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Iron effect on the fermentative metabolism of *Clostridium acetobutylicum* ATCC 824 using cheese whey as substrate

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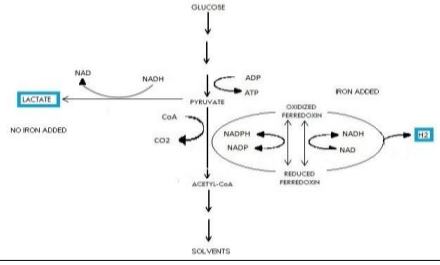
HIGHLIGHTS

- *Addition of iron was found essential to make cheese whey suitable for ABE fermentation.*
- *Lack of ferredoxin led to lactic acid production instead of the desired solvents.*
- *Addition of $FeSO_4$ improved butanol production by 65% compared to $FeCl_3$.*

GRAPHICAL ABSTRACT



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ABSTRACT

Butanol is considered a superior liquid fuel that can replace gasoline in internal combustion engines. It is produced by acetone-butanol-ethanol (ABE) fermentation using various species of solventogenic clostridia. Performance of ABE fermentation process is severely limited mostly by high cost of substrate, substrate inhibition and low solvent tolerance; leading to low product concentrations, low productivity, low yield, and difficulty in controlling culture metabolism. In order to decrease the cost per substrate and exploit a waste generated by dairy industry, this study proposes using cheese whey as substrate for ABE fermentation. It was observed that the addition of an iron source was strictly necessary for the cheese whey to be a viable substrate because this metal is needed to produce ferredoxin, a key protein in the fermentative metabolism of *Clostridium acetobutylicum* serving as a temporary electron acceptor. Lack of iron in the cheese whey impedes ferredoxin synthesis and therefore, restricts pyruvate-ferredoxin oxidoreductase activity leading to the production of lactic acid instead of acetone, butanol and ethanol. Moreover, the addition of $FeSO_4$ notably improved ABE production performance by increasing butanol content (7.13 ± 1.53 g/L) by 65% compared to that of $FeCl_3$ (4.32 ± 0.94 g/L) under the same fermentation conditions.

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1. Introduction

Butanol is an important industrial chemical considered as a superior liquid fuel with a potential to replace gasoline (Jang et al., 2012). It can be produced from petroleum, mineral fuel and biomass; the latter is conveniently denoted as biobutanol although it has the same characteristics as butanol from petroleum (Shapovalov and Ashkinazi, 2008). Compared to ethanol, the traditional biofuel, butanol has the following advantages: a) higher energy content (29.2 MJ/L, very similar to that of gasoline: 32 MJ/L), b) vapor pressure 11 times lower than that of ethanol, and is therefore safer to use (Rajchenberg-Ceceña et al., 2009), c) non hygroscopic, d) less corrosive, e) can be applied in pure form or blended in any proportions with gasoline or diesel, f) can be used in any automobile engine without modifications, g) easy to preserve and distribute, h) can be used with the existing infrastructure (Rajchenberg-Ceceña et al., 2009; Tashiro and Sonomoto, 2010), i) heat of vaporization (0.43 MJ/Kg) slightly higher than that of gasoline (0.36 MJ/Kg) avoiding cold start problems (Ranjan and Moholkar, 2009), and j) at combustion, does not produce sulfur and nitrogen oxides, advantageous from the environmental viewpoint (Shapovalov and Ashkinazi, 2008). Furthermore, it can be converted to valuable chemical compounds: acrylate, methacrylate esters, glycol ethers, butyl acetate, and *etc*. (Tashiro and Sonomoto, 2010).

