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BIOETHANOL FERMENTATION OF CHEESE WHEY POWDER (CWP) SOLUTION USING UNEXPLORED STRAIN OF KLUYVEROMYCES MARXIANUS CCT 7585

ABSTRACT

Cheese whey is a concern in biological valorization of cheese manufacturing waste due to its high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) values. Fermentation strategies for bioethanol production using lactose have been explored, and the approach can have beneficial effect on new product development such as bioethanol. Actually, these strategies are supported by the following techniques: construction of intergeneric yeast fusants, co-culture approaches, and mutation of native strains. Similarly, there is lack of data about bioethanol production from whey-borne lactose by the official strain K. marxianus CCT 7585. Would be possible to use those techniques mentioned above? For this reason, the effects of different carbon sources (mono and disaccharides), time course, and hydrolysis – the latter two in CWP solution – on bioethanol production were investigated using K. marxianus CCT 7585. The results showed that K. marxianus CCT 7585 was capable to decrease lactose content in CWP solution, which certainly will provide low COD and BOD rates. Satisfactory bioethanol yields were also observed (≈ 4% v/v). Moreover, CWP hydrolysis can be suggested as an inductor process to increase bioethanol yield (increases of around 6%). This official strain is considered to be a promising approach for using intergeneric yeast fusants, co-culture techniques, and genetic mutation to increase bioethanol yields.

Keywords: Bioethanol, cheese whey, Kluyveromyces marxianus, lactose.

1. INTRODUCTION

Depleting petroleum reserves and concerns over global climate changes make development of renewable and clean energy sources mandatory. Bioethanol as a clean and renewable fuel has gained more attention, once its combustion produces water and energy, besides reducing carbon dioxides (CO_2) emissions when compared to that derived from fossil fuels [1].

Similarly, dairy industries generate significant liquid waste, of which cheese whey is the most abundant. Whey is the liquid generated from cheese manufacture and is resulting from the coagulation of milk [2]. Cheese whey contains about 7% solids comprising of about fat (3%), minerals (8%), proteins (10-12%), the rest being lactose (74%) [3]. Whey arising from cheese manufacture has high biochemical oxygen demand and must be treated prior to disposal [4]. Thus, studies on ethanol production from whey are of great importance.

K. marxianus is a promising yeast strain for biotechnological applications [3]. Recently, studies have isolated and purified β-galactosidase from K. marxianus, suggesting that this yeast could ferment cheese whey (a wastewater) [5]. Subsequently, it was verified that K. marxianus is able to convert whey-borne lactose into ethanol (an environmentally friendly fuel) [6]. Some strategies for ethanol production from whey-borne lactose by K. marxianus have been proposed, including using intergeneric yeast fusants (K. marxianus plus Saccharomyces fragilis), co-culture approaches or using mutant strains of K. marxianus (e.g K. marxianus KD-15) [7–9]. Despite the efficiency of yeast cell fusion techniques, and co-culture and mutation approaches, there are few reports of fermentative process using native yeast strains (Table 1). These new results could offer new perspectives in dealing with yeast fusants, mutants and co-culture assays. Anyway, there is a lack of data about ethanol production from whey-borne lactose by the official strain K. marxianus CCT 7585; in addition, CWP solution is still little used in these processes (Table 2).

The present study has addressed the effect of process variables (different carbon sources - mono and disaccharides - time course, and hydrolysis – the latter two in CWP solution) on bioethanol production by K. marxianus CCT 7585.

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2. MATERIALS AND METHODS

2.1 Microorganism and Reagents

K. marxianus CCT 7585 was obtained from André Tosello Foundation. All reagents were of the highest grade available.

2.2 Media and Culture Conditions

K. marxianus CCT 7585 was first cultured on Potato Dextrose Agar (PDA), pH 5.5, at 30°C for 10 days. The biomass was inoculated into 100 mL of malt extract (15 g/L) and incubated at 30°C, pH 5.5, for 48 h with shaking at 150 rpm. After incubation, the yeast was inoculated (5% v/v) into 100 mL of culture medium containing glucose (5% w/v), galactose (5% w/v), lactose (5% w/v), glucose (2.5% w/v) + galactose (2.5% w/v), sucrose (5% w/v), and fructose (5% w/v) to evaluate the effect of different carbon sources (mono and disaccharides) on bioethanol production under shake-flask culture conditions. Kinetics of bioethanol production and lactose consumption of CWP solution under shake-flask culture conditions was measured using potassium dichromate and methylamine reaction method (see "Analytical Methods" section), respectively, and varying fermentation time. CWP was purchased from Confepar Agro-industrial Cooperativa Central®. For preparation of CWP solution, an appropriate CWP amount (5% w/v) was dissolved in distilled water and deproteinized (see "CWP Solution Deproteinization" section).

