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ORIGINAL PAPER

Production of bioethanol from organic whey using *Kluyveromyces marxianus*

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Abstract Ethanol production by *K. marxianus* in whey from organic cheese production was examined in batch and continuous mode. The results showed that no pasteurization or freezing of the whey was necessary and that *K. marxianus* was able to compete with the lactic acid bacteria added during cheese production. The results also showed that, even though some lactic acid fermentation had taken place prior to ethanol fermentation, *K. marxianus* was able to take over and produce ethanol from the remaining lactose, since a significant amount of lactic acid was not produced (1–2 g/l). Batch fermentations showed high ethanol yield (~0.50 g ethanol/g lactose) at both 30°C and 40°C using low pH (4.5) or no pH control. Continuous fermentation of nonsterilized whey was performed using Ca-alginate-immobilized *K. marxianus*. High ethanol productivity (2.5–4.5 g/l/h) was achieved at dilution rate of 0.2/h, and it was concluded that *K. marxianus* is very suitable for industrial ethanol production from whey.

Keywords *Kluyveromyces marxianus* · Cheese whey · Ca-alginate · Immobilization · Continuous fermentation

Introduction

It is a fact that the Earth is running out of fossil raw material. It is also a fact that global warming is changing our climate and that these changes are caused by an increased concentration of CO_2 in the atmosphere. It is therefore of great interest to substitute fossil fuels with renewable natural resources. Bioethanol is a renewable CO_2 reduced fuel that can be produced from raw materials rich in monosaccharides (sugar canes and sugar beets) and from crops rich in starch (corn or wheat). The sustainability of bioethanol obtained from raw materials that can also be used as food or feed (so-called first-generation bioethanol) is questionable. Therefore, it would be more advantageous if bioethanol production could be based on alternative substrates such as lignocellulosic raw materials by using second-generation conversion technologies and other byproducts from agriculture, forestry, and the food industry. Whey is a byproduct from the dairy industry. It represents a disposal problem and is an important source of environmental pollution due to its enormous global production rate all over the world (to make 1 kg cheese, 9 kg whey is generated) [13]. Bioconversion to ethanol could be an alternative use for this feedstock. The major components of whey are lactose (5–6%), protein (0.8–1%), and fat (0.06%) [13]. Lactose is a disaccharide consisting of glucose and galactose. It cannot be fermented by *Saccharomyces cerevisiae*, which is commonly used in alcohol fermentation, because this strain of yeast lacks β-galactosidase activity; it can, however, ferment the hydrolysis products of lactose: glucose and galactose. Unfortunately, acid hydrolysis can form some byproducts that may inhibit the fermentation, and enzymatic hydrolysis will add expense to the process. Another option is to use a different yeast strain, *Kluyveromyces marxianus*, which is capable of fermenting lactose to ethanol directly. *K. marxianus* has been studied extensively for utilization of whey, e.g.: the effect of multiple substrates in ethanol fermentation from cheese whey [17], ethanol production from crude whey

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[19], batch fermentation [1, 18], fed-batch fermentation [1, 9, 15], continuous fermentations [4, 11, 12], studies on cheese whey powder [6, 11–14], immobilization of thermotolerant yeast on delignified cellulosic materials [7], and alginate-immobilized yeast cells [2, 3, 10]. It has been found that, when using alginate-immobilized cells, cell flush-out is avoided and also the production of ethanol is raised compared with ethanol production from free cells [3]. In literature, no experiments have been found that study alginate-immobilized cells of *K. marxianus* in continuous fermentation of cheese whey.