Butanol has traditionally been produced by acetone-butanol-ethanol (ABE) fermentation of sugar substrates using various species of solventogenic clostridia. The fermentation occurs in two stages; the first is a growth stage in which acetic and butyric acids are produced and the second stage is characterized by acid re-assimilation into ABE solvents. During this stage, growth slows down, the cells accumulate granulose and form endospores (Green, 2011). The regular ratio of ABE solvents produced by *C. acetobutylicum* is 3:6:1 with 20 g/L being the maximum concentration achieved so far (Ranjan and Moholkar, 2009). Performance of butanol fermentation process using solventogenic clostridia is severely limited by: a) high substrate costs, b) substrate inhibition, c) low solvent tolerance (max 20 g/L of solvent), d) sluggish growth, and e) low cell density achievable during solventogenic fermentation (Jang et al., 2012). These limitations result in low final butanol concentration caused by butanol inhibition, low butanol productivity due to low cell density, low yield due to hetero-fermentation and high downstream processing cost for butanol recovery (Tashiro and Sonomoto, 2010). Unless these limitations are addressed, biological production of butanol cannot compete economically with petrochemical synthesis of these solvents (Gheshlaghi et al., 2009; Tashiro and Sonomoto, 2010; Cooksley et al., 2012; Sabra et al., 2014). Researchers have tried to solve these problems thorough various studies including: a) microbial strain development for improved butanol titer and tolerance, b) development of efficient *in situ* product recovery technologies to overcome the butanol toxicity to fermenting microorganisms, c) application of fermentation strategies to increase cell density, butanol titer, yield and productivity, and d) exploration of more economic alternative substrates (Jang et al., 2012).

The conventional ABE fermentation substrates include starch and molasses from maize, wheat, and rye. In order to reduce the cost of the product, a variety of more economic substrates have been tested including: sucrose (Parekh and Blaschek, 1999; Tashiro and Sonomoto, 2010), lignocellulosic biomass (Ranjan and Moholkar, 2009; Jang et al., 2012), domestic, agricultural and industrial waste (Jang et al., 2012; Niemistö et al., 2013), glycerol (exclusively in fermentations with *C. pasteurianum*) (Dabrock et al., 1992; Tashiro and Sonomoto, 2010; Jang et al., 2012; Sabra et al., 2014), algal biomass (Ranjan and Moholkar, 2009; Jang et al., 2012), wheat straw, corn fiber, liquefied corn starch, apple pomace, Jerusalem artichokes (Ranjan and Moholkar, 2009), cheese whey (Maddox, 1980; Bahl et al., 1986; Ennis and Maddox, 1989; Ranjan and Moholkar, 2009), among others.

Solventogenic *Clostridium* species are capable of fermenting a wide range of carbohydrates; lignocellulosic biomass has been identified as a potential substrate for inexpensive production of ABE and other fine chemicals, however, bioconversion of lignocellulosic biomass is currently plagued by a number of limitations, notably generation of microbial inhibitory compounds during pretreatment and hydrolysis of lignocellulose to mixed sugars, and inefficient utilization of the generated mixed sugars by fermenting microorganisms due to carbon catabolite repression (Ezeji and Blaschek, 2008), therefore, other economic and readily utilizable substrates, whose applications in fermentation do not require pretreatment, may prove to be a more cost-effective and efficient substrates than lignocelluloses.

Among the non-lignocellulosic substrates, cheese whey is the most widely researched for ABE production, mostly due to its abundance and high biological oxygen demand (BOD), which constitutes a major disposal problem to the dairy industry. Because of its relatively low sugar content (4-5% lactose) this waste is unsuitable for many fermentation processes without prior concentration, while considered satisfactory for the butanol fermentation where product toxicity limits the amount of sugar utilization (Maddox, 1980). All over the world cheese production and consumption has increased rapidly generating more wastes and consequently pollution problems, and this has increased the interest to use it for the production of fuels (Foda et al., 2010).

Previously published studies (Maddox, 1980; Bahl et al., 1986; Foda et al., 2010) characterize whey permeate as a substrate which can be used for ABE fermentation. In fact, Maddox (1980) and Bahl et al. (1986) described that lactose metabolism favors butanol production over acetone, adding economic incentives for butanol production. Other authors studied the specific effect of nutrient supplementation or limitation on the fermentative activity of Clostridia, including: iron (Bahl et al., 1986; Dabrock et al., 1992; Peguin and Soucaille, 1995; Vasileva et al., 2012), phosphate (Bahl et al., 1986; Dabrock et al., 1992), CO (Dabrock et al., 1992), ferredoxin substitutes (Peguin and Soucaille, 1995), flavonoids (Wang et al., 2014), reducing cofactors (Li et al., 2014), inter alia. However, fermentative conditions and nutritional requirements for optimal use of cheese whey have not been fully elucidated and further work is required.