2.3 CWP Solution Deproteinization

Deproteinization of CWP solution was carried out by lactic acid addition until pH 4.8 was reached (isoelectric point of whey protein). After that, the solution was autoclaved under continuous flow conditions (100 °C, 30 min), and the protein precipitate was removed by filtration.

2.4 Lactose Hydrolysis

The hydrolysis of lactose and CWP solution was performed using β-galactosidase solution (0.8 g/L) (MAXILACT® LX 5000) incubated at 40°C for 3 h to study the effect of lactose hydrolysis on bioethanol yield. The samples were heated in boiling water bath (8 min) to stop the enzymatic reaction.

2.5 Analytical Methods (Lactose and Bioethanol)

Lactose content was determined by methylamine reaction method [10]. For that, 4 mL of sample was homogenized with 0.5 mL Zapt solution (formula described below). After centrifugation (4000 g, 5 min), a 2.5 mL aliquot of NaOH (1N) was added to 2.5 mL supernatant. The solution was filtered, and 1.5 mL glycine-NaOH buffer (formula described below), 0.15 mL methylamine solution (0.16 M) and 0.15 mL suphite solution (0.08 M) were added to the filtrate. After homogenization, the samples were incubated for 30 min at 65 °C and transferred to an ice bath (2 min). Absorbance was measured at 540 nm using a spectrophotometer.

Bioethanol concentration was determined by potassium dichromate method after being isolated from the fermentation medium by a microdistillery (Fig. 1) [11]. An aliquot of 9 mL potassium dichromate solution (formula describe below) was added to distilled samples (10 mL) and the mixture was kept in water bath at 100°C for 15 min (glass tube method). Then, absorbance was measured at 606 nm using a spectrophotometer.

2.6 Standard Solutions

2.6.1 Zapt solution

Zinc acetate (25 g) and fosfotungistic acid (12.5 g) were dissolved in distilled water. After solubilization, 20 mL glacial acetic acid was added, followed by addition of distilled water to give a final volume of 1000 mL.

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2.6.2 Glycine-NaOH buffer

Glycine (2.47 g) and sodium chloride (1.93 g) were dissolved in distilled water to give a final volume of 150 mL. Then, NaOH (0.4 N) was gradually added until the mixture became alkaline (pH=12.8).

2.6.3 Potassium dichromate solution

Potassium dichromate (7.4 g) was dissolved in distilled water (400 mL). After that, sulfuric acid (560 mL) was added to the solution and the final volume was adjusted to 1000 mL. Procedures were carried out under dim light.

2.7 Statistical Analysis

The data are presented as mean ± SEM values. Statistical analysis was performed by one-way ANOVA, followed by Tukey's test at statistical significance level of $p< 0.05$.

3. RESULTS

Fig. 1 shows the various steps for bioethanol production from K. marxianus CCT 7585. Bioethanol yield was calculated by colorimetric method. The effect of different carbon sources (mono and disaccharides) on bioethanol production under shake-flask culture conditions are shown in Fig 2. The results demonstrated that the highest bioethanol yield was obtained on lactose. In contrast, its monosaccharide constituents (lactose and galactose), alone or in combination, did not provide better results for bioethanol production. Whereas lactose is largely found in cheese whey, the kinetics of ethanol production was performed using the CWP solution. The ethanol yield grew exponentially reaching a maximum concentration of 4.1% (v/v) from CWP (Figure 3). The lactose concentration decreased exponentially, reaching about 0.2% (w/v) after 14h. The ethanol production occurred during exponential growth phase (consistent with the marked reduction of lactose). No ethanol production was observed during the death phase.

