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Very-high-gravity Fermentation of Non-supplemented Cheese Whey Permeate by Immobilized *Kluyveromyces marxianus*

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The aim of this research was to improve the ethanol production process with very high gravity fermentation combined with entrapment cell immobilization employing non-supplemented high lactose-load cheese whey permeate (CWP) as a substrate. The effect of temperature and initial pH was tested on free and immobilized cells and the maximal allowable doses of substrate without inhibition effects were assessed. An experimental design by Response Surface Methodology (RSM) was applied to figure out the influence of gel formation parameters (alginate concentration, cell loading and bead size) on the entrapment immobilization process. Bead stability and continuous yield production was evaluated by repeated-batch recycling. The experimental data have demonstrated that non-supplemented high lactose-load CWP is an excellent low cost substrate. Lactose dosages between 170-190 g/L provided free-cell yield efficiencies reaching 95.5% with productivities higher than 1.80 g/(L·h) at 30 ºC. The optimal immobilized model was validated recycling the gel beads for 288 h, with a mean ethanol production of 83.2 g/L, a productivity of 1.6 g/(L·h) and a yield efficiency of 83.2%. Therefore, results demonstrate the feasibility of combining very high fermentation processes with *Kluyveromyces marxianus* immobilization at laboratory scale, which encourages its validation in continuous pilot plant configurations.

1. Introduction

Bioethanol is a renewable and environmentally friendly alternative to petrol. High ethanol productivity from low cost feedstock, in addition to lower investment and operation costs and reduced energy demand, are important aspects in this bioprocesses (Gabardo et al., 2014). To reduce costs and improve the economics of ethanol production, several techniques have been developed including very high gravity (VHG) fermentation or continuous fermentation configurations (Gabardo et al., 2014; Puligundla et al., 2011; Zhang et al., 2015).
VHG fermentation is associated with important water and energy savings, although the high concentrations of substrates and end products severely inhibit the performance of yeasts, limiting the production of ethanol. Therefore, the maximum allowable dose of substrate is a relevant parameter to avoid inhibitions (Zhang et al., 2015). On the other hand, cell immobilization techniques overcome most of the bioprocess restrictions, offer long-term stability of cells, increased molecular selectivity, higher resistance against inhibition, better cell protection against environmental factors, more active biocatalyst per unit of reactor volume, low loss of activity, reduced lag phase and reaction time (Eş et al., 2015). However, cell immobilization techniques also present disadvantages, such as the decrease of substrate accessibility, alterations in biocatalyst conformation and activity, biocatalyst stress problems and costly specific reactor systems (Eş et al., 2015).
Cell entrapment is a usual and effective immobilization technique and alginate hydrogel beads are commonly used due to their biocompatibility, low cost, high porosity and simplicity of preparation. However, there are challenges to overcome, such as uncontrollable gel degradation by the loss of covalent ions, mass transfer limitations, low mechanical strength or large pore size (Duarte et al., 2013). Determinant factors are: alginate concentration, cell loading and bead diameter (Duarte et al., 2013; Idris and Suzana, 2006).

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The utilization of agro-industrial wastes is of great relevance to reduce operating costs. Interesting substrates for alcoholic fermentation are cheese whey and cheese whey permeate (CWP) (Pisano et al., 2015; Sansonetti et al., 2010), even without nutrient supplementation (Gabardo et al., 2014). The use of high lactose-load CWPs is not common due to inhibitory problems, long fermentation times and low susbstrate consumption. Lactose contents about 100 g/L were reported as optimal to reduce substrate imbalance or inhibitions (Dragone et al., 2011; Gabardo et al., 2014; Ozmihci and Kargi, 2007, 2009). However, dairy industry concentrates whey up to values of 170-180 g/L to reduce transport costs. Therefore, developing VHG fermentations employing high load CWPs would be an important improvement. To achieve this, it is necessary to optimize non-inhibitory dosages and operating conditions.