The aims of this study are to find the best way of utilizing whey and to design a process for conversion of organic whey into bioethanol by fermentation using *K. marxianus*. This process is planned to be part of developing a concept for a decentralized biorefinery concept to be used in the organic agricultural industry in Denmark, by integrating energy production (biogas and bioethanol) in organic farming to increase the sustainability and self-reliance of energy utilized in this industry. This can be done by better utilization of byproducts from the farm and/or farm units in combination with byproducts from related food industries such as whey from dairy. Figure 1 shows the concept of the proposed biorefinery, where intercrops and byproducts from the agro industry are used as substrates for on-farm energy production. By combining whey produced from organic cheese production with crops produced by sustainable methods this study shows how bioethanol can be produced in a sustainable way and organic farms and/or dairies can be converted into biorefineries.

Materials and methods

Yeast strain

K. marxianus strain DSMZ 7239 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The strain was maintained at −85°C in a mixture of 50% v/v glycerol and growth medium solution, which contained per litre of demineralized water: 5 g bacto peptone, 5 g yeast extract, 30 g lactose, 2 g NH_4Cl, 0.3 g $MgSO_4 \cdot 7H_2O$, and 1 g KH_2PO_4.

Yeast cultivation

Starter culture of *K. marxianus* DSMZ 7239 was grown in 250-ml cap flasks containing 150 ml culture medium. The medium for growth of yeasts was the same synthetic lactose medium which was used for strain maintenance. The medium was sterilized at 121°C for 20 min. The flasks were incubated in an orbital shaker at 100 rpm for 24 h at 30°C.

Raw material: whey

The cheese whey used in the experiments was provided by the Thise Mejeri organic dairy, Denmark. Four different types of whey were provided, which had been treated differently in the dairy. Type 1 was raw whey taken from the cheese manufacturing process and stored cold (2–5°C). Type 2 was whey that had been stored at room temperature, which causes the lactic acid bacteria (added during the

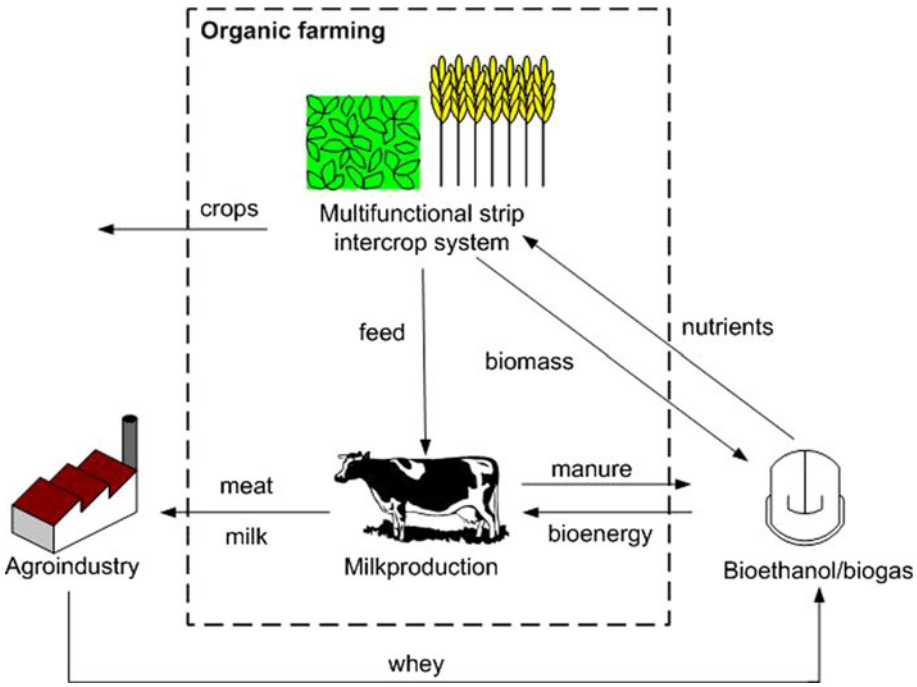

Fig. 1 Concept of bioenergy production in organic farming

cheese-making process) to convert the lactose to lactic acid. After arrival to the laboratory the lactic-acid-fermented whey was stored at 2–5°C. Type 3 was raw whey that had been pasteurized and kept frozen (−5°C), and type 4 was raw whey that had been pasteurized and stored cold (2–5°C). Table 1 gives an overview of how the four types of whey were treated in the dairy.

