FERMENTATION OF SACCHARIDES ISSUED FROM A DAIRY INDUSTRY BY A GENETICALLY MODIFIED STRAIN OF *ESCHERICHIA COLI* INTO ACETOIN AND 2,3-BUTANEDIOL

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ABSTRACT

Whey, which usually shows a high biological oxygen demand and a high chemical oxygen demand, should be treated before being rejected as waste water. The valorization of whey by chemical/physical treatments already exists. Some bioprocesses are also currently developed to transform whey into, for example, biogas. However, new performing green processes are still in development in order to obtain chemical products able to replace those issued from petroleum resources like acetoin (A) and 2,3-butanediol (2,3-BD), two important chemical platform molecules. The main objective of the present study was to evaluate the potential use of glucose, galactose and a lactose source derived from a dairy industry to produce A and 2,3-BD (ABD). The main issue of the natural producer bacteria of 2,3-BD such as *Klebsiella pneumoniae* or *K. oxytoca* during the fermentation of saccharides is their biosafety level since they are pathogen. In this way, non-pathogenic bacteria can be genetically modified to produce ABD from saccharides. In the present study, a genetically modified strain of *Escherichia coli* K12 MG1655 (non-pathogenic strain) was used. Two monosaccharides (glucose and galactose issued from lactose) at three concentrations (12.5, 25 and 50 g/L) were fermented using 0.5 L flasks for 120 h at 37°C, 1 atm, initial pH 7.4, 100 rpm and 10% (v/v) of inoculum in a synthetic culture medium (M9). All experiments showed that the fermentation of galactose was less efficient than the one of glucose (the ABD yields were around 25%, 40% and 35% lower compared to those obtained fermenting glucose at 12.5, 25 and 50 g/L at 96 h respectively). The highest ABD yield was 0.26 (g/g glucose), obtained at 96 h in the presence of 25 g/L of glucose. The ABD yields issued from glucose and galactose were compared with those derived from a dairy industry lactose.

Keywords: monosaccharide, glucose, galactose, lactose, whey, M9 culture medium, biovalorisation.

1 INTRODUCTION

Whey is a dairy effluent generated during the cheese manufacturing. It contains lactose (the main part of the dry matter of whey), proteins, etc. Due to its high biological (BOD) and chemical (COD) oxygen demands, it is necessary to treat the whey before releasing it in the environment. The valorization of whey via biotechnology has as objectives a) to decrease the BOD and COD values, and b) to generate products like 2,3-butanediol (2,3-BD). Bacteria like *Enterobacter cloacae* and *Klebsiella pneumoniae* are able to hydrolyze and transform saccharides like lactose into acetoin (A) and 2,3-BD. Although these bacteria are pathogenic, they are considered as the best natural 2,3-BD producers. Therefore, other non-pathogenic bacterial strains are being developed for the hydrolysis and the fermentation of saccharides like lactose. In this way, a non-pathogenic strain like, for example, *Escherichia coli* K12 MG1655 would be a good option. However, *E. coli* cannot produce 2,3-BD. Genetic modifications in the *E. coli* strain have to be performed in order to transfer the metabolic pathway from a natural 2,3-BD producer like *E. cloacae*.

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Escherichia coli K12 MG1655 presents several advantages compared to the previous natural producers of 2,3 BD strains: i) has a biosafety level 1 (non-pathogenic), ii) can consume a wide range of saccharides like glucose and galactose (2 monosaccharides issued from whey lactose), and iii) is easily modified to host metabolic pathways from other microorganisms [1], [2]. In order to produce A and 2,3-BD (ABD), *E. coli* has to transform both monosaccharides into pyruvic acid (PA), which occurs by different pathways: glycolysis for glucose and Leloir pathway plus glycolysis for galactose [3], [4].

Afterwards, the PA is transformed into α-acetolactate by α-acetolactate synthase (ALS). Then, α-acetolactate is transformed into A by means of α-acetolactate decarboxylase (ALDC) enzyme. Finally, A is converts into 2,3-BD by 2,3-butanediol dehydrogenase (BDH) enzyme [5]. Fig. 1 shows the metabolic pathway to obtain A and 2,3-BD from glucose and galactose.