Few microorganisms ferment lactose directly to ethanol with the exception of the genus *Kluyveromyce*s. The species *K. marxianus* presents interesting attributes for industrial uses, such as thermotolerance, high growth rate, capacity to metabolize different substrates and its GRAS status. However, not many researches evaluating ethanol production using *Kluyveromyces* sp. from substrates rich in lactose in immobilized systems have been reported (Brady et al., 1997; Gabardo et al., 2012, 2014; Ozmihci and Kargi, 2009).

The aim of this work was to improve the ethanol production process employing VHG fermentation of non-supplemented high loaded CWPs combined with cell immobilization by entrapment in alginate. In the first stage, the effect of the fermentation factors temperature (T) and initial pH (pH_0) was tested. Secondly, the maximal allowable doses of substrate without inhibition effects were assessed. Thirdly, the entrapment immobilization process was improved by optimizing three gel formation parameters [alginate concentration (Alg, %(w/v)), cell loading (Cell,%(v/v)) and bead size (D_p, mm)] by response surface methodology (RSM). Finally, bead stability and continuous yield production was evaluated by repeated-batch recycling.

2. 2. Material and methods

2.1 Microorganisms and culture conditions

A lyophilized *K. marxianus* DSM 5422 strain provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) was used. The reactivated culture was maintained on nutrient agar and stored at 4 ºC. A loop-full of a slant culture was transferred to sterilized growth medium [50 g/L lactose, 0.3 g/L $MgSO_4 \cdot 7H_2O$ (Sigma Aldrich, Steinheim, Germany), 5 g/L yeast extract, 2 g/l NH_4Cl (Fluka-Sigma Aldrich, Steinheim, Germany), 10 g/L peptone (Fluka, Buchs, Switzerland) and 1 g/L KH_2PO_4 (Panreac, Castellar del Vallès, Spain)]. The medium was incubated in an orbital shaker Infors HT Minitron (Bottmingen, Switzerland) at 35 ºC with a constant shaking at 120 rpm during 7 h in order to obtain exponential-phase cells.

2.2 Cell immobilization

Sodium alginate (Sigma Aldrich) was dissolved at different concentrations in 0.9% w/v NaCl (Fluka-Sigma Aldrich) and sterilized by autoclaving at 121 ºC for 15 min. The different tested cell suspensions (% v/v) were mixed to the sterile alginate solution. The mixture was immediately dripped trough different gauge needles to get beads of different diameters using a peristaltic pump into a flask containing a 2% (w/v) $CaCl_2$ solution (Sigma Aldrich). Immobilized beads were left to harden for 1 h at 35 ºC with an agitation of 150 rpm. These beads were rinsed twice with sterile distilled water to remove residual calcium ions and free cells.

2.3 Cheese whey and fermentation media

The substrate was cheese whey permeate obtained from a mixture of cow and sheep milk after a concentration process by ultrafiltration (provided by Quesería Entrepinares SAU, Valladolid, Spain). The CWP was pasteurized by heating at 80 ºC for 30 min to avoid the effects of endogenous microorganisms. Lactose and protein content were respectively 170 g/L and 34 g/L. The CWP presented an initial pH of 5.8.

2.4 Analytical methods

Lactose and ethanol were measured using Agilent LC1200 HPLC equipment with a refractive index detector and an Aminex HPX-87-H column (Bio-Rad, Hercules, California, USA) with a 5 mM H_2SO_4 mobile phase.

At the end of each run, fermentation kinetic parameters were determined: lactose consumption rate (ΔL, %) (defined as the percentage of lactose consumed), ethanol conversion yield ($Y_{E/L}$, g/g) (defined as the ratio between ethanol produced and lactose consumed), ethanol production efficiency (η, %) (defined as the ratio between the actual yield and the theoretical yield expressed as a percentage (considering a theoretical value of 0.538 g/g)) and ethanol production rate (W_E, g/(L·h)) (defined as the ratio between ethanol concentration (g/L) and fermentation time (h)).