Trying to recycle wastes of the local dairy industry, this study investigated the suitability of using cheese whey as fermentation broth for ABE production emphasizing on covering the nutritional requirements of *C. acetobutylicum*, especially iron supplementation (no iron, $FeSO_4$ and $FeCl_3$), for routing metabolism toward the production of the desired solvents.

2. Material and method

2.1. Strains and culture maintenance

C. acetobutylicum ATCC 824 was grown anaerobically at 37 °C for 48 h in the *C. acetobutylicum* medium as described by Atlas (2004) with modifications. The medium contained 40 g potato flakes, 6 g glucose, 2 g $CaCO_3$, 0.5 g L-cysteine, 1 mg resazurin and 15 g bacteriological agar, diluted to 1 L of distilled water, pH 7.0. Cultures were kept at 4 °C and reseeded into fresh media every 14 d.

2.2. Fermentation

Cheese whey was used as fermentation medium (lactose being the main carbon source with an initial concentration of approximately 55 g/L of reducing sugars). More specifically, it was first deproteinized followed by adjusting pH value to 5.2 with 1M HCl and autoclaving (14 psi-115 °C/15 min). The cold whey was then filtered through cheesecloth and filter paper (10 μm mesh) under sterile conditions. Yeast extract (5 g/L) and $CaCO_3$ (18 g/L) were added to the filtrate, and the pH value was readjusted to 7.0 using 1 M NaOH. Three sets of fermentation experiments were performed: Group 0: without added iron; Group $FeSO_4$: added with 20 mg/L Fe^{+2}, supplemented as $FeSO_4$-$7H_2O$; and Group $FeCl_3$: added with 20 mg/L Fe^{+3}, supplemented as $FeCl_3$-$6H_2O$. Prepared media was stored at 4°C. Anaerobiosis was achieved by sparging nitrogen to remove dissolved oxygen. Inocula were generated by seeding the bacteria in 80 mL of fermentation medium contained in 125 mL flasks covered with rubber septa and incubated under anaerobic conditions in an orbital shaker (125 rpm) at 37 °C for 72 h. After 72 h, the content of each flask was emptied into a fermentation bottle. One inoculum was required for each batch fermentation, which was carried out under anaerobic conditions using capped glass bottles of 500 mL, containing 320 mL of fresh media for a total fermentation volume of 400 mL. Fermentations were incubated at 37 °C in orbital shaking at 125 rpm for 7 d. A total of 3 fermentations were carried out per each tested condition. Samples were taken periodically every 12 or 24 h; a 2 mL sample of the broth was taken from each experiment using sterile syringes. The broth samples where

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centrifuged at 11,920 ×g for 10 min in a micro17TR microcentrifuge (Hanil Science Industries, South Korea), the supernatant was then filtered through a 45 µm acrodisc (Merck Millipore, Darmstadt, Germany). Filtrated samples were cooled and stored at 4 °C in sealed vials until analysis.

2.3. Analytical methods

2.3.1. Lactose concentration

Total sugar concentration was determined using dinitro salicylic acid (DNS) reagent (Sumner, 1921) with lactose as standard.

2.3.2. pH

Direct measurement on the sample of culture broth at each sampling time was performed using a potentiometer pH/mV meter UB1-10 ultrabasic (Denver Instruments, Colorado, US).

2.3.3. Solvents and acids quantification

An HP 6890 series gas chromatograph (Hewlett Packard, California, US) equipped with a flame ionization detector (FID) and a HP-Innowax capillary column (30 m long; 0.53 mm Ø; 1.00 µm film) (Agilent J&W GC Columns, California, US) was used for solvents and acids quantification. For the assays, 1 µL of each sample was injected into the gas chromatograph, then heated from 60 to 150°C (10 °C/min) and maintained at 150 °C/5min. Injector and detector temperatures were maintained at 250°C. Gas injection was constant at 4 mL/min, and gas pressures were: He 60 psi; H_2 40 psi; dry air 60 psi. Concentration of each product was calculated by integrating the area under the curve of the peak generated using the software Peak Simple 3.21® (SRI Instruments, California, US).