In the present study, a genetically modified strain of *E. coli* K12 MG1655 (*E. coli* JFR1) was used to host the metabolic pathway of 2,3-BD from *E. cloacae* in order to test the ability of *E. coli* JFR1 to produce ABD from glucose and galactose. Three monosaccharide concentrations (12.5, 25 and 50 g/L) were fermented at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm and the ABD yields were compared. The culture medium, M9, was used in a preliminary study and was selected as the best culture medium to obtain ABD from glucose [8]. Lactose was also fermented, and the ABD yield was compared with those from glucose and galactose.

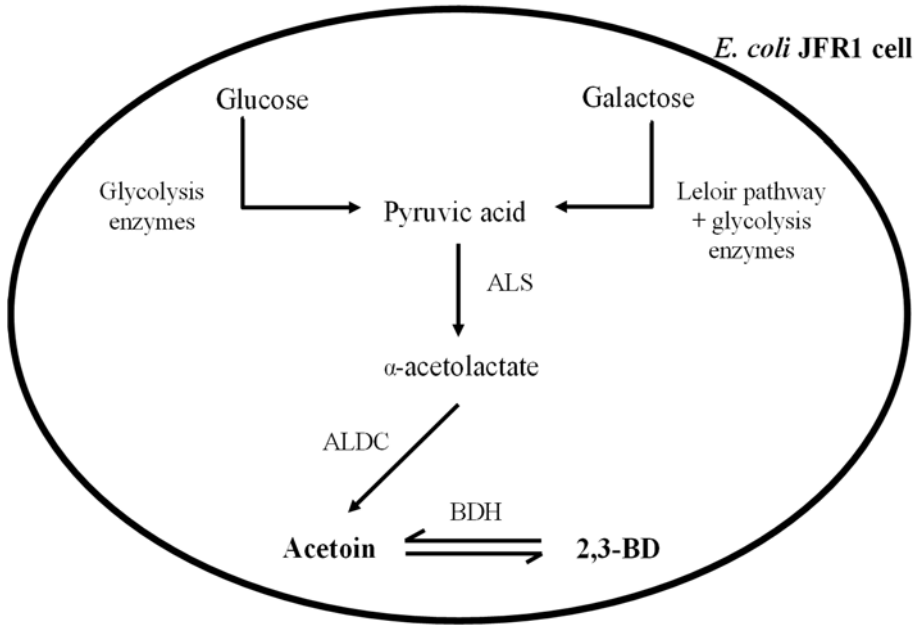

Figure 1: Metabolic pathway to produce 2,3-BD from glucose and galactose fermentation in the presence of a genetically modified strain of *Escherichia coli*. Relevant reactions to transform pyruvic acid into 2,3-BD are represented by the names of the corresponding enzymes from *Enterobacter cloacae*: α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC) and 2,3-butanediol dehydrogenase (BDH) [3]–[7].

2 MATERIALS AND METHODS

2.1 Microorganisms

The genetically modified strain of *E. coli* K12 MG1655 hosts the metabolic pathway of 2,3-BD to produce ABD from *E. cloacae*. The biosynthetic pathway of fermentative D-lactate dehydrogenase (*ldhA*) was blocked to avoid the formation of lactic acid. The modified strain was *E. coli* K12 MG1655/D*ldhA* + *bud*ABC, named as *E. coli* JFR1. The conservation of *E. coli* JFR1 was performed at -81ºC in a blend (50:50, v/v) of glycerol and lysogeny broth (LB) culture medium.

2.2 Culture media and conditions

Escherichia coli JFR1 was grown in LB culture medium since it is suitable for recombinant *E. coli* strains [9]. The composition of LB culture medium was as follows: 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of sodium chloride (NaCl) and distilled water [10]. The pH of LB for growing *E. coli* JFR1 was adjusted at 6.5. The fermentation of glucose, galactose and lactose by *E. coli* JFR1 was tested using M9, which was made as follows: 12.8 g/L of sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), 3 g/L of potassium dihydrogen phosphate (KH_2PO_4), 1 g/L of ammonium chloride (NH_4Cl), 0.5 g/L NaCl, 15 g/L urea ($(NH_2)_2CO$), 0.49 g/L of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 0.01 g/L of calcium chloride ($CaCl_2$) and distilled water [8].

The seed culture medium was prepared as follows: a sample of *E. coli* JFR1 from the conservation blend was taken with a tip and mixed with 10 mL of fresh LB medium in a test tube (15 mL). The test tube was incubated overnight at 37ºC (pre-culture medium). Then, 2 mL of the pre-culture medium were transferred into a 0.5 L Erlenmeyer flask containing 0.2 L of fresh LB medium and incubated in a rotary shaker incubator (Fermentation Design inc, Allentown, PA) at 37ºC, 1 atm, initial pH 6.5 and 100 rpm to reach a bacterial population of $6.5x10^8$ colony-forming unit (CFU)/mL in 9 h. The seed culture medium was used to inoculate M9 culture medium.