2.5 Statistical analysis

The RSM for experimental design was generated and interpreted with the software Minitab 16 (Minitab Inc., State College, PA, USA). Comparisons among treatments were assessed with Mann-Whitney U tests with the software Statistica 7 (StatSoft Inc., Tulsa, OK, USA); differences were considered significant when $p < 0.05$.

2.6 Experimental design

This study had a twofold aim. On one hand, to evaluate the effect of key factors such as initial lactose concentration (L_0), T, initial pH (pH_0) and fermentation time on the efficiency of bioethanol production from CWP during VHG fermentation, employing free and immobilized cells. On the other hand, to improve immobilized fermentation yield optimizing alginate bead formation parameters to maximize ethanol production. The optimized model was validated by repeated-batch fermentations, prolonged for 288 h (6 cycles of 48 h). The whole experimentation was carried out in 100-mL Erlenmeyer flasks containing 50 mL of CWP at different lactose concentrations without any nutrient supplementation. Flasks were plugged with foam stoppers to guarantee aeration and maintained with a constant shaking at 150 rpm in an orbital shaker.

3. Results and discussion

3.1 Effect of temperature and initial pH

The effect of T and pH_0 on free and immobilized cell fermentation is shown in Table 1. Significant differences ($p < 0.05$) were observed for temperature and type of fermentation (free or immobilized cells) but not for pH_0. The highest ethanol production ($\eta > 85\%$; $W_E = 1.23$ g/(L·h)) was obtained employing free cells at 30 ºC. With immobilized cells, the performance was lower ($\eta = 75\%$; $W_E = 1.08$ g/(L·h)), working also at 30 ºC. Lactose consumption was total in all cases. The worst performance was obtained by immobilized cells due to diffusion problems, because entrapped cells have a poorer effective contact with essential nutrients in the broth (Nikolić et al., 2010). These data are in accordance with previous works, which fixed 30 ºC as optimal fermentation temperature for *K. marxianus* strains employing CWP as a substrate in ranges between 30 and 45 ºC (Gabardo et al., 2012), with a productivity of 0.96 g/(L·h) and an efficiency of 83.3%, respectively.

Table 1. Fermentation results for temperatures and pH_0 evaluation (L_0 = 120 g/L, time = 44 h)

T (ºC)	pH	Type	E_f(g/L)	ΔL (%)	$Y_{E/L}$ (g/g)	η (%)	W_E (g/(L·h))
30.0	5.5	Free	54.19±0.33	100.00±0.00	0.460±0.003	85.50±0.52	1.35±0.008
37.0	5.5	Free	48.65±2.04	99.10±1.55	0.413±0.002	76.76±3.21	1.22±0.051
30.0	6.3	Free	53.86±0.84	100.00±0.00	0.461±0.000	85.73±0.02	1.34±0.021
37.0	6.3	Free	50.79±0.62	100.00±0.00	0.432±0.005	80.21±0.98	1.27±0.015
30.0	5.5	Immobilized	48.11±0.34	100.00±0.00	0.408±0.003	75.90±0.54	1.09±0.008
37.0	5.5	Immobilized	45.33±0.92	100.00±0.00	0.385±0.008	71.51±1.45	1.03±0.021
30.0	6.3	Immobilized	47.12±0.72	99.27±1.26	0.403±0.001	74.95±0.25	1.07±0.016
37.0	6.3	Immobilized	44.68±0.41	100.00±0.00	0.380±0.003	70.55±0.64	1.01±0.009