Screening of the four types of whey in flask fermentations

Inoculum (1 ml) was added to 100 ml whey in 250-ml shake flasks equipped with yeast locks. The flasks were incubated at 30°C at 100 rpm, and samples for lactose and ethanol analysis were taken once a day for 3 days.

Batch fermentation of whey

Two batch fermentations were performed in 2.5-l fermentor (Minifors, Infors HT, Switzerland) containing 2 l nonsterilized whey. In the first experiment 25 ml inoculum [1.25% (v/v)] was added to the whey. The temperature was controlled at 30°C, and pH was maintained at 4.5 by addition of 1 M HCl and 1 M NaOH throughout the fermentation (150 h). In the second batch experiment only 5 ml inoculum [0.25% (v/v)] was added, temperature was controlled at 40°C, and no pH control was applied. The fermentation time was 170 h. Agitation was 500 rpm in both experiments, and samples were withdrawn from the fermentor for analysis of lactose, ethanol, lactic acid, and acetic acid.

Continuous fermentation of whey with Ca-alginate-immobilized *K. marxianus*

Cells of *K. marxianus* were immobilized by suspending 2.6 g centrifuged washed wet cells in 250 ml 4% sodium-alginate gel. The yeast–alginate mixture was extruded as drops into a 4% calcium chloride solution kept on ice. For extrusion a pump and a Pasteur pipette were used, which resulted in uniform round beads of approximately 2 mm in diameter. The beads were washed with sterile 0.1% salt-water and stored in a sterile synthetic lactose medium at 4°C until use. The continuous fermentation was performed in a 300-ml fluidized bed reactor with an outside water flow for temperature control. Beads (100 ml) with immobilized cells were filled in the bottom of the reactor, and the reactor was filled to the overflow with whey. The temperature was kept at 32°C using water pumped from a temperature-controlled water bath to the outside of the reactor. Fermentation was initiated in batch mode for the first 3 h. After 3 h the substrate flow was turned on at low dilution rate ($\mu_{max}/10$). The dilution rates were based on the maximum specific growth rate (μ_{max}) of *K. marxianus* in this type of whey. The nonsterilized whey was kept on ice and pumped to the fermentor using a pump (Masterflex L/S 07534—04, USA). Samples were withdrawn five times in the first 48 h and every 24 h thereafter and analyzed for lactose and ethanol content. Productivity was calculated by multiplying the dilution rate by the actual ethanol concentration.

Table 1 Treatment in the dairy of the four types of whey used in the study

Whey	Pasteurization	Cooling	Freezing	Lactic acid fermentation
1	−	+	−	−
2	−	+	−	+
3	+	−	+	−
4	+	+	−	−

Analytical methods

Growth rate of *K. marxianus* was followed by measuring the optical density at 600 nm using a spectrophotometer (Spectrophotometer 6305; Buch & Holm A/S, Denmark).

The concentrations of lactose, glucose, ethanol, lactic, and acetic acid in the samples were determined by high-performance liquid chromatography (HPLC) (Shimadzu Corp., Kyoto, Japan) using a Rezex ROA column (Phenomenex, Torrance, CA, USA) at 63°C and 4 mM H_2SO_4 as eluent at flow rate of 0.6 ml/min, equipped with a refractive index detector (Shimadzu Corp.). Samples were pH-adjusted to 2.0–2.3 and filtered through a 0.45-μm membrane prior to injection into the vials.

