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Batch and Fed-Batch Ethanol Fermentation of Cheese-Whey Powder with Mixed Cultures of Different Yeasts

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Abstract: Eight yeast strains of *Lachancea thermotolerans*, *Kluyveromyces marxianus*, and *Kluyveromyces waltii* have been tested for their ability to ferment lactose into ethanol in mashes containing 10% (*w*/*v*) cheese whey powder (CWP). The *K. marxianus* NCAIM Y00963 achieved 3.5% (*v*/*v*) ethanol concentration at 96–120 h of fermentation. The ethanol production by the selected lactose-positive strains and the well-known ethanologenic *Saccharomyces cerevisiae* (Levuline Fb) in mixed culture was also investigated at different CWP concentrations and inoculation techniques in batch mode. The mixed culture in an equal ratio (1:1) of cell counts of *K. marxianus* and *S. serevisiae* showed an increase in lactose conversion rate. The two yeast strains in a ratio of 3:1 (three-quarters of *K. marxianus* and a quarter of *S. cerevisiae* in a total of 4.5×10^{10} cells) resulted in 72.33% efficiency of lactose bioconversion and 7.6% (*v*/*v*) ethanol production at 17.5% (*w*/*v*) of CWP concentration. In the repeated inoculation process, with the addition of three-quarter part of 3:1 ratio of mixed culture (3.3×10^{10} cells of *K. marxianus*) into 150 mL CWP mash at initiation and the rest quarter part (1.2×10^{10} cells of *S. cerevisiae*) at 24 h, 8.86% (*v*/*v*) ethanol content with 87.5% efficiency of lactose conversion was reached. Both the ethanol concentration and efficiency of bioconversion were increased to 10.34% (*v*/*v*) and 92%, respectively, by combination with fed-batch fermentation technology. Our results can serve a very good basis for the development of industrial technology for the utilization of cheese whey.

Keywords: cheese whey; bioethanol; mixed culture; fermentation; lactose conversion

1. Introduction

Whey is a by-product of cheese manufacturing industries. Generally, the production of 1 kg of cheese will result in about 9 kg whey; thus a significant amount of this by-product is generated annually [1,2]. Sustainable bio-utilization of cheese whey is a challenge due to its environmental

problems of high biochemical oxygen demand (approximately from BOD 40,000 to BOD 60,000) and chemical oxygen demand (approximately from COD 50,000 to COD 80,000), which are caused primarily by lactose [3–5]. Generally, about 50% of milk solids are retained in cheese whey, comprising carbohydrates, mostly lactose (4.5–5.5%, *w*/*v*), functional proteins and peptides (1.0–1.2%, *w*/*v*), minerals (0.8%, *w*/*v*), lipids (0.3%, *w*/*v*), and other components like lactic and citric acids, non-protein nitrogen sources, and B group vitamins in small quantities [6–8]. Lactose has a very high potential for use in biotechnological, food, and medical processes for the production of numerous value-added products such as antibiotics, enzymes, single-cell proteins, surfactants, or even bio-fuels for transportation [4,9]. To date, ethanol production from cheese whey has been studied by some groups due to its high carbohydrate content and the availability of cheese whey [10–15]. *Saccharomyces cerevisiae* is one of the most applied ethanologenic yeasts for ethanol production due to its high ethanol tolerance, as well as the resulting high yields. However, it is unable to ferment lactose because it lacks both lactose permease and β-galactosidase, which transport lactose into the cytoplasm and hydrolyse it into glucose and galactose; thus, enzymatic or acid hydrolysis is needed prior to lactose fermentation [16]. Some species of lactose-positive yeasts, especially *Kluyveromyces* (*Kluyveromyces fragilis*, *K. marxianus*, etc.) and *Candida* (*Candida kefir*, formally *C. pseudotropicalis*), are promising in lactose hydrolysis and fermentation [17]. Unfortunately, the conversion capacity of released sugars (glucose, galactose) into ethanol by these yeasts is lower, and thus, the cost for the distillation of dilute fermentation broths (about 2–3% ethanol) is high [18,19]. Consequently, whey or whey permeate fermentation is not economically competitive when compared to mature technologies such as sugar cane- or starch-based ethanol. Cheese whey powder (CWP), i.e., a dried and concentrated form of cheese whey which contains high concentrations of lactose and other nutrients, may be suitable material for ethanol production [14,20]. Many fermentation strategies have been applied to increase the efficiency of the conversion of lactose into ethanol [11–21]. Substrate inhibition can be reduced by using mixed culture, while the ethanol concentration can be increased up to 8–10% (*v*/*v*) using the fed-batch process [14,21]. Furthermore, improved ethanol fermentation can be achieved by optimizing various parameters, like temperature, pH, substrate concentration, etc. [22]. The aim of this research is focused on the selection of a lactose-fermenting strain and the measurement of the effect of the selected strain in mixed culture with *S. cerevisiae* on ethanol production from the CWP-containing medium in batch and fed-batch fermentation processes.

