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Effect of Nutrient Supplementation on Biobutanol Production from Cheese Whey by ABE (Acetone–Butanol–Ethanol) Fermentation

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Cheese whey is a liquid effluent obtained from the cheese manufacturing process, presenting a high volumetric production and high organic loads related to its lactose content. The aim of this research was to increase the productivity of ABE fermentation with the bacterial strain *Clostridium beijerinckii* CECT 508, when using nanofiltered sheep cheese whey as a substrate. Thermal sterilization of the whey was essential to avoid the proliferation of lactic acid bacteria. An experimental design including Plackett-Burman and Response Surface Methodology (RSM) was applied to figure out which additional nutrients were necessary for the fermentation process and their optimum concentrations. For this specific whey, it was established that 1 g/L yeast extract, 5 g/L $CaCO_3$, 0.019 g/L $FeSO_4 \cdot 7H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 2.1 g/L NH_4Cl needed to be added. Under optimal nutrient conditions, the highest butanol production (9.11 g/L) was obtained with an initial lactose concentration of 57 g/L; achieving 49% lactose consumption and 0.311 g/g $Y_{B/L}$ yield. However, the best lactose consumption rate (87%) was recorded for lactose initial concentrations of 30 g/L, which also allowed satisfactory fermentation values (8.51 g/L butanol and 0.328 g/g $Y_{B/L}$ yield). On the other hand, the best $Y_{B/L}$ yields (0.428 g/g; which is close to the theoretical value) were obtained for initial lactose concentrations of 40 g/L, attaining a value of 52% lactose consumption and 8.91 g/L butanol. Therefore, the ABE fermentation could be a feasible solution to treat cheese whey and remove lactose, obtaining acceptable amounts of butanol.

1. Introduction

Biocatalytic processes are presented as a real alternative to face new social challenges of a post-oil era. Since the 1980s, there has been an ongoing search for alternative fuels to be used either directly or mixed with fossil fuels. Interest in butanol production is increasing (Meesukanun and Satirapipathkul, 2014), because this alcohol presents higher energy content, lower volatility, lower hygroscopicity and it is less corrosive than ethanol. Additionally, butanol is an important chemical compound with many applications, like the production of solvents, resins and plasticisers.

The Acetone-Butanol-Ethanol (ABE) fermentation with *Clostridium* strains was used worldwide at industrial scale for the production of acetone and butanol from the early twentieth century until the 1960s (Jones and Woods, 1986), but this fermentation was replaced by petrochemical processes with higher profitability. The major factors that compromised ABE fermentation economic feasibility included: the high cost of traditional substrates (corn, molasses, etc.), low concentration of butanol in fermented broth (associated with strong product inhibition affronted by microorganisms) (Ezeji et al., 2004), low butanol yield, low volumetric productivity with typical values lower than 0.5 g/(L·h) (Ezeji et al., 2013) and, finally, the high cost of solvent recovery processes, not only due to the diversity of solvents produced, but also to the high boiling point of butanol (118 °C) (Qi et al., 2014). Under batch conditions, ABE fermentation takes place in two main phases:

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a first phase called acidogenesis, associated with rapid cell growth and secretion of carboxylic acids (acetate and butyrate) and a mixture of H_2 and CO_2 as gaseous substances, and a second phase involving the conversion of acid intermediates into solvents, called solventogenesis.

With the aim of reducing fermentation costs, the utilization of an agro-industrial waste with high polluting potential is of great relevance (Pisano et al., 2015). An interesting substrate for ABE fermentation is whey. This liquid effluent obtained from the cheese manufacturing process presents high volumetric production and high organic loads. Typical chemical and biological oxygen demand (COD and BOD) values are 50–102 kg/m^3 and 27–60 kg/m^3, respectively (Ergüder et al., 2001). This high organic load is mainly due to its lactose content (48–60 g/L), which may be too low for most industrial fermentation processes, but results optimal in the case of butanol production. Cheese whey is also characterised by high protein content in the range of 33–67 g/L and fat content in the range of 8–10 g/L, which also contributes to its high organic load.

ABE fermentation has been extensively studied as an alternative for whey valorization. Most of these studies have been conducted in batch conditions resulting in higher acetone/butanol/ethanol production ratios than those typically obtained when fermenting carbohydrate substrates, such as starch or molasses (3:6:1). Reported solvent concentrations range between 5–15 g/L, with typical butanol productivities and yields ranging from 0.1–0.3 g/(L·h) and 0.23–0.40 $g_{solvents}/g_{substrate}$ (Alam et al., 1988; Napoli et al., 2010; Welsh and Veliky, 1984), respectively.