All fermentations were performed in triplicate at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm in 0.5 L flask (0.2 L of working volume).

2.3 Analytical methods

The analysis of glucose, galactose, lactose, 2,3-BD and A was determined by high performance liquid chromatography (HPLC) as shown in a previous study [8].

2.4 Statistical analysis

The ABD formation may be influenced by the type and concentration of the substrate. In this way, the effect of glucose, galactose and lactose concentration on ABD yield was statistically determined by means of an analysis of variance (ANOVA) at $p < 0.05$. In addition, Dixon's Q test was carried out to estimate and rule out the outlier values of ABD yield at a confidence level of 95% [11].

3 RESULTS AND DISCUSSION

The fermentation of glucose and galactose was performed in M9 culture medium in order to produce ABD by *E. coli* JFR1. The effect of 3 concentrations (12.5, 25 and 50 g/L) of glucose and galactose were tested.

Fig. 2 (a) and (b) shows the glucose and galactose conversion for 3 initial concentrations (12.5, 25 and 50 g/L) of glucose and galactose ($[Glu]_0$ and $[Gal]_0$, respectively) as a function of time. At 24 h, for a $[Glu]_0$ of 12.5 g/L, the glucose conversion was 100%, whereas for a $[Glu]_0$ of 25 and 50 g/L conversions were 77% and 56%, respectively. Glucose conversion was 100% for a $[Glu]_0$ of 25 g/L at 72 h, while the conversion was 70% fermenting 50 g glucose/L. The maximum conversion for a $[Glu]_0$ of 50 g/L was 77%, obtained at 120 h (Fig. 2(a)).

Similarly, the galactose conversion was 100% for a $[Gal]_0$ of 12.5 g/L at 24 h; whereas it was 75 and 55% in the presence of 25 and 50 g/L of galactose, respectively. At 72 h, the galactose conversion was 96% using a $[Gal]_0$ of 25 g/L and it was 72% for a $[Gal]_0$ of 50 g/L. The maximum galactose conversion for a $[Gal]_0$ of 50 g/L was 76%, which was reached at 120 h (Fig. 2(b)).

As observed in Fig. 2(a) and (b), the trend of glucose and galactose conversions was similar for both monosaccharides whatever the concentration used. On the other hand, the use of a high concentration (50 g of monosaccharide/L) led to a low conversion (77%) even after 120 h of fermentation. No change in galactose conversion was observed after 96 h of