2. Materials and Methods

2.1. Microorganisms and Maintaining

Different strains of yeast *Lachancea* (*L. thermotolerans* Y00702, Y00715, Y00775, Y00798, Y00873, Y00959), as well as *Kluyveromyces* (*K. marxianus* Y00963 and *K. waltii* Y01184), were kindly provided by the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary). The yeast strains were maintained on malt agar slants (10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract and 15 g/L bacterial agar) at 30 ± 2 °C for a week, and then stored at 4 °C for further use.

2.2. Culture Medium

The compositions of cultivation media for strains of yeast *Lancancea* and *Kluyveromyces* were 20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone in distilled water. Before inoculation, the cultivation medium was sterilized at 121 °C for 20 min. The inoculum for fermentation was prepared by transferring a loopful of yeast cells from freshly-grown culture into 250 mL Erlenmeyer flasks containing 50 mL of culture medium. The flasks then were incubated at 30 ± 2 °C for 18–20 h in a rotary shaker incubator operating at 160 rpm. The *S. cerevisiae* (Levuline FB type dried yeast provided by Kokoferm Ltd., Gyongyos, Hungary) was rehydrated in 10 g/L glucose solution. The cell number of inoculum cultures was counted directly using Burker-chamber under the microscope (Olympus CX 31) at 40-times magnification before initiation of the ethanolic fermentation process.

2.3. Ethanol Fermentation

Cheese whey powder (CWP) as a substrate was obtained from Dénes Nature Kft. (Hungary). It contains about 76% (*w*/*v*) carbohydrates (lactose), 11% (*w*/*v*) proteins, 23% (*w*/*v*) salts, and 15% (*w*/*v*) lipids. A general mashing technique was used. Briefly, different amounts of CWP were scaled into 500 mL Erlenmeyer flasks and dissolved in 150 mL tap water. Then, the pH of the mashes was adjusted to 4.0 ± 0.2 by 0.1 n hydrochloric acid, and supplemented with chemical manure (Uvavital, 25–30 g/hL) before the initiation of ethanol fermentation. Two fermentation strategies, i.e., batch, and fed-batch, were performed. The concentration of CWP varied between 10–30% (*w*/*v*) and about 15% (*w*/*v*) in the case of batch and fed-batch, respectively. Fermentation processes, except for repeated inoculation techniques, were initiated by the addition of a total of about 4.5×10^{10} yeast cells (correspondence to around 15 mL inoculum, 10% inoculum size) to the prepared mashes. The experiments were carried out at 30 ± 2 °C for 168 h. In the case of fed-batch, feeds of CWP solution were performed twice, i.e., at 24 h and 48 h of fermentation [21].

All experiments were carried out at 30 ± 2 °C for 168 h without shaking. The fermentation runs were monitored by periodic sampling (at 24 h intervals) to determine the lactose consumption and ethanol production. All experiments were performed in triplicate, and mean values are given.