Results and discussion

Screening of the four types of whey in flask fermentations

The whey was treated in four different ways at the dairy (Table 1) before being used for ethanol fermentation by *K. marxianus* in the laboratory. The chemical compositions of the four resulting whey types were analyzed with regards to sugars, ethanol, and organic acid content (Table 2). The composition of type 2 significantly differed from other types. This untreated whey sample, as expected, had much lower content of lactose and high content of lactic acid, due to the natural lactic acid fermentation taking place at these conditions. The composition of types 3 and 4, which had been pasteurized and frozen or kept cool, respectively, did not differ significant from type 1, which had just been kept cool, showing that

Table 2 Chemical composition of the four different types of whey

Concentration (g/l)	Whey 1	Whey 2	Whey 3	Whey 4
pH	5.67	3.46	6.85	6.62
Lactose	46.8	19.3	48.6	48.6
Glucose	0	0.22	0.16	0.15
Xylose	0.16	0.16	0.18	0.17
Acetic acid	0.13	0.25	0.28	0.02
Lactic acid	1.06	9.19	0.21	0.16
Formic acid	0.11	0.28	0.02	0.20
Ethanol	0	0.19	0	0

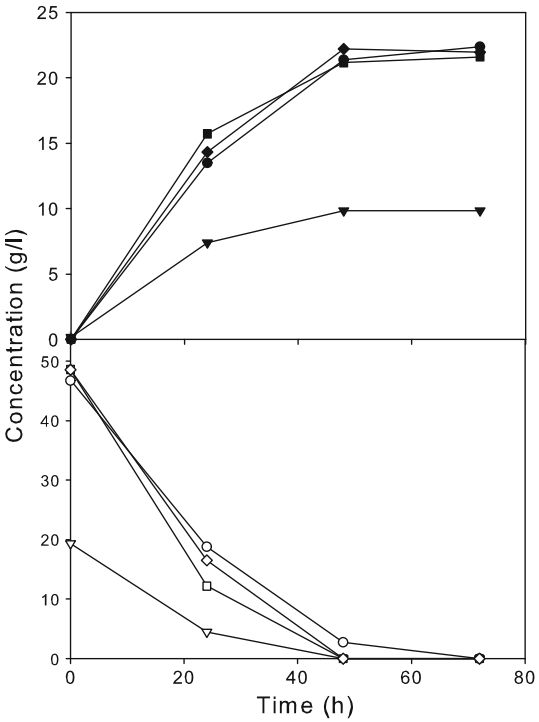

Fig. 2 Ethanol production (*closed symbols*) and lactose consumption (*open symbols*) in flask fermentations of the four different types of whey: type 1 (*circles*), type 2 (*inverted triangles*), type 3 (*squares*), and type 4 (*diamonds*)

pasteurization of the whey was not necessary in order to keep the lactose from being fermented. However, pasteurization might be necessary in order to prevent the lactic acid bacteria (present from the cheese production) from taking over during ethanol fermentation of the whey; this was examined in flask fermentations.

The four types of whey were fermented in flask fermentations with *K. marxianus* to examine the potential ethanol production by this strain and to choose the type of whey to use in subsequent experiments (Fig. 2).

The lactic-acid-fermented whey (type 2) gave the lowest ethanol concentration due to the lower lactose content. Also, ethanol was produced at a lower rate, which could be due to the lactic acid present in this substrate. However, the highest ethanol yield per gram of sugar was achieved in this experiment (0.51 g ethanol/g lactose), which can be explained by the low pH in the whey, which forces the yeast to use a lot of energy pumping H^+ ions out of the cell instead of using the energy on biomass formation. Consequently, this gives a higher ethanol yield, because more lactose is used for production of energy instead of formation of biomass. Similar ethanol yields were obtained in the other three types of whey: 0.48, 0.44, and 0.45 g ethanol/g lactose for types 1, 3, and 4, respectively. Figure 2 depicts that lactose was utilized and the ethanol concentration reached a steady level after 48 h. No lag phase was observed in any of the experiments.