2.4. Statistical analyses

One-way ANOVA was performed to compare butanol and total ABE production using different iron sources. $P< 0.05$ was considered as significant. LSD Multiple Range Tests (at 95.0 % confidence level) were performed to detect significant differences between the productive capacities. All analyses were performed using STATGRAPHICS Centurion XVI® (Statpoint Technologies, Virginia, US).

3. Results and discussion

Cheese whey fermentations supplemented with iron (Group $FeSO_4$ and Group $FeCl_3$) showed the typical behavior of a two-stage ABE fermentation (Fig. 1 and 2, respectively). On the first stage, lasting approximately 48 h, lactose uptake occured and acetic and butyric acids were produced lowering the pH value until it was substantially constant around a value of 5.5. Between 48 and 60 h of fermentation the second stage started, diminishing lactose uptake and promoting re-assimilation of acids into the desired solvents: acetone, butanol and ethanol, reaching maximum solvents concentration after 168 h of fermentation.

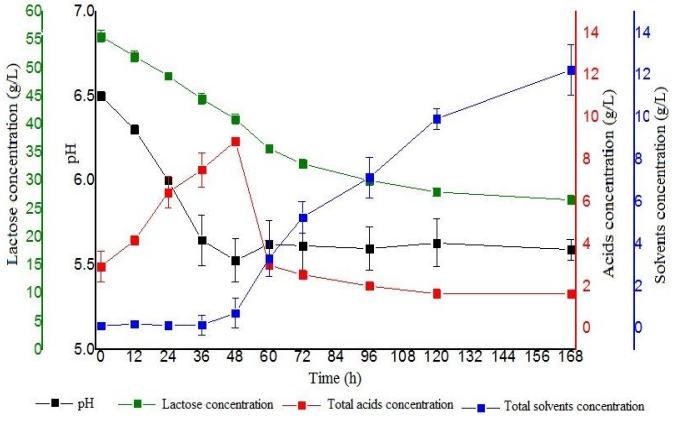

Fig.1. Time course profiles of average pH value, lactose concentration, total acids (acetic and butyric) and total solvents (acetone, butanol and ethanol) production from Group $FeSO_4$ fermentations. Bars show standard deviation (n=3).

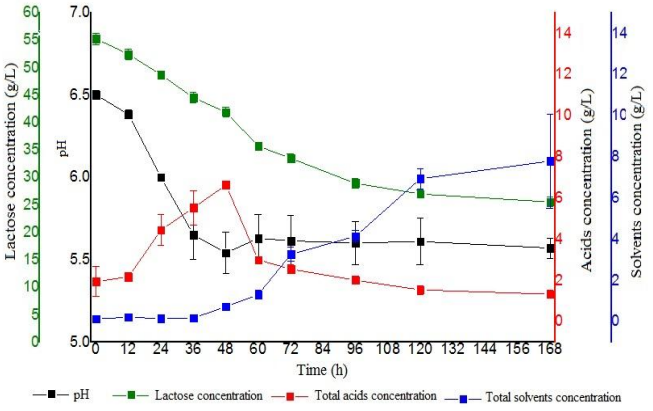

Fig.2. Time course profiles of average pH value, lactose concentration, total acids (acetic and butyric) and total solvents (ABE) production from Group $FeCl_3$ fermentations. Bars show standard deviation (n=3).

The main product of the fermentation was butanol, followed by ethanol, and almost negligible amounts of acetone were detected in the fermentation broths. This could be ascribed to two facts: 1) it has been proved that the use of lactose as the carbon source favors butanol production over acetone (Maddox, 1980; Bahl et al., 1986), and 2) culture conditions, specifically agitation and temperature (37° C), promoted acetone volatilization making it difficult to detect in the culture broth.

Time required to complete the fermentation (7 d) and for inoculum preparation (72 h) was longer than that reported by Foda et al. (2010) (75 h) and Napoli et al. (2008) (96 h) due to the presence of citrate in the culture medium (≈10 g/L). Citrate at high concentrations (> 2.5 g/L) inhibits *C. acetobutylicum* growth; the presence of citrate contributes to unusual prolonged time required to complete whey fermentation (Bahl et al., 1986).

Group 0 fermentations did not show the typical two-stage behavior (Fig. 3). Lactose uptake occurred but acetic and butyric acid productions were remarkably low (less than 4.5 g/L combined), instead, substantial amounts of lactic acid were produced (5.49 ± 0.69 g/L) (Table 1). No re-assimilation of acids was observed but ethanol production occurred.