2.4. Analytical Methods

The amounts of carbohydrates (glucose, galactose, lactose) were monitored by Surveyor HPLC (Thermo Fisher Scientific Corporation, Waltham, MA, USA). Samples were centrifuged at 14,000 rpm for 15 min and the supernatant was analysed. The analytical column used Aminex-87H of Bio-Rad (USA) and carbohydrates, as well as ethanol, was detected by a refractive index detector (RI). The mobile phase consisted of 0.005 mol/L sulfuric acid as eluent at 45 °C with a flow rate of 0.6 mL/min. A quantitative analysis of reducing sugars was undertaken using the Somogyi-Nelson method [22,23]. The BÜCHI Distillation Unit K-350 for the rapid and complete distillation of fermentation broth was used with Anton Paar DMA 35N portable density meter. All measurements were performed in triplicate.

2.5. Experimental Design and Statistical Analysis

Central Composite Design (CCD) was used for the optimization of ethanol production in batch fermentation. The CWP concentration (X_1) and ratio of yeast *K. marxianus* NCAIM Y00963 to *S. serevisiae* (X_2, a total cell count was about 3×10^8 cells/mL medium) were selected for the independent variables, as shown in Table 1. Ethanol concentration (Yi) was used as the dependent output variable.

Table 1. Independent variables in the experimental design.

Independent Variable	Symbol	Coded Levels				
		$-\sqrt{2}$	−1	0	1	$\sqrt{2}$
Initial CWP concentration % (w/v)	X_1	7.5	10.0	15.0	20.0	22.5
The ratio of yeast K. marxianus to S. serevisiae *	X_2	0.3	0.5	1.0	1.5	1.7

* total cell count was about 3×10^8 cells/mL mash.

The statistical analysis of the data, as well as the second-order polynomials (Equation (1)), were calculated with a statistical package (STATISTICA 9.0, StatSoft Inc., Tulsa, OK, USA) to estimate the response of the dependent variable.

$$Yi = b_0 + b_1X_1 + b_2X_2 + b_{11}{X_1}^2 + b_{22}{X_2}^2 + b_{12}X_1X_2 \quad (1)$$

where Y is the response variable, X_1 and X_2 are the independent variables, b_0 is the interruption coefficient, b_1 and b_2 are the coefficients of the linear effects, b_{11} and b_{22} are the coefficients of the quadratic effects, and b_{12} is the coefficient of the interaction effect.

2.6. Statistical Analysis

All data except those in part of the experimental design are presented as the mean and standard deviation (SD). One-way analysis of variance (ANOVA), and unpaired and paired Student's t-tests were done using the Statistica v9.0 software package (StatSoft, Tulsa, OK, USA) for process experimental data. Generally, only $p < 0.05$ was accepted as the statistical significance level.

3. Results and Discussion

3.1. Selection of Lachancea and Kluyveromyces Yeast Strains

Six *L. thermotolerans* strains, one *K. marxianus*, and one *K. waltii* strain were screened for ethanol fermentation in the mashes contained 10% (*w*/*v*) CWP as substrate. The results are summarised in Table 2.

Table 2. Ethanol concentrations and bioconversion rates at batch fermentation using *Lancancea* and *Kluyveromyces* yeast strains.

Strains	CWP % (*w*/*v*)	Ethanol % (*v*/*v*)	Y_{ET} %
L. thermotolerans Y00702	10	2.4 ± 0.11	48.15 ± 1.92
L. thermotolerans Y00715	10	3.0 ± 0.17	56.41 ± 2.39
L. thermotolerans Y00775	10	3.4 ± 0.15	61.67 ± 3.61
L. thermotolerans Y00798	10	2.8 ± 0.11	50.98 ± 2.62
L. thermotolerans Y00873	10	3.3 ± 0.18	63.22 ± 3.41
L. thermotolerans Y00959	10	2.6 ± 0.15	49.98 ± 2.15
K. marxianus Y00963	10	3.5 ± 0.18	62.33 ± 2.99
K. waltii Y01184	10	3.4 ± 0.16	62.04 ± 2.44