Type 1 was chosen as the type of whey to use in subsequent experiments, since it gave the highest ethanol production as well as a slightly higher ethanol yield (excluding the lactic-acid-fermented whey). Furthermore, these experiments showed that the yeast had no problem competing with the live lactic acid bacteria present in the nonpasteurized whey, and it is advantageous that no pasteurization of the whey is needed before ethanol fermentation.

Batch fermentation of whey with *K. marxianus*

Two different batch experiments of whey (type 1) were performed, at (1) 30°C, pH 4.5 (Fig. 3) and (2) 40°C, without pH control (Fig. 4). Figure 3 illustrates the ethanol production and lactose utilization in the fermentation performed at 30°C and pH 4.5. The low pH was chosen to overcome bacterial contamination. Lactose utilization started within 24 h, and all lactose was utilized after 72 h. The ethanol concentration continued to increase until approximately 140 h, when a concentration of 20 g ethanol/l was achieved, corresponding to a yield of 0.47 g ethanol/g lactose (calculated based on the initial lactose content determined at the beginning of the fermentation). This value (43 g/l) is lower than that shown in Table 1 (46.8 g/l), due to inoculation causing dilution. Slight decrease in lactose content during storage was also observed due to the activity of microorganisms present in the whey, originating from the cheese-making process. No lag phase in ethanol production was observed in this experiment, and the large inoculum size (25 ml) and low pH efficiently controlled lactic acid bacteria, so that no lactic acid was produced.

Batch fermentations were also carried out at 40°C, since our future aim is to apply cheese whey together with different byproducts from organic farming in a biorefinery concept in a simultaneous saccharification and fermentation (SSF) process which is usually carried out at 40°C. Figure 4 shows the ethanol production and lactose

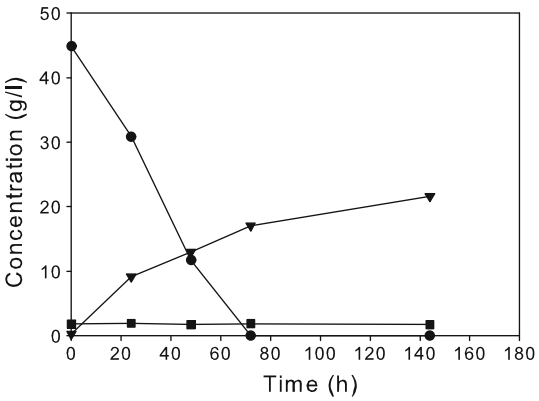

Fig. 3 Lactose consumption and ethanol and lactate production in batch fermentation of nonsterilized whey (type 1) performed at 30°C and pH 4.5 with 1.25% (v/v) inoculum: lactose (*closed circles*), ethanol (*closed inverted triangles*), and lactate (*closed squares*)

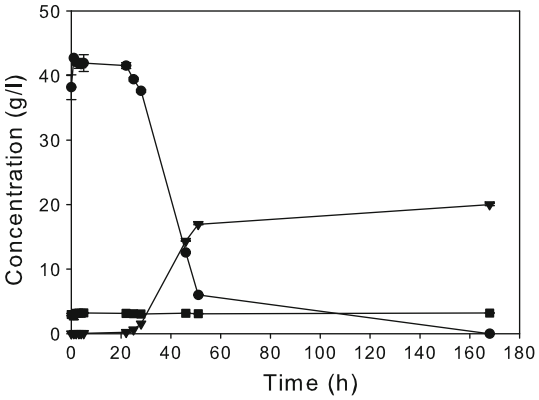

Fig. 4 Lactose consumption and ethanol and lactate production in batch fermentation of nonsterilized whey (type 1) performed at 40°C without pH control with 0.25% (v/v) inoculum: lactose (*closed circles*), ethanol (*closed inverted triangles*), and lactate (*closed squares*)

