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Kluyveromyces Marxianus Biofilm in Cheese Whey Fermentation for Bioethanol Production

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The feasibility of anaerobic fermentation of cheese whey using *Kluyveromyces marxianus* DSMZ 5422 biofilm on particle support was studied. Firstly the effect of the concentration of cheese whey powder solution and of the amount of inoculum used was tested. Results indicate that both these parameters have limited effect on substrate conversion and the concentration of the produced ethanol. Experiments of cheese whey fermentation in presence of natural supports, namely olive pits and walnut shells, was carried out to observe the holding capacity of *K. marxianus* on these supports. Results confirm the cell adhesion on the surface of olive pits and walnut shells and the suitability of this system for the development of biofilm technology fermentation.

1. Introduction

Fuel ethanol production by fermentation of cheese whey solution (mainly lactose) can be a sustainable route for the production of liquid fuels from renewable source due to high volume whey production. Furthermore, it may solve the problem of whey disposal due to its high demand of BOD (40-50 g L^{-1}) and COD (60 - 80 g L^{-1}). Fermentation of cheese whey by using free and immobilized yeasts *Saccharomyces cerevisiae* (Lewandowska *et.al*, 2007), *Kluyvromyces fragilis* (Gianetto *et.al*, 1986), *Candida psudeotropicalis* (Ghaly and El-Taweel, 1997), *Kluyveromyces marxianus* (Ozmihci and Kargi, 2007, Zafar *et al.*, 2005) has been widely reported. Several technical problems are highlighted in the literature such as the poor substrate lactose utilization *S. cerevisiae* yeasts, lactose and ethanol inhibition affecting the final ethanol concentration in batch processes and the ethanol productivity in continuous processes.

Biofilm technology has been extensively applied in wastewater treatment, but its potential application in bioethanol production has not been explored yet. In general, advantages of biofilm processes include 1) selective substrate and product diffusion due to layered microbial structure, 2) prevention of cell wash out due to the immobilization effect of EPS (extra polymeric substance) formation in the film, 3) operational stability due to higher resistance to external environment. This latter advantage is in particular sought in the bioethanol fermentation process.

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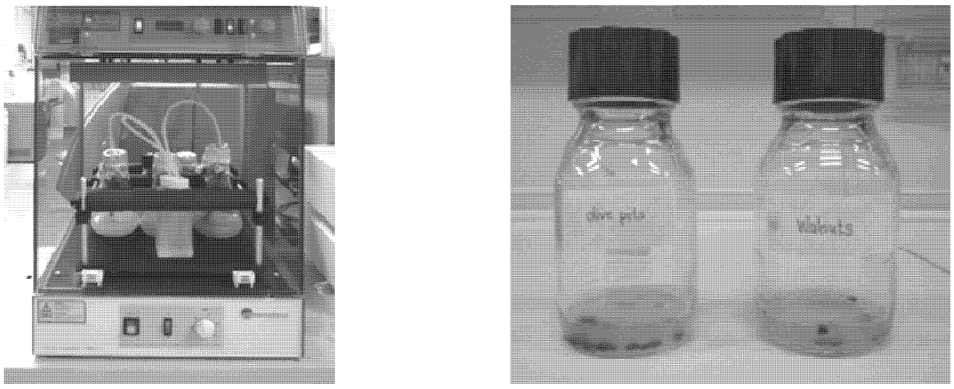

Figure 1: Experimental setup for: a) homogeneous batch fermentation, b) fermentation with natural support for K. marxianus.

K. marxianus yeast strains has shown excellent ethanol tolerance (free and immobilized) and biofilm forming ability without any selective preference of support in the cited literature (Li *et al.*, 2007). The present research work involves studying the feasibility of anaerobic fermentation of cheese whey using *K. marxianus* DSMZ 5422 in batch and repeated batch mode. The first phase of the research involves characterization of the selected *K. marxianus* DSMZ 5422 strain by batch fermentation of cheese whey powder solution at different concentration and different inoculum amount. Furthermore, repeated batch cheese whey fermentation in presence of natural supports, namely olive pits and walnut shells, was carried out to observe the holding capacity of these supports with *K. marxianus* DSMZ 5422 and, simultaneously, the fermentation performance.