All investigated strains were able to take up lactose directly from the mash, hydrolyse it, and convert it to ethanol. The ethanol concentrations in the mashes varied from 2.4% (*v*/*v*) to 3.5% (*v*/*v*) with bioconversion rates of approximately 48–63%. Four strains *L. thermotolerans* Y00775, Y00873, *K. marxianus* Y00963, and *K. waltii* Y01184 resulted in about 3.3% (*v*/*v*) ethanol after 7 days of fermentation. The highest alcohol content (3.5% *v*/*v*) was detected in the case of *K. marxianus* Y00963 strain. Figure 1 showed that the main part of the lactose substrate (about 90%) was consumed by yeast at 96 h, while the alcohol content reached 3% at 72 h in the case of the *K. marxianus* Y00963 strain. In the cases of other yeast strains, at least 5 days or even more (data are not shown) were needed. The residual sugar content such as lactose, galactose, and glucose was about 3.5 g/L at the end of the fermentation process.

Some other strains *L. thermotolerans* Y00775, Y00879 and *K. waltii* Y01184 strains also showed similar trends of lactose utilization, but these strains required about 168 h. In those cases, 10.8–15.3 g/L sugars remained in the fermentation medium. Silveira et al. [13] did the flux analysis of oxidoreductase metabolism as a function of lactose concentration and oxygen levels in the production of ethanol from cheese whey permeate by *K. marxianus* UFV-3 strain, and they found that the maximum ethanol concentration was about 80 g/L; even the initial lactose concentration was up to 240 g/L. This means that *Lanchancea* and *Kluyveromyces* yeasts may have some limitations in ethanol production from lactose substrate. Based on these results, the *K. marxianus* Y00963 strain was selected for further studies.

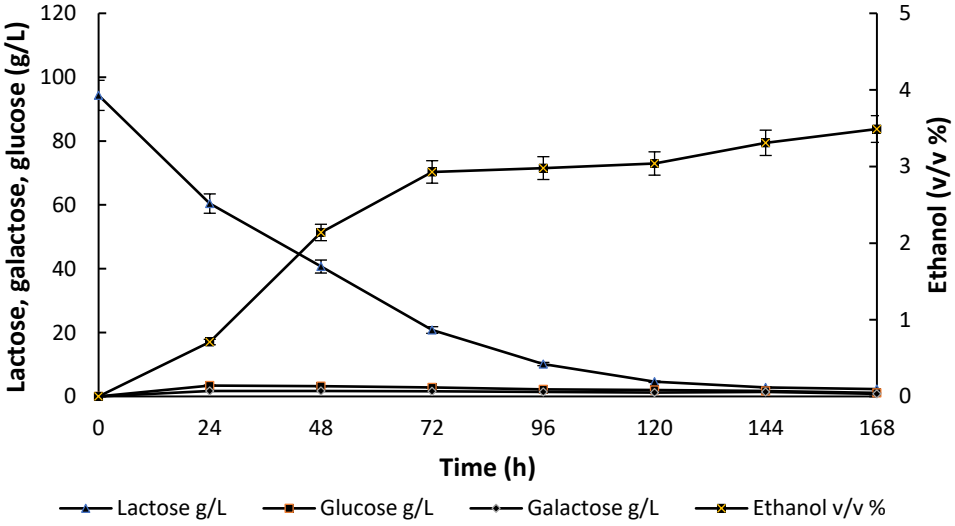

Figure 1. Fermentation profile of *Kluyveromyces marxianus* Y00963 strain.

3.2. Batch Fermentation Processes with the Mixed Culture

The effect of CWP concentration on the capacity of lactose conversion by mixed culture of yeast *K. marxianus* Y00963 strain and Levuline FB type dried *S. cerevisiae* was investigated. The ratio of the cell count (total cell count was about 3×10^8 cells/mL mash) of the yeasts was set to 1:1. The ethanol concentrations, bioconversion rates, and residual lactose contents with CWP concentration are shown in Table 3.