utilization in the fermentation performed at 40°C without pH control. In this experiment a lag phase of approximately 24 h was observed, which can be explained by the lower inoculation volume used (5 ml). It can be seen from the figure that the initial lactose concentration is slightly lower than in the first experiment (Fig. 3) and that the lactic acid concentration is slightly higher. This could indicate that lactic acid fermentation was initiated during start-up of the fermentor. However, even under these conditions the yeast was able to take over and efficiently convert lactose to ethanol, after the initial lag phase, and no lactic acid was produced during ethanol fermentation. Furthermore, the initial conversion rate was slightly faster at these conditions, which can be explained by the fact that 40°C is closer to the optimal growth temperature of *K. marxianus*, which has been found to be 36°C in our previous experiments (unpublished data). The final ethanol yield was 0.47 g ethanol/g lactose in this experiment (based on initial lactose content in the fermentation). Both experiments showed that *K. marxianus* was capable of adapting to a changing environment very quickly and was able to control the fermentation in the nonsterilized whey.

Continuous fermentation with alginate-immobilized cells of *K. marxianus*

Continuous fermentation was carried out using alginate-immobilized cells. No pH control was applied, and the temperature was kept constant at 32°C. pH in the medium stayed between 4.26 and 4.76 throughout the fermentation. The continuous fermentation was initiated in batch mode (3 h), and the dilution rate was doubled two times until 0.2/h (approximately half the maximum specific growth rate of *K. marxianus*). Figure 5a shows the lactose consumption and ethanol/lactic acid/acetic acid production during the continuous fermentation.

During the first 3 h there was no flow of whey, and the fermentation ran in batch conditions (not shown in the figure). During the first 17 h the dilution rate was set to 0.04/h, which gave a flow rate of 0.2 ml/min. During the following 5 h the dilution rate was increased to 0.08/h, which resulted in flow rate of 0.4 ml/min. After 22 h of the experiment, the dilution rate was changed to 0.2/h and the flow rate to 1 ml/min. This dilution rate remained constant until the end of the experiments.

During the initial phase with low dilution rate (0.04–0.08/h) lactose was efficiently utilized and ethanol production of 17.6 g/l was achieved. The dilution rate was increased to 0.2/h after 22 h, and still very efficient ethanol production was observed. However, after 28–78 h at this dilution rate the lactose in the effluent started to increase and less efficient ethanol production was observed. Nevertheless, this was overcome by the microorganisms, and for the last 100 h of fermentation all lactose was utilized and high ethanol concentrations were measured. No lactic acid was produced during any stages of the fermentation, but towards the end of the fermentation (after approximately 200 h), as the ethanol productivity decreased slightly, some acetic acid was produced. This could be due to changes in the metabolism of *K. marxianus*. The average ethanol yield calculated at dilution rate of 0.2/h was 0.48 g ethanol/g lactose, and during the last stages of the fermentation a very high yield of 0.59 g ethanol/g lactose was measured. Figure 5b shows the productivity at different stages of the fermentation. At the highest dilution rate of 0.2/h the productivity varied between 2.5 and 4.5 g/l/h and stabilized around 4 g/l/h towards the end of the fermentation. Other studies have reported productivity of 0.7 g/l/h by *K. marxianus* in continuous fermentation of whey with free cells [13], 2.9 g/l/h in fed-batch fermentation on lactose medium [9], and 1.3 g/l/in batch fermentation of cane