The objective of this work was to improve lactose transformation in ABE fermentation of nanofiltered sheep cheese whey. In the first place, the composition of the fermentation medium (including the necessity of nutrient addition) was optimised for a maximal butanol production. Secondly, the effect of initial lactose concentration was studied in order to increase the fermentation yield and favour lactose depletion, thus reducing the polluting potential of the resulting effluent.

2. Material and methods

2.1 Microorganisms and culture conditions

The strain used was *Clostridium beijerinckii* CECT 508 (NCIMB 8052), provided by the Spanish Collection of Type Strains (CECT, Paterna, Spain). The lyophilised cells were resuspended in synthetic medium consisting of 19 g/L Reinforced Clostridial Medium (Oxoid, Basingstoke, UK) and 10 g/L lactose (Sigma-Aldrich, Steinheim, Germany) and exposed to a thermal shock (2 min at 80 ºC in a water bath and 5 min in ice). They were subjected to sporulation according to the CECT protocol. Then, 500 µl spores were added to 100 mL of the above-mentioned synthetic medium, which was placed in glass bottles capped with a rubber septum, and exposed to a thermal shock. Afterwards, gaseous N_2 was bubbled into the headspace of the closed bottles during 5 min to obtain anaerobic conditions. The bottles were incubated for 20 h at 35 °C and were employed as inocula, containing an approximate bacterial density of $6 \cdot 10^8$ cells/mL.

2.2 Cheese whey and fermentation media

All fermentations were performed with sheep cheese whey provided by the cheese factory Quesería Artesanal del Río Carrión S.L. (La Serna, Palencia, Spain). This whey was nanofiltered, resulting in an increase in the concentrations of its components (Table 1). Whey ultrafiltration or nanofiltration is sometimes performed at the cheese producer's facilities in order to reduce transport costs if the whey is to be treated at a different locality. In the present work, the use of nanofiltered whey allowed for the possibility of making dilutions to obtain different lactose concentrations for the experiments.

Table 1: Composition of the nanofiltered sheep cheese whey employed in the experiments

	Concentration		Concentration		Concentration
Lactose (g/L)	137	Mg (mg/L)	207	Cl^- (mg/L)	1181
Proteins (g/L)	5.5	Cu (mg/L)	<1.0	Br^- (mg/L)	33
Fats (%)	< 0.05	Fe (mg/L)	<5	NO_2^- (mg/L)	<1
K (mg/L)	2524	Mn (mg/L)	<8	NO_3^- (mg/L)	<1
Na (mg/L)	751	Zn (mg/L)	<1	SO_4^{2-} (mg/L)	222
Ca (mg/L)	193	F^- (mg/L)	139	PO_4^{3-} (mg/L)	1907

Previous works indicate that ABE fermentation is properly developed up to lactose levels of 50-60 g/L (Qureshi and Maddox, 2005). Therefore, in the present work the nanofiltered whey was diluted with distilled water in order to obtain initial lactose concentrations which were more suitable for the development of *C. beijerinckii*. An adequate balance of organic and inorganic nutrients is required for growth and solvent production by *Clostridium* sp. Current studies confirm the fact that nutrient supplementation, particularly the addition of yeast extract, is essential for solvent production (Ezeji et al., 2013). In the present work, the need

for addition of supplementary nutrients was studied with the following substances: yeast extract (Fluka, Buchs, Switzerland) as vitamin source, KH_2PO_4 and $KHPO_4$ (Sigma-Aldrich) as phosphorus source, NH_4Cl (Panreac, Castellar del Vallés, Spain) as nitrogen source, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich) as mineral sources, $CaCO_3$ (Sigma-Aldrich) as pH buffer, and cysteine (Sigma-Aldrich) as reducing agent.
Therefore, fermentation media used in this study consisted on cheese whey supplemented with one or more of the above mentioned substances at different concentrations. For fermentation experiments, 3 mL of inoculum were added to 97 mL of fermentation medium in rubber-capped bottles. The initial pH was adjusted to 6.0 with NaOH. Gaseous N_2 was bubbled into the bottom of the closed bottles during 5 min. Fermentation bottles were incubated at 35 ºC and 100 rpm in an Infors HT Minitron orbital shaker (Infors AG, Bottmingen, Switzerland) during 92 h. All experiments were performed in triplicate.
Fermentation media (before inoculation), bottles, control instruments and any material in contact with microorganisms susceptible to contamination were autoclaved for 15 min at 121 °C. The only exceptions were $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and cysteine, which were added to the fermentation media as solutions filtered through syringe nylon filters with 0.20 µm pore size and 25 mm diameter (Auxilab SL, Beriáin, Spain). It was experimentally observed that cheese whey must be autoclaved before the inoculation of *C. beijerinckii*, regardless whether the whey has been previously pasteurised or nanofiltered, since lactic acid bacteria can survive these latter processes and inhibit ABE fermentation.