Table 3. Batch fermentation with the mixed culture of *S. cerevisiae* and *K. marxianus* in a ratio of 1:1 at different CWP concentrations.

CWP % (*w*/*v*)	Ethanol % (*v*/*v*)	Y_{ET} %	Residual Lactose g/L
10	3.9 ± 0.15	69.26 ± 3.43	3.03 ± 0.11
15	5.8 ± 0.22	82.64 ± 4.25	9.71 ± 0.37
20	7.3 ± 0.29	70.35 ± 3.22	29.19 ± 0.93
25	5.3 ± 0.25	32.75 ± 1.27	61.86 ± 2.45
30	3.1 ± 0.11	11.62 ± 0.42	87.32 ± 4.12

In the case of 100 g/L substrate, the ethanol concentration was 3.9% (*v*/*v*) at 120 h of fermentation meaning 69.3% bioconversion rate. In comparison with the results mentioned above, it is evident that the *K. marxianus* strain may quickly hydrolyse lactose in whey to galactose and glucose, while *S. cerevisiae* effectively converted these sugars into ethanol. The mixed culture resulted in higher lactose consumption and ethanol production than those of yeast monocultures. This phenomenon has also been observed by other authors [24–27]. The amounts of glucose and galactose were minimal during ethanol fermentation. An increase in the initial concentration of CWP up to 15–20% (*w*/*v*) resulted in a maximal bioconversion efficiency of lactose (82.64% and 70.35%), as well as ethanol concentration (5.8–7.3% *v*/*v*). The residual lactose contents in these cases were about only 9.71–29.19 g/L, which is economically acceptable. An increase in the concentration of CWP to above 20% (*w*/*v*) negatively influenced the ethanol production. These results were also confirmed by Silviera et al. [13] and Díez-Antolínez et al. [28]. High CWP concentrations repressed sugar utilization, probably due to the high osmotic pressure [17]. The ethanol concentration was between 5.3% (*v*/*v*) and 3.1% (*v*/*v*), with a bioconversion rate of 32.8–11.6%.

Optimization of the substrate concentration of CWP (X_1) and inoculation ratio of lactose-positive yeast in mixed culture (X_2) were performed using the central composite design. The fermentative production of ethanol (Y) was selected as the dependent variable. An experimental set with a total of

10 runs was carried out using different combinations of the two independent variables at five levels (Table 4). Central points were made in duplicate.

Table 4. Experimental design and results for batch CWP fermentation.

Run No.	X_1	X_2 *	Y (%)
1	22.0	1.0	5.8
2	20.0	1.5	6.5
3	15.0	0.5	5.1
4	15.0	2.4	6.2
5	15.0	0.3	4.4
6	10.0	1.5	3.5
7	10.0	0.5	2.8
8	8.0	1.0	2.3
9	15.0	1.0	4.9
10	15.0	1.0	4.6

* Total cell counts were about 3×10^8 cells/mL mash.

The CWP concentration significantly affected the change of ethanol concentration ($p \leq 0.05$), while the interaction between these independent variables on lactose conversion into ethanol were found to be relatively weak ($p \geq 0.05$) in the interval with ±95% confidence (Table 5). The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.944, indicating that 94.4% of the variability in the response could be explained by the model. The significance and adequacy of the second-order equation were statistically checked by analysis of variance (ANOVA, Table 6).

Table 5. ANOVA for ethanol concentration (R^2 = 94.4).

Factor	Seq SS	DF	Adj M	F	*p*-Value
X_1	10.5615	1	10.5615	95.8273	0.0006
X_1*X_1	0.7809	1	0.7809	7.0853	0.0562
X_2	0.6847	1	0.6847	6.2129	0.0672
X_2*X_2	0.0399	1	0.0399	0.3619	0.5798
X_1*X_2	0.0211	1	0.0002	0.1916	0.6841
Error	0.4408	4	0.4058		
Total Seq SS	17.7290	9			

Seq SS: a sequential sum of squares; DF: degrees of freedom; Adj SS: the adjusted sum of squares; Adj M: adjusted mean square.