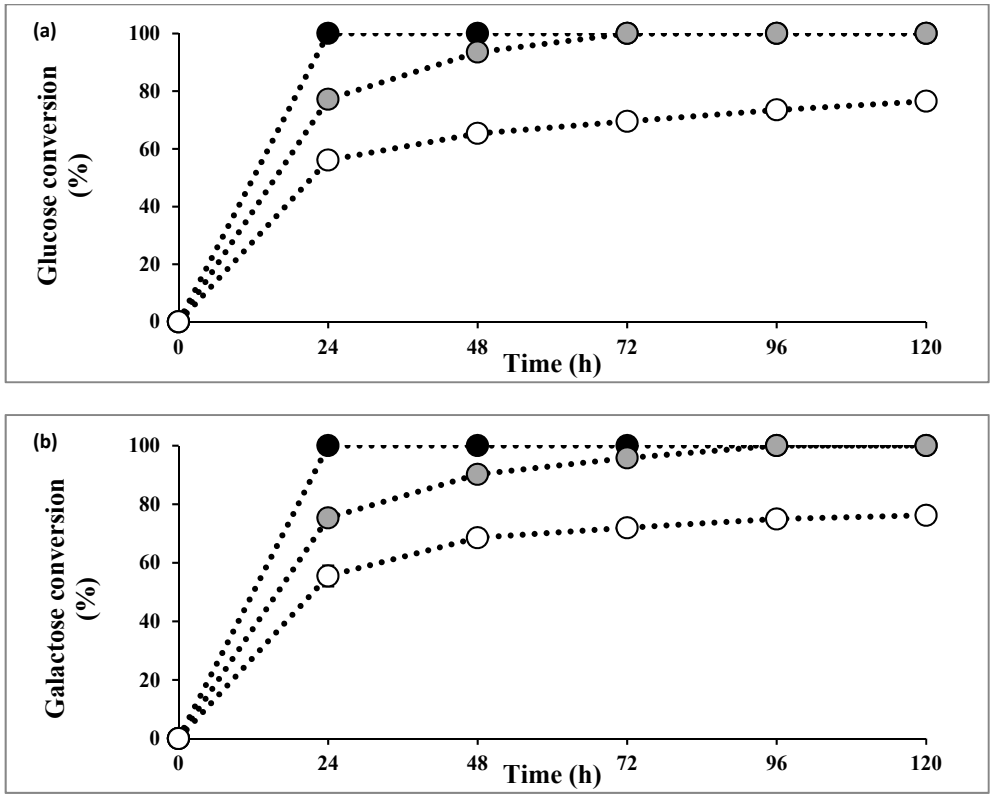

Figure 2: Glucose (a) and galactose (b) conversion as a function of time. Results are means ± SD of 3 replications performed in flasks at 37ºC, 1 atm, initial pH 7.4, 100 rpm and 10% (v/v) of inoculum in the presence of *E. coli* JFR1 in M9 culture medium (0.2 L of working volume) with 12.5 (●), 25 (●) and 50 (○) g/L of monosaccharide.

fermentation. Bacteria present a threshold concentration to which the bacterial population might be affected if it is exceeded, causing a slower bacterial growth as suggested by Chan et al. [12]. In the case of *E. coli* JFR1, this threshold concentration seems to be between 25 and 50 g/L since the 100% conversion was reached using 25 g/L for both monosaccharides between 72 and 96 h; however, it was not achieved for 50 g/L at 120 h reaching a plateau after 96 h.

Fig. 3 (a) and (b) shows the ABD yield for 3 initial concentrations (12.5, 25 and 50 g/L) of glucose and galactose ($[Glu]_0$ and $[Gal]_0$), respectively as a function of time. For the $[Glu]_0$ of 12.5 g/L, the maximum ABD yield (0.18 g/g glucose) was obtained at 24 h and remained nearly constant up to 120 h. For a $[Glu]_0$ of 25 g/L, the ABD yield increased reaching a plateau at 0.26 g/g glucose at 96 h and remained constant up to 120 h; whereas with the $[Glu]_0$ of 50 g/L, the ABD yield increased up to 0.15 g/g glucose at 120 h ($p < 0.05$) as shown in Fig. 3(a).

In the case of galactose, for a $[Gal]_0$ of 12.5 g/L, the ABD yield reached a plateau at 0.13 g/g galactose at 24 h (Fig. 3(b)) and then remained constant. Using a $[Gal]_0$ of 25 g/L, the ABD yield increased up to 0.16 g/g galactose ($p < 0.05$) at 72 h and remained constant until