2.3 Analytical methods

Periodic samples were collected from the fermentation bottles using aseptic techniques to determine lactose and metabolite concentration. Samples were centrifuged at 5,000 rpm in a refrigerated microcentrifuge for 5 min. The supernatant was filtered through a 0.20 µm filter and analysed by high performance liquid chromatography (HPLC) and gas chromatography (GC). Acetone, butanol, ethanol, acetic acid and butyric acid were determined by GC using an Agilent 7890 GC equipped with a flame ionization detector (FID) and using a column HP Innowax 30 m x 0.530 mm, 1.00 µm (Agilent Technologies, Santa Clara, CA, USA). Lactose and lactic acid were determined by an Agilent LC1200 HPLC equipment with a refractive index detector and an Aminex HPX-87-H column (Bio-Rad, Hercules, California, USA), where the mobile phase was 5 mM H_2SO_4.
Fermentation yields and productivity were calculated at the end of each run. Fermentation yield ($Y_{i/L}$, g/g) was calculated as the ratio between the metabolite (i) produced and lactose consumed. The metabolite productivity rate (W_i, g/(L·h)) was calculated as the ratio of metabolite (i) expressed in concentration (g/L) and the time of fermentation (h).

2.4 Statistical analyses

For the optimization step, experimental designs, such as Plackett-Burman and Response Surface Methodology (RSM) were generated and interpreted with the software Minitab 16 (Minitab Inc., State College, PA, USA). Comparisons among treatments were assessed with a one-way ANOVA and the Tukey HSD test using the software Statistica 7 (StatSoft Inc., Tulsa, OK, USA); differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1 Optimization of nutrient supplements in cheese whey

In the first place, it was necessary to figure out which additional nutrients were essential for a correct ABE fermentation with cheese whey. It must be noted that the optimal nutrient content and fermentation parameters depend on the specific cheese whey used, since whey chemical composition varies among cheese factories. In order to select those relevant compounds, a Plackett-Burman experimental design was performed with the independent variables and value ranges shown in Table 2. Twelve experiments were performed in which the composition of the fermentation media was different in each run and was a combination of the conditions given in Table 2. The variable *Initial lactose in whey* refers to the concentration of lactose in whey after diluting with water (since nanofiltered whey had a too high lactose content). The variable *Added lactose* represents the addition of commercial lactose to the fermentation media. The response variables (i.e. the dependent variables) analysed in every experiment were butanol concentration (g/L), $Y_{B/L}$ (g/g) and consumed lactose (%), after 92 h of fermentation.

Table 2: Independent variables included in the Plackett-Burman experimental design with their respective minimum and maximum values, and effects of each independent variable on the three response variables. Note: Asterisks indicate a significant effect (p < 0.05)

Independent variables		Response variables (dependent variables). Effects.		
Name	Range	Butanol (g/L)	$Y_{B/L}$ (g/g)	Consumed lactose (%)
Yeast extract (g/L)	1-5	-0.302	-0.0057	1.903
$FeSO_4 \cdot 7H_2O$ (g/L)	0-0.01	2.908 *	0.0509 *	12.32
$MgSO_4 \cdot 7H_2O$ (g/L)	0-0.2	0.712	0.0156	7.717
Cysteine (g/L)	0-0.5	-1.038	-0.0207	-6.893
NH_4Cl (g/L)	0-2.1	0.485	0.0102	10.29
K_2HPO_4 (g/L)	0-1	-0.525	-0.0085	0.300
KH_2PO_4 (g/L)	0-1	-2.158	-0.0388	-4.123
$CaCO_3$ (g/L)	1-8	1.348	0.0216	11.33
Initial lactose in whey (g/L)	38-54	-0.118	-0.0103	-5.34
Added lactose (g/L)	0-15	0.742	0.0059	-3.68
Temperature (ºC)	30-37	0.265	0.0089	-0.617

The results for butanol concentration and $Y_{B/L}$ showed parallelism. There were six independent variables with a positive effect on the response variables ($FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, NH_4Cl, $CaCO_3$, added lactose and temperature). However, the only nutrient with a significant ($p < 0.05$) effect on butanol concentration and $Y_{B/L}$ was $FeSO_4 \cdot 7H_2O$ (Table 2). All those nutrients with a negative effect (yeast extract, cysteine, K_2HPO_4, KH_2PO_4) were considered to be detrimental for the ABE process, and they were therefore set at the lower values of their ranges for the subsequent experiments. The effects on the variable *Consumed lactose* were slightly different, since this variable is more dependent on the initial lactose concentration.
The following step consisted on a RSM experiment where only three variables were optimized, whereas the others were kept at fixed values, according to Table 3.