2. Batch fermentation experiments

2.1 Microorganisms strain, source and preservation

K. marxianus strain DSMZ 5422 was obtained from the Deustche Sammlung von Mikrooranismen un Zellkulturen GmBH (DSMZ) in lyophilized form. Stock culture was maintained on agar slants with composition lactose (20 g L^{-1}), peptone (10 g L^{-1}), yeast extract (5 g L^{-1}), malt extract (5 g L^{-1}) and agar (20 g L^{-1}). A 24h incubation period was provided before preserving at a temperature of 4°C for further use.

2.2 Inoculum preparation

The medium used for cultivation of the inoculum consists of lactose (50 g L^{-1}), yeast extract (5 g L^{-1}), peptone (5 g L^{-1}), NH_4Cl (2 g L^{-1}), KH_2PO_4 (1 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.3 g L^{-1}), Na-thioglycolate (200 mg L^{-1}). The pH of the medium was adjusted to 5. The medium was autoclaved at 121°C for 15 min. The yeast culture maintained on agar slant was used for inoculation. The medium was kept at 37°C and 120 rpm in an incubator shaker.

2.3 Experimental system

Batch experiments were performed by using sterile Erlenmeyer flasks and a incubator shaker. 500ml of Erlenmeyer flasks were loaded with 198 and 190 ml sterilized cheese whey solution containing desired concentration of cheese whey (50, 100, 150 g L^{-1}) for 1 and 5% (v/v) inoculum respectively (Figure 1a). The initial pH of the media was adjusted to 5 using 0.1N H_2SO_4. Sodium thioglycolate (200 mg L^{-1}) was added as the reducing agent. The flasks were autoclaved at 121°C for 15 min prior to the addition of inoculum. Inoculated flasks were placed in an incubator shaker at 37°C and 120 rpm. Samples were withdrawn from the experimental flasks periodically for the analysis of total sugar, ethanol and biomass concentration.

2.4 Analytical methods

The samples were removed from the flasks periodically and centrifuged at 6500 rpm to remove solids from the liquid media. Total reducing sugar concentrations were measured by using the phenol-acid method (Dubois *et al.*, 1956). Ethanol concentration was measured using dichromate method (Williams and Resse, 1950). Biomass was determined by weighing, after centrifugation, washing with distilled water and drying overnight at 100°C.

2.5 Results

Results for batch fermentation are reported in Figure 2. The most relevant figures of the batch experiment are reported in Table 1.

It is noteworthy that the final ethanol concentration is almost independent of both the inoculum. This feature is common also in the literature for *K. marxianus* (e.g. Ozmihci and Kargi, 2007) where the maximum concentration of ethanol is generally around 4.5 % (v/v), and can be explained with product inhibition. Instead, the biomass produced always grows with the initial substrate concentration but less significantly at high substrate concentrations. With 1% inoculum it appears also that the final biomass concentration is reached at longer times than the compete conversion of lactose indicating the use of some other substrate present in the system. The rate of biomass growth seems larger for the test at higher initial inoculum. It has also to be recalled that, in our test we did not control pH which is one of the sensitive parameters that controls fermentation performance.

Table 1 Comparative accounts of kinetic parameters for different inoculums and cheese whey concentration.

Inoculum volume %	Initial lactose (g L^{-1})	Final ethanol (% v/v)	Maximum biomass (g L^{-1})	Lactose conversion (%)	Ethanol yield $g_{EtOH}/_{glactose}$
1	50	4.25	2.26	92.1	0.711
1	100	4.21	3.28	91.5	0.354
1	150	4.17	3.28	92.0	0.233
5	50	4.28	2.15	90.0	0.725
5	100	4.54	2.09	93.4	0.339
5	150	4.31	4.12	86.6	0.238