3.2 Effect of substrate concentration

The hypertonic environment caused by excessive levels of substrate could weaken the viability and fermentation ability of yeasts (Zhang et al., 2015). Significant differences were observed on all kinetic fermentation parameters at the tested lactose dosages. As shown in Figure 1, it could be inferred that 170 g/L is the optimal substrate concentration, because at this dosage, yield efficiency (η) reached 95.5% in 48 h, with an ethanol production of 86.6 g/L and the highest productivity ($W_E = 1.80$ g/(L·h)). Ethanol concentration increased up to 91.3 g/L with an initial lactose concentration of 190 g/L, but productivity decreased to 1.40 g/(L·h) with a yield efficiency of 90%. From this point on, either ethanol production is blocked and lactose consumption is interrupted or substrate is used for other purposes, independently of the duration (fermentations were prolonged more than 96 h), resulting in a drastic decrease of productivity. This fact might be due to the severe decrease in cell membrane fluidity for long-exposure to hypertonic solutions, mass transfer becoming difficult (Thomas and Ingledew, 1992). Therefore, additional carbon sources are consumed by the yeast to maintain the activity of the transport system of essential materials instead of being fermented to produce ethanol (Zhang et al., 2015). Therefore, employing a CWP with 170 g/L lactose, commonly produced by dairy industry, is very convenient for the economy of ethanol industry, taking the advantages of VHG fermentation (Puligundla et al., 2011) without wasting resources.

To the best of our knowledge, this is the first time that *K. marxianus* is described to work with such a high lactose concentration. Many authors have reported inhibitory problems working with substrate concentrations higher than 100 g/L (Dragone et al., 2011; Gabardo et al., 2014; Ozmihci and Kargi, 2007, 2009). This study concludes that *K. marxianus* DSM 5422 did not suffer important substrate inhibition below 230 g/L lactose. However, at lactose concentrations higher than 190 g/L, worse sugar exploitation was obtained with a drastic reduction of ethanol productivity, so its use would not be feasible.

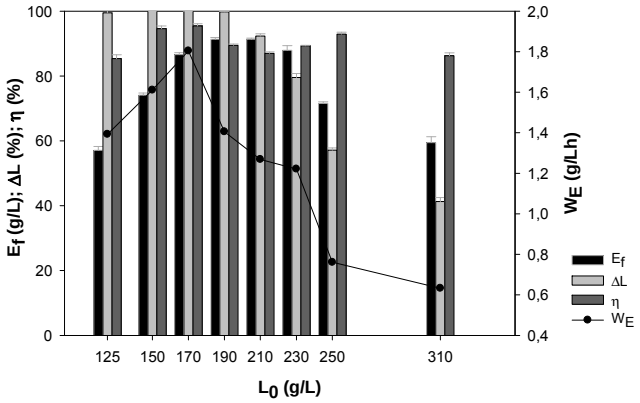

Figure 1: Effect of CWP lactose content on E_f, ΔL, η and W_E variables.

3.3 Response surface (RSM) analysis for the optimization of immobilization parameters

A central composite design (CCD) of three factors was used (X_1: Alg, %; X_2: Cell, %; X_3: D_p, mm). The design had 20 experiments and included 8 cube points, 6 axial points and 6 central point replications (α = 1.68). Final ethanol concentration (E_f, g/L) and lactose consumption rate (ΔL; %) were selected as response variables. Fermentation conditions were established at L_0 = 170 g/L; T = 30 ºC; pH_0 = 6.3. In all cases 10 g of cell immobilized beads were employed per 50 mL of fermentation medium. Table 2 shows randomized experimental conditions and observed responses (E_f and ΔL). The RSM showed that the studied parameters affected significantly both responses. The empirical models in terms of uncoded factors for E_f and ΔL are given in Eq (1) and (2), respectively (Note: Significant coefficients are marked with an asterisk).

$$E_f = 82.01 - 1.51x_1^* + 1.26x_2^* - 5.15x_3^* + 0.41x_1^{2*} + 0.06x_2^{2*} + 0.24x_3^{2*} + 0.06x_{12}^* - 0.05x_{23} - 0.21x_{13} \quad (1)$$

$$\Delta L = 107.17 - 1.18x_1^* + 2.77x_2^* - 4.37x_3^* + 0.20x_1^{2*} - 0.04x_2^{2*} + 0.58x_3^{2*} - 0.02x_{12}^* - 0.17x_{23} - 0.57x_{13} \quad (2)$$

