-1}) production (Table 2) and the highest yield of ethanol by fermentation time (0.22 g L^{-1}), according to *Qp* analysis (Figure 3a). These results could be attributed to possible synergistic effects between the two species (*K. marxianus* and *S. cerevisiae*) that are caused by a positive symbiosis between the yeast strains. Researchers have found that for other co-cultures, especially in yeast/bacteria symbioses, the yeasts provide the bacteria with growth factors, such as vitamins. This relationship consequently leads to increased metabolite production because the yeasts use the bacterial end-products as energy sources (Plessas et al., 2008). Another possible explanation for the increased yields may be that the deproteinized cheese whey substrate results in an ethanol yield approximately four times greater than that achieved during the fermentation of non-deproteinized cheese whey by *K. marxianus* (Dragone et al., 2011).

The *S. cerevisiae* and *K. marxianus* (UFLA KF22) yeast strains were able to grow in relatively high concentrations of ethanol (ca. ~16 g L^{-1}), which demonstrates the ability of these two ethanol-tolerant yeast strains to withstand osmotic stress. Dragone et al. (2011) asserted that alcohol tolerance is an advantage when a yeast species is being considered for industrial use, especially where ethanol is being produced, because the high concentrations of ethanol in the medium are difficult to avoid during fermentation.

According to the results of this study, the yeasts co-culture system could be a technology for obtaining higher ethanol yields from deproteinized cheese whey fermentations. This process could be an alternative method for reducing organic wastes and producing value-added products from wastewater drained during the cheese manufacturing process.

Previous reports on ethanol production from cheese whey found that the ethanol concentration proportionally increased with the initial sugar concentration (Dragone et al., 2011). The maximal ethanol concentration in the present study was ~16 g L^{-1}, with an initial sugar concentration of 46 g L^{-1} and yield of 0.22 g L^{-1}. Therefore,

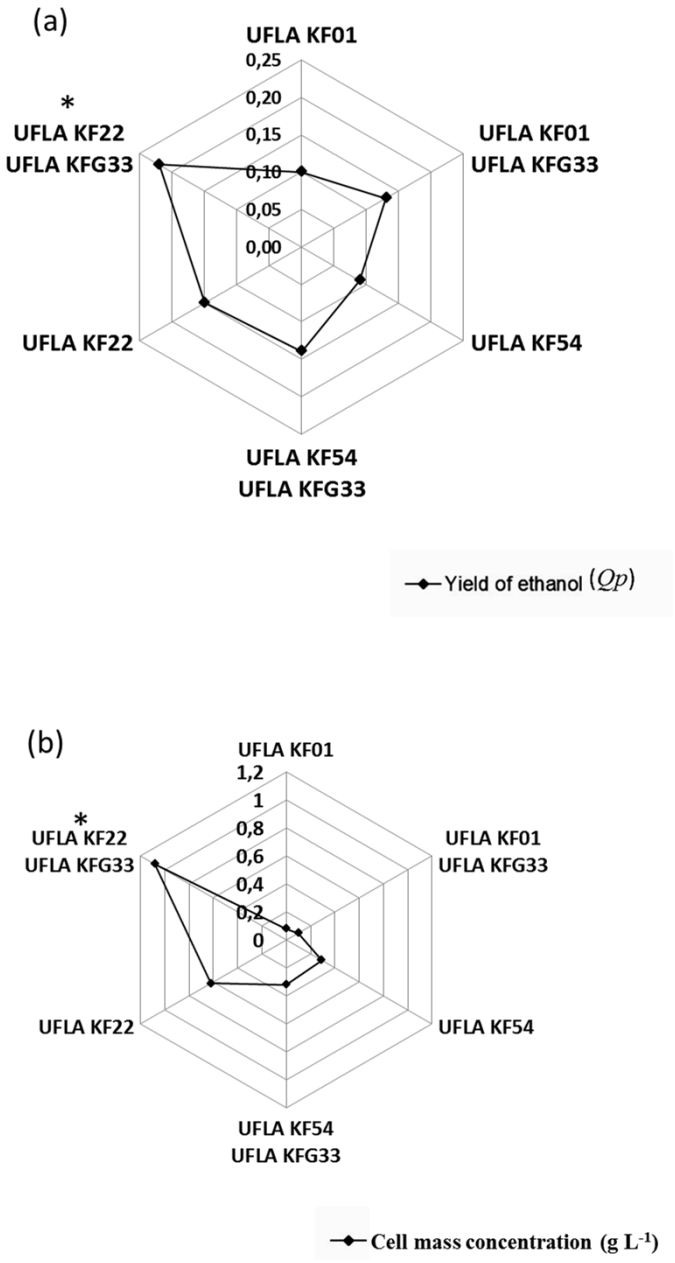

Figure 3. (a) Volumetric productivity or yield of ethanol (Qp) in g L h-1. (b) Cell mass concentration (g L-1). Yeast strains: UFLA KF01, UFLA KF54 and UFLA KF22 = *Kluyveromyces marxianus*; UFLA KFG33 = *Saccharomyces cerevisiae*. Asterisk – Significant difference ($p < 0.05$) between ethanol yield values and cell mass concentration.

further studies could be conducted with increased amounts of lactose and the *K. marxianus* UFLA KF22 and *S. cerevisiae* UFLA KFG33 co-culture system.

The difference in final concentration of the cell mass in each fermentation was significant (Figure 3b). The co-culture of *S. cerevisiae* UFLA KFG33 (ethanol over-producer) and *K. marxianus* (UFLA KF22) showed the highest value cell mass concentration in final fermentation (1.02±0.01 g L^{-1}). Similar observations were reported with immobilized mixed culture of *K. marxianus* and *S. cerevisiae* in cheese whey fermentation (Guo et al., 2010).

Conclusion

This study highlights an application of a co-culture of *K.marxianus* and *S. cerevisiae* for ethanol production from deproteinized cheese whey on an industrial scale. The ethanol yield high of ethanol was achieved by co-culture of *Saccharomyces cerevisiae* UFLA KFG33 (ethanol over-producer) and *Kluyveromyces marxianus* (UFLA KF22). Another possible application of cheese whey could be the production of lactic acid and acetic acid, analyzed in this study.

Furthermore, the methodology employed for the use of deproteinized cheese whey is reported, representing an interesting alternative to decrease the distillation costs of ethanol production, besides being a method for deproteinization.

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