Table 3: Conditions of the RSM experiment. In the case of the RSM variables, axial values are indicated

RSM variables		Fixed parameters			
$CaCO_3$ (g/L)	0-10	Yeast extract (g/L)	1	K_2HPO_4 (g/L)	0
Initial lactose (g/L)	42-70	$MgSO_4 \cdot 7H_2O$ (g/L)	0.2	KH_2PO_4 (g/L)	0
$FeSO_4 \cdot 7H_2O$ (g/L)	0.008-0.03	NH_4Cl (g/L)	2.1	Cysteine (g/L)	0

The number of variables to be optimized was reduced to three in order to simplify the process, and they were chosen according to the sign and value of their effects in the Plackett-Burman test. The RSM design had 20 experiments and included 8 cube points, 6 central points and 6 axial points (α=1.68179). The initial lactose concentration in each experiment was obtained by diluting the nanofiltered whey with distilled water. The fermentation was performed at 35ºC during 92 h, and the studied responses were butanol concentration and $Y_{B/L}$. It was decided to prioritize butanol concentration and, according to the RSM mathematical estimations, an optimal of 10.09 g/L butanol would be theoretically obtained when 5.35 g/L $CaCO_3$, 56.7 g/L initial lactose and 0.019 g/L $FeSO_4 \cdot 7H_2O$ are used. Contour plots can be seen in Figure 1.

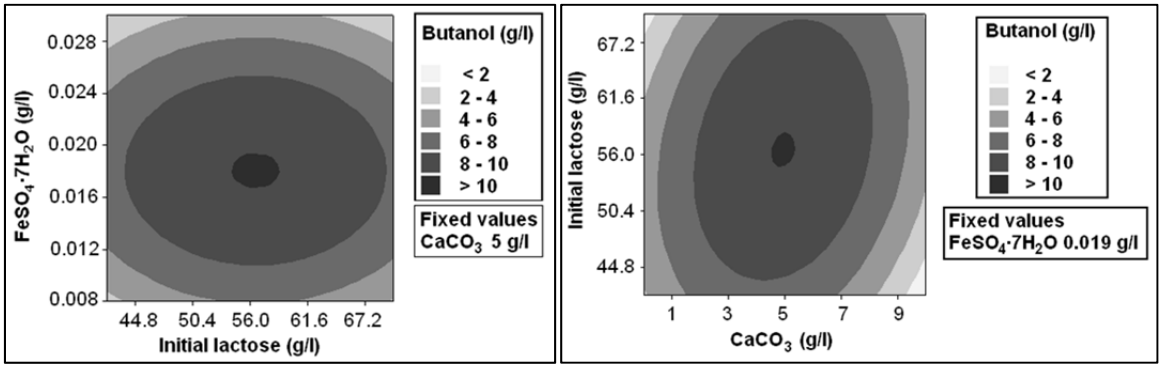

Figure 1: Contour plots for butanol production in the RSM experimental design.

Therefore, the final composition of the whey was established as follows: an initial concentration of lactose at 57 g/L (obtained by dilution with water), and the addition of 1 g/L yeast extract, 5 g/L $CaCO_3$, 0.019 g/L $FeSO_4 \cdot 7H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 2.1 g/L NH_4Cl.
To validate the RSM model, a fermentation was performed in triplicate at 35 ºC and using the optimized composition of the whey (lactose initial concentration and nutrient addition). After 92 h of fermentation, 9.11±0.66 g/L butanol, 1.44±0.24 g/L acetone, 0.13±0.01 g/L ethanol, 1.47±0.14 g/L acetic acid and 1.12±0.06 g/L butyric acid were recorded. The consumption of lactose attained 48.5±4.2 %, the $Y_{B/L}$ yield was 0.3105±0.0081 g/g and the productivity rate W_B was 0.0979±0.0071 g/(L·h). Concentrations of butanol and ABE solvents ranging from 5.2 to 8.1 g/L and 8.5 to 11.3 g/L, respectively, have been reported by different authors (Ennis and Maddox, 1985; Qureshi and Blaschek, 2001; Qureshi and Maddox, 2005) under batch conditions at 50 g/L of lactose and using *C. acetobutylicum*.