Table 6. Effects of substrate concentration and inoculation ratio on ethanol concentration ± interval with 95% confidence.

Factor	Effect	Standard Error	*t*-Value	*p*-Value	Conf. Limit (−95%)	Conf. Limit (+95%)
Constant	4.9237	0.2022	24.3396	0.0000	4.3620	5.4853
X_1 (L)	**2.6584**	**0.2715**	**9.7891**	**0.0006**	**1.9044**	**3.4124**
X_1 (Q)	−0.8684	0.3262	−2.6618	0.0562	−1.7742	0.0373
X_2 (L)	0.6866	0.2754	2.4925	0.0672	−0.0782	1.4514
X_2 (Q)	0.0902	0.1500	0.6016	0.5798	−0.3263	0.5069
1L by 2L	0.1863	0.4255	0.4377	0.6841	−0.9953	1.3679

Ethanol production was described by the following second-order polynomial Equation (2).

$$Yi = 4.4342 - 0.2875\,X_1 - 0.9111{X_1}^2 + 0.3457X_2 - 0.0578{X_2}^2 - 0.0106X_1X_2 \quad (2)$$

where Yi is ethanol concentration, X_1 is CWP concentration, and X_2 is inoculation ratio of yeasts.

The lactose conversion into ethanol increased linearly with an increase in CWP concentration up to 17–20 (*w*/*v*) % and 2.5:1–3:1 ratio of *K. marxianus* NCAIM Y0963 and *S. cerevisiae* in mixed culture (Figure 2). A maximum ethanol concentration of about 8–10 (*v*/*v*) % was predicted when using these parameters in their optimal range in ethanol fermentation.

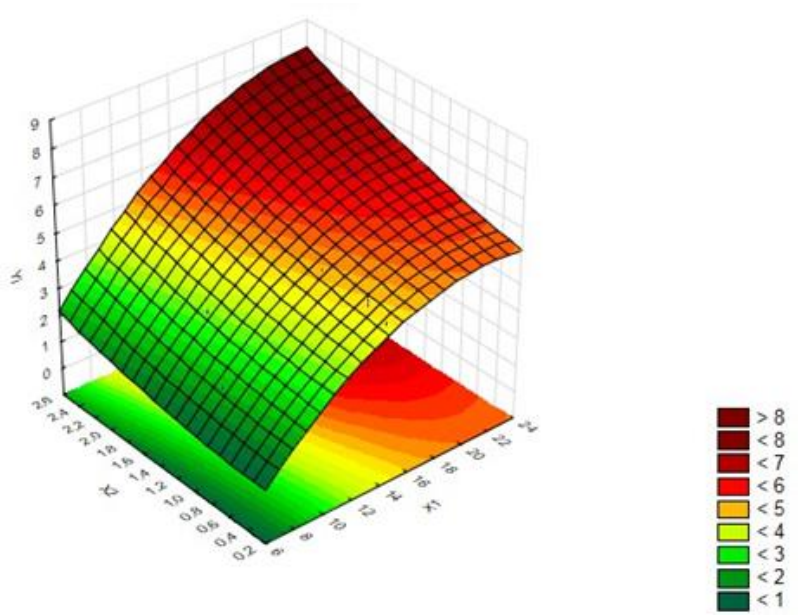

Figure 2. Response surface of ethanol production as a function of the concentration of CWP and inoculum ratio of *K. marxianus* NCAIM Y00963 in mixed culture.

The ethanol production at optimal conditions was validated experimentally in laboratory-scale batch fermentation for 168 h. The results are shown in Table 7.

Table 7. Model validation and confirmation using the optimal range of two independent variables.