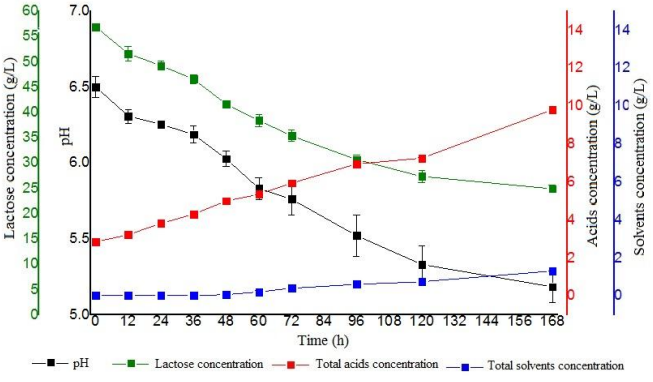

Fig.3. Time course profiles of average pH value, lactose concentration, total acids (lactic, acetic and butyric) and total solvents (ABE) production from Group 0 fermentations. Bars show standard deviation (n=3).

These results differ from those presented by Maddox (1980), who claimed that using cheese whey supplemented only with yeast extract, *C. acetobutylicum* N.C.I.B. 2951 could produce up to 15 g/L of butanol while requiring 5 d to reach this maximum concentration. Also cheese whey without any supplementation could achieve a maximum butanol concentration of 13 g/L within 7 d. However, in later studies, Ennis and Maddox (1986), using a different strain of *C. acetobutylicum* (P262), prepared semi-synthetic media simulating cheese whey, supplemented

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with ferrous sulfate and other components, despite having yeast extract added, but no explanation for these additions were given.

Table 1.
Performance of ABE fermentations (168 h).

Product	Group 0	Group Fe^{+2}	Group Fe^{+3}
Acetate (g/L)	2.61 ± 0.34	0.972 ± 0.08	1.44 ± 0.28
Butyrate (g/L)	1.70 ± 0.53	0.650 ± 0.12	1.27 ± 0.35
Lactate (g/L)	5.49 ± 0.69	Not detected	Not detected
Acetone (g/L)	0.04 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
Butanol (g/L)	0.06 ± 0.10	7.13 ± 1.53	4.32 ± 0.94
Ethanol (g/L)	1.20 ± 0.10	5.11 ± 1.65	3.46 ± 1.37
Total solvents (g/L)	1.30 ± 0.21	12.24 ± 3.18	7.78 ± 2.30
Lactose utilization (%)	56.24 ± 0.65	52.28 ± 0.62	53.80 ± 0.60
ABE yield (gABE/g lactose consumed)	0.04 ± 0.01	0.42 ± 0.10	0.25 ± 0.07
A:B:E Ratio	0.31:0.46:9.23	0.0:5.87:4.13	0.0:5.61:4.39

Moreover, Bahl et al. (1986), Peguin and Soucaille (1995), and Vasileva et al. (2012) agree on the importance of iron in the fermentative metabolism of *C. acetobutylicum*, asserting that in an iron-deficient environment lactate was the main product obtained from the fermentation, observing no re-consumption of acetic and butyric acids.

As shown in Figure 4, *C. acetobutylicum* has the ability to break down lactose by hydrolyzing it into glucose and galactose which are then metabolized by the Embden Meyerhof Pathway (EMP) to generate pyruvate. Then pyruvate has to be oxidized to produce acetyl CoA which plays a central role in the metabolism of *C. acetobutylicum* as it serves as a precursor for the generation of all desired products. Oxidation of pyruvate occurs in a reaction that is coupled to the reduction of ferredoxin, using hydrogen as the ultimate electron acceptor. Sufficient iron is required to produce enough ferredoxin to complete this oxidation (Lee et al., 2008). In an iron deficient environment formation of molecular hydrogen does not take place and metabolism changes from pyruvate to lactate instead of producing acetyl CoA (Gheshlaghi et al., 2009).