3.2 Effect of initial lactose concentration on $Y_{B/L}$ yield and lactose consumption

Although butanol concentrations were satisfactory when using the optimized supplemented whey described in Section 3.1, lactose consumption was not very high. Depending on the particular circumstances and the industrial objectives, and given the polluting nature of cheese whey (especially regarding COD), it could be desirable to maximize lactose consumption by microorganisms instead of prioritizing high butanol productions. This can be made by reducing initial lactose concentrations; however, too low lactose concentrations hinder the solventogenesis phase (Welsh and Veliky, 1986).
In order to find a compromise between lactose consumption and the feasibility of ABE fermentation, four different initial lactose concentrations were tested (30 g/L, 40 g/L, 50 g/L and 60 g/L, obtained by dilution of the nanofiltered whey), maintaining the addition of supplementary nutrients established in Section 3.1. (1 g/L yeast extract, 5 g/L $CaCO_3$, 0.019 g/L $FeSO_4 \cdot 7H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 2.1 g/L NH_4Cl). The samples were fermented during 92 h, providing the results shown in Table 4.

Table 4: Fermentation results for different initial lactose concentrations. Note: For the dependent variables marked with an asterisk (*) some letters are given between brackets, which are related to the results of the ANOVA. If two treatments share one of the letters, there are no significant differences between treatments (i.e. between initial lactose concentrations); if they do not have any letter in common, there are significant differences ($p < 0.05$*) between treatments. In the case of those dependent variables without an asterisk, no significant differences appeared between treatments*

	Initial lactose concentration (g/L)			
Dependent variables	30	40	50	60
Acetone (g/L)	1.01 ± 0.22	1.19 ± 0.13	1.27 ± 0.33	1.69 ± 0.33
Butanol (g/L)	8.51 ± 0.17	8.91 ± 0.48	8.47 ± 1.21	9.15 ± 2.32
Ethanol (g/L)	0.12 ± 0.00	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.03
Acetic acid (g/L) *	0.69 ± 0.06 (a)	1.15 ± 0.09 (b)	1.24 ± 0.18 (b)	1.64 ± 0.22 (c)
Butyric acid (g/L)	1.44 ± 0.34	1.01 ± 0.15	0.98 ± 0.15	1.28 ± 0.23
Lactose consumption (%) *	86.8 ± 5.4 (a)	52.2 ± 4.1 (b)	52.0 ± 9.0 (b)	50.7 ± 12.8 (b)
$Y_{B/L}$ (g/g) *	0.328 ± 0.018 (a)	0.428 ± 0.019 (b)	0.327 ± 0.011 (a)	0.301 ± 0.006 (a)
W_B [g/(L·h)]	0.093 ± 0.002	0.097 ± 0.005	0.092 ± 0.013	0.099 ± 0.025

Lactose consumption was significantly larger (86.8%) when the initial lactose concentration was at its lowest level (30 g/L); whereas the best $Y_{B/L}$ yields were obtained for initial lactose concentrations of 40 g/L, attaining a value of 0.428 g/g, which is considerably high, since the theoretical $Y_{B/L}$ value is 0.41 g/g for lactose (Napoli, 2009). As Madihah et al. (2001) concluded, limited nitrogen concentrations or low carbon/nitrogen ratios result in better yields and productivities. A higher lactose content in the medium increases fermentation time and reduces lactose exploitation, therefore reducing solvent product rates. In addition, an increase in the production of acetic acid was observed with the increase in the initial value of lactose concentration (Table 4). The production of metabolites (acetone, butanol, ethanol, acetic acid and butyric acid) is directly related to lactose utilisation due to the stoichiometry of the reaction. Initial lactose concentrations also affected A:B:E ratios. Hence, the following ratios were calculated: 8:71:1 for 30 g/L, 9:66:1 for 40 g/L, 10:64:1 for 50 g/L and 12:64:1 for 60 g/L. These values are similar to those reported in literature for whey but vary significantly from those traditionally reported when using glucose as a substrate (ABE ratio of 6:3:1) (Qureshi and Blaschek, 2001). In fact, Bahl et al. (1986) also obtained variations in these ratios by altering nutritional factors affecting growth conditions.

4. Conclusions

Nutrient addition and optimization is essential when subjecting cheese whey to ABE fermentation. This optimization must be performed with each type of whey, since its composition and fermentation suitability differ depending on the cheese manufacturer. Experimental design methodologies, like Plackett-Burman and RSM, can simplify the optimization process and provide reliable results. In addition, lactose consumption and $Y_{B/L}$ yield can be maximized and yet without a loss in butanol concentration.

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