Run no.	CWP % (*w*/*v*)	Inoculation ratio of *K. marxianus* to *S. cerevisiae*	Ethanol % (*v*/*v*)	Y_{ET} %
1	17.5	3:1	7.25 ± 0.22	72.33 ± 2.85
2	20.0	2.5:1	6.40 ± 0.18	63.48 ± 2.48
3	22.5	3:1	5.25 ± 0.17	55.21 ± 1.64
4	25.0	3.5:1	4.85 ± 0.17	44.07 ± 1.93

An ethanol concentration of 7.25% (*v*/*v*) was detected at 17.5% (*w*/*v*) CWP concentration and 3:1 ratio of two yeasts (Table 7); this was very close to the predicted value (8.02%). The bioconversion efficiency of lactose was 72.33%, while the fermentation time reduced from 120 h to 72 h. This result is in agreement with one published by Dragone et al. [29]; those authors optimized the initial lactose concentration, temperature, and inoculation size to maximize ethanol production by yeast *K. fragilis*. Maximum 80.93 kg/m^3 was obtained when using an initial lactose concentration of 200 kg/m^3.

3.3. Effect of Repeated Inoculation Techniques

The effect of different inoculation procedures at an initial 17.5% (*w*/*v*) CWP containing medium and different ratios of the *K. marxianus* NCAIM Y0963 and the *S. cerevisiae* in mixed cultures was investigated (Figure 3). The total cell counts were kept at 3×10^8 cells/mL mash.

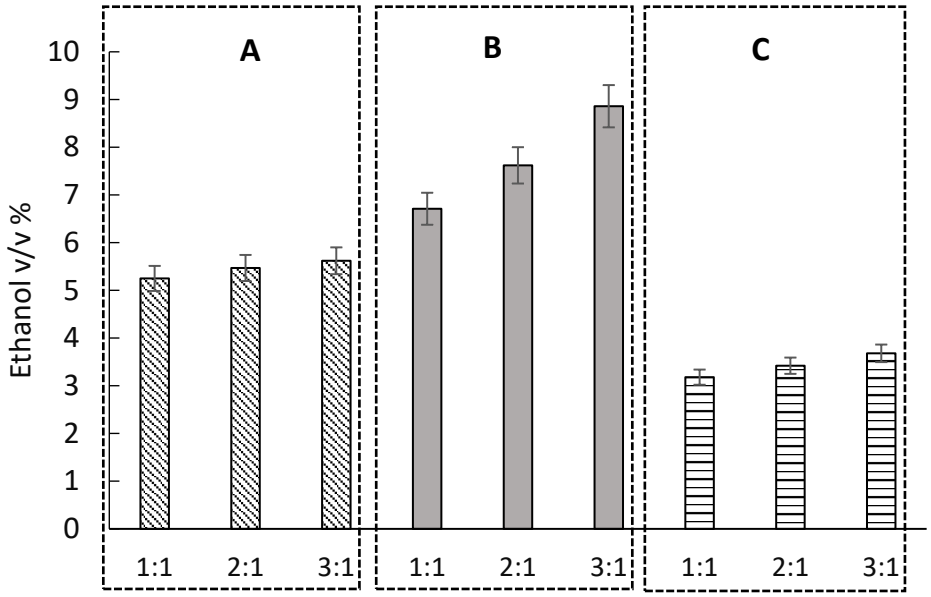

Figure 3. Effects of repeatedly feeding mixed culture. The total cell count was 3×10^8 cells/mL mash. (**A**): a $\frac{1}{2}$ part of the mixed culture (around 7.5 mL inoculum 2.25×10^{10} cells) was inoculated at the initial time and another $\frac{1}{2}$ part (around 7.5 mL inoculum 2.25×10^{10} cells) at 24 h; (**B**): a $\frac{3}{4}$ part of the mixed culture (around 11.5 mL inoculum 3.35×10^{10} cells) was inoculated at the initial time, and another $\frac{1}{4}$ part (around 3.5 mL inoculum 1.15×10^{10} cells) at 24 h; (**C**): *K. marxianus* (around 7.5 mL inoculum 2.25×10^{10} cells) was inoculated at an initial time and *S. cerevisiae* (around 7.5 mL inoculum 2.25×10^{10} cells) at 24 h.