Bahl et al. (1986) indicated that limiting the amount of iron in the culture broth helped increase the proportion of butanol/acetone (from 2:1 to 8:1). They argued that ferrous ions had the most drastic effect on that ratio; however, at iron-limited conditions lactate became the main product, instead of producing acetate and butyrate, adversely affecting the amount, yield and productivity of butanol. Increasing the butanol/acetone proportion could facilitate the recovery and purification processes of the product, but iron-limitation backfires by decreasing the amount of butanol produced.

Moreover, Peguin and Soucaille (1995) suggest that limiting the available iron in glucose culture broths, using *C. acetobutylicum* ATCC824, helps modulate carbon and electron flows. For desirable fermentation results, a compound (methyl viologen) that replaces the functions of ferredoxin must be provided to the culture media, because ferredoxin could not be properly synthesized due to iron deficiency. By limiting the amount of iron present in the medium, dehydrogenase activity involved in the conversion of acetyl-CoA into β-hydroxybutyrate is limited. Methyl viologen function as a better substrate for ferredoxin-$NAD(P)^+$ reductase than ferredoxin itself, creating an artificial electron transport chain. Both effects: reduction of the dehydrogenase activity and increase of ferredoxin-$NAD(P)^+$ reductase activity made possible to obtain higher yields of butanol that those obtained with regular ABE fermentation, reaching a maximum 13 g/L butanol concentration. Nevertheless adding methyl viologen causes long lag growth phase affecting the fermentation productivity. Increasing the production of alcohols by limiting iron may sound favorable, but to be obligated to supplement compounds who can serve as electron acceptors to prevent the formation of lactate may be more costly for the process and more problematic for the recovery of products. In this paper, it has been shown that by adding adequate amounts of iron (20 mg /L) butanol production improvement could be achieved, favoring the desired normal behavior of the metabolic pathway.

Metabolic development of Group 0 fermentations demonstrate the effect of iron deficiency when cheese whey is used as substrate, effect that was not observed by Napoli et al. (2008) while they also worked with synthetic lactose medium simulating cheese whey enriched with yeast extract and $CaCO_3$ and without any additional iron source, reporting the typical two-stage ABE fermentation behavior with a 3 g/L butanol production and no lactate detected, results that were reproduced in our laboratory too (data not shown).