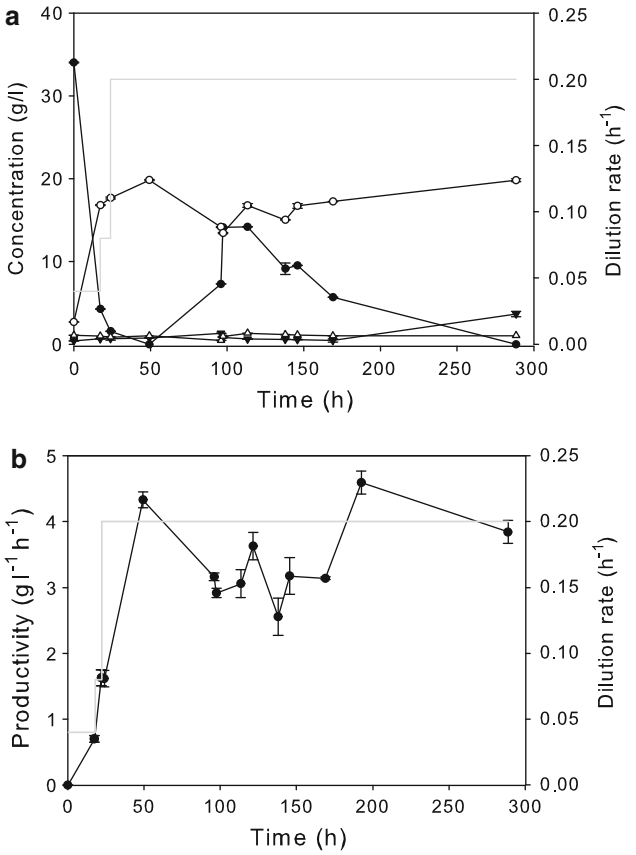

Fig. 5 **a** Lactose consumption and production of ethanol, acetic acid, and lactic acid at different dilution rates in continuous fermentation of nonsterilized whey (type 1) with Ca-alginate-immobilized *K. marxianus*: lactose (*closed circles*), ethanol (*open circles*), acetic acid (*closed inverted triangles*), lactic acid (*open triangles*), and dilution rate (*dash*). **b** Productivity at different dilution rates in continuous fermentation of nonsterilized whey (type 1) with Ca-alginate-immobilized *K. marxianus*: productivity (*closed circles*) and dilution rate (*dash*)

juice [8]. Studies have been carried out using engineered flocculating *S. cerevisiae* on lactose medium, reporting a productivity of up to 2 g/l/h in continuous fermentations [5]. In comparison with these previous studies it seems that immobilization of *K. marxianus* in Ca-alginate gel is a promising method for achieving high ethanol productivity. However, since these productivities were achieved at dilution rate of 0.2/h even higher productivity should be possible, since immobilized systems should be able to run close to or even above the maximum specific growth rate of the microorganism, which has been found to be 0.4/h for *K. marxianus* in this whey medium.

A more suitable immobilization method, e.g., flocculation, should be explored for industrial use, and the system should be optimized to be less fluctuating. Although high biomass loadings can be obtained by gel-entrapment immobilization methods (such as in Ca-alginate), this approach has received less attention in the fermentation industry because of several drawbacks such as diffusion limitations of nutrients, metabolites due to the gel matrix and the high cell densities in the gel beads, chemical and physical instability of the gel, and the nonregenerability of the beads, making this immobilization approach rather expensive [16]. Use of flocculating yeast is very attractive, due to its simplicity and low cost. However, flocculation is affected by numerous parameters, such as nutrient conditions, agitation, Ca^{2+} concentration, pH, fermentation temperature, yeast handling, and storage conditions.

Conclusions

Ethanol production by *K. marxianus* in different kinds of whey from organic cheese production was examined in batch and continuous mode. The results showed that pasteurization was not necessary prior to the process, which is a great advantage from an industrial point of view, where pasteurization/sterilization of the whey would add expense to the process. Batch fermentation of the nonsterilized whey showed high ethanol yields (~0.50 g ethanol/g lactose) at both 30°C and 40°C using low pH (4.5) or no pH control. Continuous fermentation of nonsterilized whey was performed using Ca-alginate-immobilized *K. marxianus*. High ethanol productivity (4.5 g/l/h) was achieved at dilution rate of 0.2/h, and *K. marxianus* was capable of maintaining high productivity at low pH in nonsterilized whey. *K. marxianus* was able to take over lactic acid bacteria present in the whey and was found to be a very robust microorganism capable of producing ethanol at high temperature and low pH in whey.

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