The highest ethanol yield was about 8.86% (*v*/*v*), and lactose utilization was about 87.5%, by adding three-quarters at 24 h and one-quarter at 48 h (Figure 3). This value was significantly higher than in other cases (5% and 3%). These results are promising for further experiments. As we have stated, this is the first study carried out to investigate this fermentation technique.

The ethanol concentration of fermented mash increased to 10.34% (*v*/*v*) with about 92% conversion rates when the fed-batch technology (30 g CWP/L was fed at 24 and 48 h of fermentation) was used in combination with repeated inoculation at an initial CWP concentration of 150 g/L. This ethanol concentration is very promising for the development of an economic distillation process. Guimarães et al. [30] gave an excellent review of the current status of integrated solutions for the valorization of cheese whey, and different results were summarized. In our study, despite the fact that neither the *K. marxianus* nor *S. serevisiae* yeasts were engineered and selected as biocatalysts for the valorisation of cheese whey, our results were definitely in line with the others shown in Table 8. Our study showed that the new culturing technique of mixed-culture of yeasts has the potential to improve the efficiency of the whey-to-ethanol process. These experimental data are essential in engineering work on the development of ethanologenic and lactose-positive strains for the valorization of cheese whey; this was also an important aspect in the aforemetioned review [30].

Table 8. Comparison of ethanol production and lactose consumption under various fermentation process using ethanologenic yeast strains in monoculture and mixed culture.

Yeast	Fermentation Media	Bioreactor Type	Ethanol Titer (g/L)	Ethanol Yield (%)	Lactose Consumed (%)	References
Monocultures						
K. fragilis	CW permeate (240 g/L lactose)	3 L static bottles	80	70	89	[31]
	CW permeate (240 g/L lactose)	14 L stirred tank	72	91	61	[32]
	CWPS (150 g/L lactose) with peptone supplementation	1 L stirred flasks	71	87	100	[33]
K. marxianus	CW (46 g/L lactose)	1 L stirred tank	8.6	37	93	[21]
	CW (48 g/L lactose) with yeast extract supplementation	shake flasks	7.9	32	-	[34]
	CWPS (60 g/L lactose) with yeast extract and salts supplementation	5 L stirred tank	26	82	100	[35]
	CWPS (75 g/L lactose)	shake-flasks	41	100	100	[14]
	CW (100 g/L lactose) with yeast extract and salts supplementation	2 L stirred tank	43	>80	>95	[36]
	CWPS-permeate (170 g/L lactose)	1 L stirred flasks	76-80	>94	>91	[13]
	CWPS (150 g/L lactose)	shake-flasks	80	100	98	[17]
Candida pseudotropicalis	CW and lactose powder (150 g/L lactose)	5 L stirred tank	45	98	78	[37]
	CWP (100 g/L lactose)	500 mL shake flasks	30	60	95	[38]
	CW (100 g/L lactose)	shake-flasks	41	78	>99	[38]
Mixed cultures						
K. marxianus *S. cerevisiae* (free cells)	CWP (100 g/L lactose)	500 mL shake flasks	36	71	98	[39]
***K. marxianus* *S. cerevisiae* (free cells)**	**CWP (150 g/L lactose)**	**500 mL shake flasks**	**80–82**	**86**	**92**	**our results**
K. marxianus *S. cerevisiae* (immobilized cells)	CWP (100 g/L lactose)	500 mL shake flasks	42	80	>99	[39]

CW: cheese whey, CWP: cheese whey powder, CWPS: cheese whey permeate supernatant (after centrifugation).

4. Conclusions

Cheese whey constitutes an abundant, inexpensive, and nutritionally-rich dairy industry was product, which could be a potential source for value-added products such as ethanol. Mixed culture fermentation of CWP resulted in higher lactose conversion into ethanol compared to using only lactose-positive microorganisms, especially the *Kluyveromyces* or *Lachancea* yeast strains. Additionally, bioconversion efficiency can be improved by a shared culturing technique using a mixed culture. Overall, our results could serve a very good basis for the development of fermentation technology for ethanol production from cheese whey.

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