Table 2. Experimental central composite design (CCD) runs and responses

	Independent factors			Responses			Independent factors			Responses	
Run	X_1	X_2	X_3	E_f (g/L)	ΔL (%)	Run	X_1	X_2	X_3	E_f (g/L)	ΔL (%)
1	3.50	2.00	3.75	68.10	97.11	11	3.50	2.00	4.84	66.43	95.25
2	5.00	1.00	3.10	68.16	96.48	12	5.00	1.00	4.40	67.28	95.00
3	3.50	2.00	3.75	68.01	96.60	13	5.00	3.00	3.10	70.16	98.82
4	5.00	3.00	4.40	67.18	95.46	14	2.00	3.00	4.40	68.22	97.09
5	3.50	3.68	3.75	68.97	97.93	15	3.50	2.00	3.75	68.32	97.04
6	2.00	3.00	3.10	69.71	99.41	16	6.02	2.00	3.75	67.46	96.25
7	3.50	0.32	3.75	67.16	95.45	17	2.00	1.00	4.40	67.29	96.14
8	0.98	2.00	3.75	70.66	99.40	18	3.50	2.00	3.75	67.93	95.55
9	3.50	2.00	3.75	67.5	96.69	19	2.00	1.00	3.10	69.23	97.37
10	3.50	2.00	2.66	70.73	99.31	20	3.50	2.00	3.75	66.70	95.23

From an ANOVA analysis (Table 3), it was inferred that predicted models were adequate for both responses. However, the E_f model predictability could be questioned (pred-R^2 = 5.99%). Therefore, reduced models were proposed to try to improve model predictability and obtain quadratic model significance. The reduced quadratic models for E_f and ΔL responses are shown in Eq(3) and Eq(4), respectively.

$$E_f = 82.01 - 1.61x_1^* + 0.46x_2^* - 5.87x_3^* + 0.18x_1^{2*} + 0.56x_3^2 \quad (3)$$

$$\Delta L = 113.49 - 1.83x_1^* + 0.73x_2^* - 6.03x_3^* + 0.19x_1^{2*} + 0.58x_3^2 \quad (4)$$

In the reduced models, regression, linear and quadratic significance were obtained (Table 3). For both responses, the F-value increased after eliminating not significant terms, p-value for lack of fit was also greater, improving accuracy. R^2 and R^2_{adj} were slightly affected; however, model predictability was heavily improved.

Table 3. Analysis of variance- ANOVA- of the predicted models for E_f and (ΔL) responses

	E_f Model		ΔL Model		E_f reduced Model		ΔL reduced Model	
Source	F	p-value	F	p-value	F	p-value	F	p-value
Regression	5.67	0.006	9.41	0.001	12.89	0.000	17.28	0.000
Linear	14.71	0.001	24.48	0.000	18.89	0.000	25.94	0.000
Square	2.06	0.170	2.71	0.101	3.89	0.045	4.28	0.035
Interaction	0.26	0.851	1.05	0.412				
Lack of fit	1.92	0.426	0.35	0.864	1.21	0.438	0.43	0.869
R^2 (%)	83.6		89.4		82.1		86.0	
R^2_{adj} (%)	68.9		79.9		75.8		81.1	
pred- R^2	6.0		67.9		51.3		73.1	