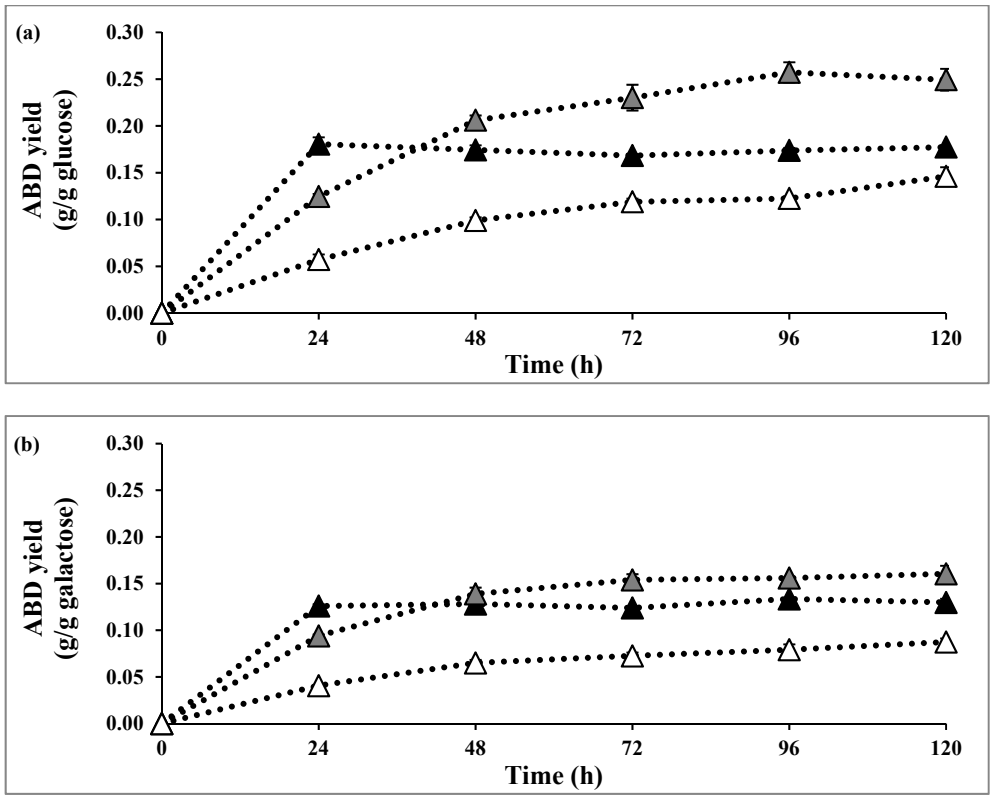

Figure 3: ABD yield from glucose (a) and galactose (b) as a function of time. Results are means ± SD of 3 replications performed in flasks at 37ºC, 1 atm, initial pH 7.4, 100 rpm and 10% (v/v) of inoculum in the presence of *E. coli* JFR1 in M9 culture medium (0.2 L of working volume) at 12.5 (▲), 25 (▲) and 50 (Δ) g/L of monosaccharide.

the end of the experiment (120 h); whereas in the presence of $[Gal]_0$ of 50 g/L, the ABD yield increased to 0.08 g/g galactose at 96 h and remained nearly constant up to 120 h ($p < 0.05$).

Comparing the 3 glucose concentrations, the maximum ABD yield was 0.26 g/g glucose at 96 h in the presence of 25 g/L of glucose, which was around 48% and 110% higher compared to the maximum obtained using 12.5 and 50 g/L of glucose, respectively. In the case of galactose, the highest ABD yield (0.16 g/g galactose) was reached in the presence of a $[Gal]_0$ of 25 g/L at 72 h. However, this ABD yield (0.16 g/g galactose) was only improved by 25% compared to the one at 12.5 g/L of galactose, obtained at 24 h, a fermentation time 3-fold shorter. Therefore, the best ratio galactose concentration-fermentation time in order to obtain ABD was 12.5 g/L since this yield was around 0.13 g/g galactose at 24 h.

The difference between both monosaccharides in terms of ABD yield is that the use of glucose was more efficient than galactose since the ABD yields were 30%, 65% and 55% higher using glucose at 12.5, 25 and 50 g/L respectively compared to galactose at 96 h. As mentioned previously, the use of a high monosaccharide concentration (50 g/L) might lead to the inhibition of the bacterial growth, which affects the formation of ABD as suggested by Chan et al. [12], Krämer [13] and Priya et al. [14]. This phenomenon is clearly observed in the present study at 50 g/L of monosaccharide in the presence of *E. coli* JFR1 since the ABD yield at a $[Glu]_0$ of 25 g/L was more than double compared to the one obtained at a $[Glu]_0$ of 50 g/L at 96 h; the ABD yield was near 100% higher at a $[Gal]_0$ of 25 g/L compared to a $[Gal]_0$ of 50 g/L at 96 h.

As observed in Fig. 3 (a) and (b), the ABD yield reached a maximum and was nearly constant whatever the monosaccharide and concentration used. As shown in Fig. 1, the diol (2,3-BD) is an end product which is in equilibrium with A, its precursor. Acetoin is transformed into 2,3-BD by the BDH enzyme. Mazumdar et al. [6] suggested that 2,3-BD can be used by *E. coli* and, thus, can be transformed into A. This might explain why using *E. coli* JFR1, the ABD yield was nearly constant in the present study after reaching its maximum.

To the best of our knowledge, *E. coli* has not been used to ferment glucose and galactose in order to obtain ABD. However, other studies reported a comparison of glucose and galactose in the presence of bacteria like *Enterobacter aerogenes*, *Geobacillus* XT15 *Paenibacillus polymyxa* and *Saccharomyces cerevisiae* [15]–[18]. For example, Xiao et al. [18] reported an ABD yield of 0.34 and 0.21 g/g monosaccharide (ABD yield calculated from the data provided by the authors) fermenting 20 g/L of glucose and galactose respectively at 55ºC and 170 rpm (pH and time non-defined) in the presence of *Geobacillus* sp. XT15 (5% (v/v) of inoculum). Although the yields reported by this author were superior to those obtained in the present study, the difference of ABD yield between both monosaccharides was 1.6-fold higher using glucose than galactose; a similar difference in terms of ABD yield in the presence of *E. coli* JFR1 using 25 g/L of monosaccharide at 96 h was also observed. Therefore, the use of glucose seems to be more suitable than galactose in order to produce ABD in the presence of *E. coli* JFR1.