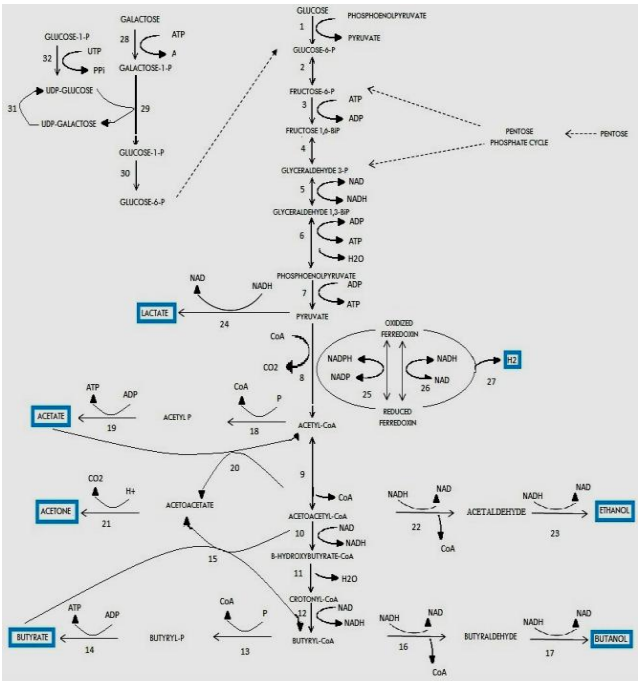

Fig.4. Metabolic pathways of glucose and galactose in *C. acetobutylicum*. Extracellular products are highlighted in blue boxes. Numbers indicate the enzymes involved: 1: phosphotransferases system; 2: glucose-6-phosphate isomerase; 3: phosphofructo kinase; 4: aldolase; 5: glyceraldehyde-3-phosphate dehydrogenase; 6: phosphoglycerate kinase 7: pyruvate kinase; 8: pyruvate-ferredoxin oxidoreductase; 9: thiolase; 10: β-hydroxybutyryl CoA dehydrogenase; 11: crotonase; 12: butyryl-CoA dehydrogenase; 13: phosphotransbutyrylase; 14: butyrate kinase; 15: acetoacetyl-CoA Butyrate-CoA transferase; 16: butyraldehyde dehydrogenase; 17: butanol dehydrogenase; 18: phosphotransacetylase; 19: acetate kinase; 20: acetoacetyl-CoA acetate-CoA transferase; 21: acetoacetate decarboxylase; 22: acetaldehyde dehydrogenase; 23: ethanol dehydrogenase ; 24: lactate dehydrogenase; 25: ferredoxin-NADP reductase NADPH-ferredoxinoxidorreductase; 26: ferredoxin-NAD reductase NADH-ferredoxinoxidorreductase; 27: ferredoxin hydrogenase; 28: galactokinase; 29: UDP-glucose glucose-1-P uridyl transferase; 30: phosphoglucomutase; 31: UDP-galactose epimerase; 32: UDP-glucose phosphotransferase.

In fact, yeast extract contains iron in amounts up to 20 mg/100 g that may be sufficient to produce enough ferredoxin for routing of metabolism to the production of acetyl CoA and later generation of acetone, ethanol and butanol when it is added to a synthetic fermentation media; but, when cheese whey is used an extra amount of iron is necessary because of the presence of lactoferrin (20- 200 µg/ml) (Law and Reiter, 1977), an iron-binding protein that binds to iron reversibly with a high affinity. This blocks iron and impedes ferredoxin formation and, therefore, restricts solvents production.

The fermentation performance of each group is summarized in Table 1. Analyses of variance were conducted to assess whether the factor studied (iron source) had an effect on ABE production. LSD Multiple Range Test was conducted to assess which fermentation condition tested was better than the others in order to obtain a higher butanol concentration and higher total solvents production, being butanol as the main product. One way ANOVA indicated statistically significant differences in the production of total solvents between fermentations with added iron and unsupplemented ($p < 0.005$). Multiple range tests showed that the total production of solvents presents statistically significant difference between cheese whey without iron supplementation and the same substrate with an extra iron source, however, no statistically significant difference existed when comparing the two different iron sources i.e. $FeSO_4$ and $FeCl_3$.

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Evaluating only butanol production, one way ANOVA indicated that there is a statistically significant difference between the total amount of butanol produced by each fermentation condition tested, ($p<0.001$). LSD Multiple Range Test indicated that, for butanol production, there is statistically significant difference between the three groups. Both iron sources tested showed practically nil production of acetone; while butanol proportion was higher using $FeSO_4$ (0.0:5.87: 4.13) than $FeCl_3$ (0.0: 5.61: 4.39). Of particular interest is the fact that the use of $FeSO_4$ as iron source increased butanol production by 65% compared to what achieved by employing $FeCl_3$. This could be explained by the presence of iron and sulfur, both essential components of ferredoxin.

Additionally Group $FeSO_4$ showed higher ABE yield (0.42 ± 0.10) than Group $FeCl_3$ (0.25±0.07), despite almost equal sugar consumptions. These results showed that sugar conversion to acids and acids reassimilation developed best when the iron source also included sulfur, under conditions tested.

These results showed that whey supplemented appropriately, can serve as a good substrate for ABE fermentation. Cheese whey is a readily available and widely-produced inexpensive substrate which requires low pretreatment to serve as fermentation substrate. These make it more economic than other substrates used such as lignocellulosic materials, algal biomass or different industrial and domestic wastes. Cheese whey tends to favor the production of butanol over acetone and therefore by improving fermentative conditions to fully utilize lactose in whey could lead ABE fermentation to economic competitiveness.

4. Conclusion

Cheese whey is a suitable substrate for ABE fermentation as long as it is properly prepared and supplemented. Addition of an iron source is strictly necessary for cheese whey to be a viable substrate and the lack of iron in cheese whey impedes ferredoxin synthesis and restricts pyruvate-ferredoxin oxidoreductase activity leading to the production of lactic acid instead of ABE. Furthermore, the addition of $FeSO_4$ improved butanol production by 65% reaching a concentration of 7.13 ± 1.53 g/L, compared to what obtained with $FeCl_3$ under the same fermentation conditions. This indicates that $FeSO_4$ is an ideal iron source for improving butanol production under conditions tested.

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