Optimum conditions were mathematically determined through the maximization of both responses. At the optimal point (X_1 = 0.98%; X_2 = 3.68%; X_3 = 2.66 mm), E_f achieved 71.22 g/L with a yield efficiency of 80% and total lactose consumption. Smaller beads yielded a better performance, due to an increase in the surface-volume ratio. The obtained values of alginate about 1% are in accordance with reported studies immobilizing *S. cerevisiae* (Idris and Suzana, 2006). However, these authors pointed out that bead alginate concentrations lower than 2% are highly susceptible to compaction and disintegration during the process in bioreactor systems due to the internal mechanical loading on the beads. As it was confirmed experimentally, the use of alginate concentration above 2% generates an ethanol decrease probably due to the lower diffusion efficiency of the beads. Therefore, a concentration of 2% of sodium alginate will be considered as optimal. An improvement of 5% in efficiency was obtained with the optimization versus the results obtained in Section 3.1 (Table 2. Run 5).

3.4 Repeated batch fermentation

The optimal immobilized model (%Alg: 2; % Cell: 3.68; D_p: 2.66 mm) was validated by recycling the gel beads for 6 cycles of 48 h, employing non-supplemented CWP (L_0 = 170 g/L) as a substrate to assess continuous fed-batch fermentation performance. The evolution of fermentation kinetic parameters is shown in Figure 2.

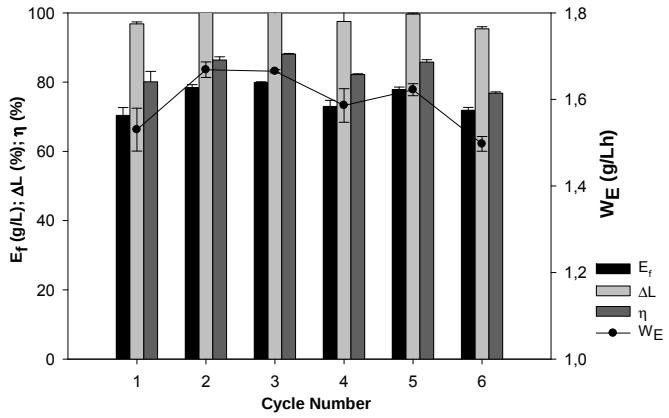

Figure 2. Kinetic fermentation parmeters (E_f, ΔL, η and W_E) at the end of each cycle.

In the first cycle, final ethanol concentration, efficiency and productivity were lower than in the next cycles. This could be due to the need of cells to adapt to the immobilization stress (Duarte et al., 2013). During the 288 h of experimentation, there was a mean ethanol production of 83.2 g/L, with a productivity of 1.6 g/(L·h) and a yield efficiency of 83.2%. A stable ethanol production can be observed throughout the cycles with a very good performance without contamination; therefore, these immobilization technique could be employed in continuous fermentation configurations with tubular bioreactors. A minimum degree of compaction was observed, but beads did not experience disaggregation during operation. When the beads were created (t = 0) they had a spherical shape (r = 1.30 mm, V = 9.20 mm^3) and contained $1.99 \cdot 10^3$ cell/mm^3. After six batch cycles the amount of yeast cells inside the beads was only slightly lower ($1.16 \cdot 10^3$ cell/mm^3). In addition, at the end of every cycle cells were also detected in the CWP outside the bead. For instance, after the sixth cycle, the yeast concentration in CWP was $9.48 \cdot 10^4$ cell/mm^3. The average final volume of each bead was 6.39 mm^3 and they had an ellipsoidal shape (a = b = 1.25 mm, c = 0.98 mm).

4. Conclusions

High lactose-loaded cheese whey permeate is an excellent low cost medium for alcoholic fermentation employing *Kluyveromyces marxianus*. The CWP did not need nutrient supplementation to maximize ethanol production, which improves significantly the economic feasibility of the process. The combination of VHG fermentation with cell entrapment immobilization turned out to be a very promising approach to ethanol production: very low substrate cost, high ethanol efficiency, high productivity, total substrate consumption, and great stability throughout time without contamination. Experiments were performed under a fed-batch configuration at laboratory scale which should be scaled up in continuous bioreactors in pilot plants.

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