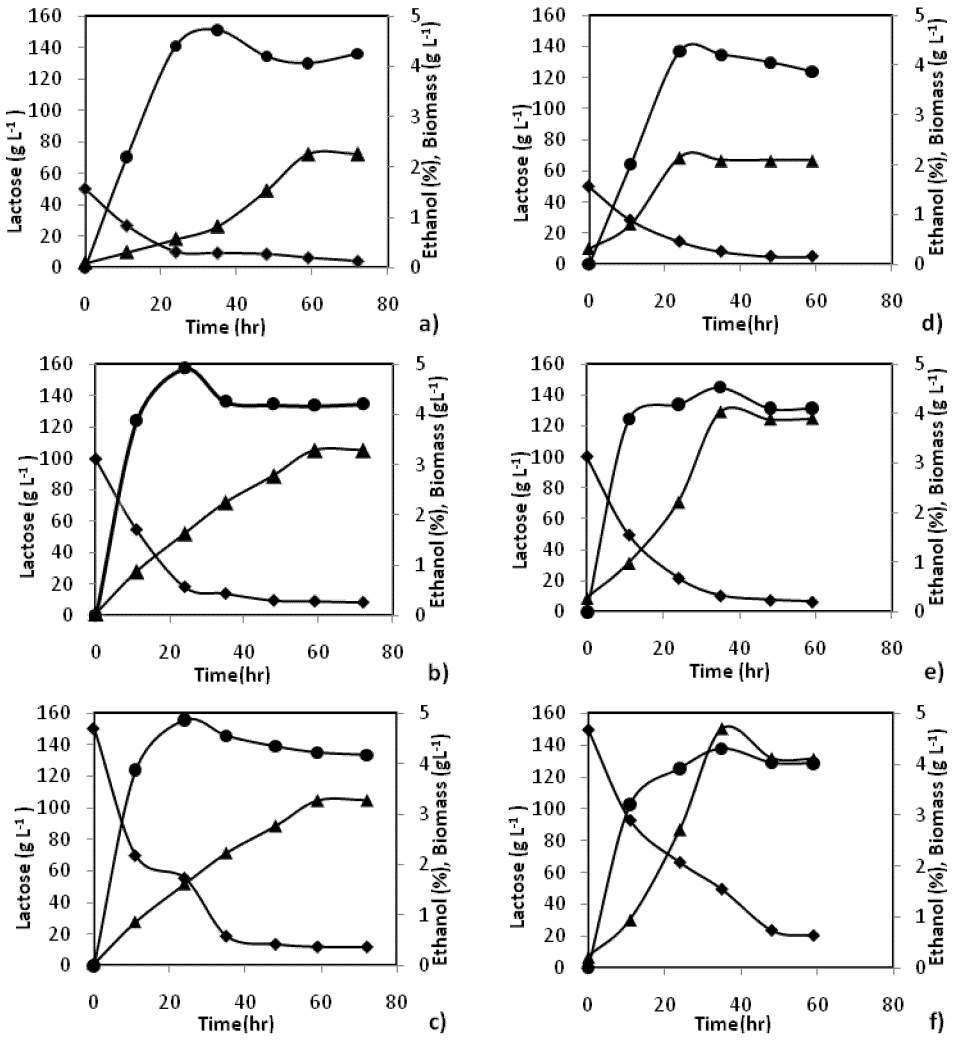

Figure 2: Batch experiments of cheese whey conversion at different initial inoculum to batch volume ratio, i, and substrate concentration, s: a) i=1%, s=50 g L^{-1}; b) i=1%, s=100 g L^{-1}; c) i=1%, s=150 g L^{-1}; d) i=5%, s=50 g L^{-1}; e) i=5%, s=100 g L^{-1}; f) i=5%, s=150 g L^{-1}. ◆,lactose; ●, ethanol; ▲, biomass.

3. Cheese whey fermentation with supported yeast

3.1 Preparation of supports

.Fragments of olive pits and of wall nut shells were selected within the size range 1-2mm. Before the experiment, the olive pits and walnut shells were soaked for one hour in 2% (w/v) detergent solution at 80°C and finally rinsed three times for 10min with distilled water to remove the contamination present on the surface (Brugnoni *et al.*, 2007). The washed pits and shells were divided in two groups; one was heated at 100°C and the other at 200°C respectively. The scope of thermal treatment on the supports is to