Table 1 presents the ABD yields obtained after fermentation of 3 substrates, i.e. glucose, galactose and lactose (lactose being the whey disaccharide composed of galactose and glucose) at 2 concentrations: 12.5 and 25 g (substrate)/L at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm in 0.5 L flask (0.2 L of working volume). Lactose was only fermented at 12.5 and 25 g/L since these 2 concentrations of glucose and galactose gave the highest ABD yields.

As can be seen in Table 1, whatever the concentration of 12.5 or 25 g (substrate)/L at 24 h of fermentation, the ABD yield from lactose is similar to the one from glucose (for example, at 24 h, and for a saccharide concentration of 12.5 g/L, the ABD yield is 0.18 g/g saccharide

Table 1: ABD yield (g/g saccharide) for glucose, galactose and lactose as a function of fermentation time. Results are means ± SD of 3 replications performed in flasks at 37ºC, 1 atm, initial pH 7.4, 100 rpm and 10% (v/v) of inoculum in the presence of *E. coli* JFR1 in M9 culture medium (0.2 L of working volume) with 12.5 and 25 g/L of saccharide.

Saccharide concentration: 12.5 g/L				Saccharide concentration: 25 g/L		
Time (h)	Glucose	Galactose	Lactose	Glucose	Galactose	Lactose
24	0.18	0.13	0.18	0.12	0.09	0.12
72	0.17	0.12	0.17	0.23	0.16	0.19

(glucose or lactose); the ABD yield relative to galactose being always inferior to the ABD yields obtained with glucose or lactose. A similar behavior is observed at 72 h of fermentation for 12.5 g lactose/L. An ABD yield of 0.19 g/g lactose, superior by 19% is noticed at 25 g lactose/L and 72 h compared to that one using galactose. These ABD yields were higher than those obtained in other studies while fermenting lactose in the presence of bacteria belonging to risk level 1. For instance, Kallbach et al. [19] fermented 57 g/L of lactose in the presence of *Bacillus atrophaeus, B. licheniformis, B.mojavensis* and *B. vallismortis* at 39ºC and 700 rpm (pH not provided) for 47 h. The ABD yields (g/g lactose) using these strains were 0.00 (*B. atrophaeus* and *B. mojavensis*), 0.01 (*B. licheniformis*) and 0.08 (*B. vallismortis*). On the other hand, during the fermentation of lactose in presence of *E. coli* JFR1, galactose present in lactose does not seem to affect the ABD yield. Lactose issued from whey has also been fermented but for confidentiality reasons, the promising results cannot be presented yet [20].

4 CONCLUSION

The present study was based on the fermentation of 2 monosaccharides in order to produce acetoin and 2,3-butanediol (ABD) in the presence of a genetically modified strain of *Escherichia coli*: *E. coli* JFR1. Glucose and galactose at different concentrations (12.5, 25 and 50 g/L) in M9 culture medium at 10% (v/v) of inoculum, 37ºC, 1 atm, initial pH 7.4 and 100 rpm in 0.5 L flask (0.2 L of working volume) were fermented.

The use of whatever glucose concentration provided better results in terms of ABD yield than the fermentation of galactose. The ABD yields were between 30% and 65% higher in the presence of glucose for the range of concentrations tested compared to galactose at 96 h. The highest ABD yield was 0.26 g/g glucose at 96 h and 25 g/L of glucose; whereas the best ABD yield was 0.16 g/g galactose at 72 h and 25 g/L of galactose. The ABD yields obtained during the fermentation of glucose or lactose were near similar at 24 h and 72 h under the same operating conditions.

This study demonstrated particularly that galactose can be used by *E. coli* JFR1 in order to produce ABD. Hence, an in-depth study using galactose should be considered in the presence of *E. coli* JFR1 with the aim of improving the ABD yield. On the other hand, the current study has provided evidences that lactose can be transformed by a green bioprocess into ABD in the presence of *E. coli* JFR1 and thus whey has a huge potential as a lactose source to be valorized.

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