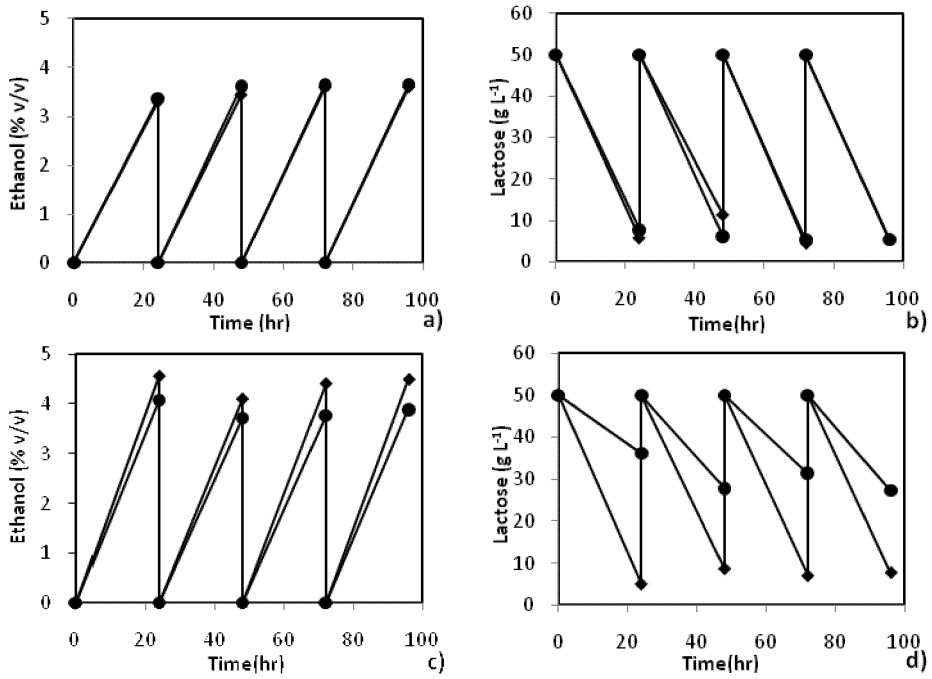

Figure 3: Production of ethanol , a) and c), and consumption of lactose b) and d) in repeated batches in presence of washed olive pits and walnut shells as microorganism supports and inoculated with free K. marxianus only for the first cycle: a) and b) supports pre treated at 100°C; c) and d) supports pre treated at 200°C. ◆, olive pits; ●, walnut shells.

verify its effect on cell adhesion caused by increase in porosity due to removal of volatiles present in the supports. The pits and shells were autoclaved at 121°C for 15 min prior to the fermentation.

3.2 Experimental setup

The experiments were carried out in two phases: 1) Activity and fermentation performance on olive pits and walnut shells heated at 100°C; 2) Activity and fermentation performance on olive pits and walnut shells heated at 200°C. 20ml sterilized cheese whey solution (50 g L^{-1} lactose) was taken in 250 ml volumetric flask containing the supports. The initial pH of the solution was adjusted to 5 using a 0.1N water solution of H_2SO_4. The fermentation was started initially by inoculating cheese whey solution with 5% (v/v) *K. marxianus* DSMZ 5422 culture for 24 hr at 37°C and 120 rpm. After 24 hr, the fermentation broth was removed and centrifuged at 9000 rpm to remove free cells. The supports were washed with distilled water three times to remove the free cells. A 20 ml fresh cheese whey solution was poured in each flasks without addition of further inoculum and kept for 24 hr to observe the activity of *K. marxianus* DSMZ 5422 (Figure 1b).This procedure was carried out for the total time of

96hr. The centrifuged samples were then analyzed for total reducing sugar and ethanol concentration.

3.3 Results

Results of the experiments are reported in Figure 3. The consistent ethanol formation observed in absence of inoculum during repeated batch fermentation confirms the cell adhesion of *K. marxianus* DSMZ 5422 on the supports. No significant increase in ethanol concentration was observed using thermal treatment. However, some reduction in lactose conversion was observed for both the supports heated at 200^0C.

4. Conclusions

Substrate concentration and inoculum quantity have limited effect on substrate conversion and final ethanol concentration. Cell adhesion of *K. marxianus* on the surface of olive pits and walnut shells is verified.

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