Figure 4 shows the effect of hydrolysis on CWP, as well as the influence of other organic and inorganic components when lactose was used alone. The results showed that hydrolysis of CWP and lactose may be an effective tool to increase bioethanol production ($p<0.05$). Organic and inorganic components from CWP showed a positive effect (10%) on bioethanol production when compared to lactose.

4. DISCUSSION

Cheese whey is a main pollutant of the dairy industry characterized by high COD (60-80 g/L) and BOD (30-50 g/L) [12]. Bioconversion of cheese whey into value-added products such as bioethanol by fermentative process provides an environmentally benign and near term solution to fulfill the demand of clean energy [1,2]. The main objective of this study was to evaluate the process variables (effect of different carbon sources - mono and disaccharides - time course and hydrolysis – the latter two in CWP solution) on bioethanol production by K. marxianus CCT 7585. The basic difference between the previous papers and the present study is that we have improved some fermentation parameters about bioethanol production to the official strain K. marxianus CCT 7585 using CWP solution. Moreover, these results provide new perspectives in dealing with yeast fusants, mutants and co-culture assays to produce bioethanol, besides the development of a descriptive methodology to use CWP solution, once there are few reports on CWP solution in literature [7–9]. In addition, cheese whey powder (CWP) is an alternative way of using fresh whey with certain advantages such as high lactose content, long-term stability, easy transportation and storage [9].

Evaluation of the effect of different carbon sources (mono and disaccharides) on bioethanol production allows knowing their potential to explore new wastes or byproducts (Table 3). The question is whether a microorganism without ability to ferment fructose, for example, can properly develop into a pineapple or orange peels wastes with consequent production of desired product [13,14]. Thus, the effects of mono and disaccharides on the yield of the desired product/metabolite should be evaluated in advance, as a potentially indicator to

disclose a particular use of wastes and byproducts. The effect of different carbon sources (mono and disaccharides) on bioethanol production under shake-flask culture conditions using K. marxianus CCT 7585 is shown in Figure 2.

The results of the present study show that lactose – a disaccharide composed of two hexose units, galactose and glucose – exhibited the highest bioethanol concentration when compared to other mono/disaccharides. Interestingly, its monosaccharide constituents (glucose and galactose), used alone or in combination, did not provide good results in the bioethanol production, which was expected since no synergistic effect was observed – glucose → glycolysis → bioethanol production plus galactose → galactose-1-phosphate → glucose-1phosphate → glycolysis → bioethanol production (Figure 5 A) [15,16]. When glucose and galactose were used in combination, a statistically significant reduction in bioethanol production was observed. Probably, lactose may have influenced an enzyme induction - lactose permease and β-galactosidase – which did not occur when glucose and galactose were used in combination. Lactose permease is responsible for transporting lactose into the cytoplasm, and β-galactosidase hydrolyzes lactose into glucose and galactose. In other words, when lactose is used, a higher concentration of galactose and glucose is observed within the cell [17]. Thus, the bioethanol production from CWP solution (high lactose content) using K. marxianus CCT 7585 seems to be reasonable and logical (Figure 3).

This experiment (Figure 3) has proven that K. marxianus CCT 7585 was able to significantly decrease lactose content, which certainly will provide low COD and BOD rates. Satisfactory bioethanol yields were also observed (≈ 4% v/v) when compared with other studies on bioethanol production by K. marxianus strains (Table 1) [9,18].

The effect of the enzymatic hydrolysis on bioethanol production using CWP and lactose is shown in Figure 4. The magnitude of influence of unhydrolizate CWP (high lactose content) on bioethanol production was lower than the hydrolyzate CWP (high glucose and galactose). This change was not expected, once preliminary results have shown an opposite behavior (Figure 5 B). To our knowledge, yeasts possess a sophisticated mechanism for sensing glucose and responding it appropriately. Glucose transporters (HXTs) play a part in the glucose signaling [19]. Recently, whey protein showed to have a significant increase in glucose transporter translocation to plasma membrane [20]. In addition, enzymatically hydrolyzed CWP has obviously an increase of glucose and galactose units. Thus, considering the direct effects of whey protein on glucose transporter translocation, it is assumed that the glucose levels remain significantly higher (within yeast) in hydrolyzate CWP than in unhydrolyzate CWP (Figure 6). This explains why the bioethanol production was improved in hydrolyzed CWP, as well as the lactose group (without whey protein) had lower bioethanol yields (Figure 4).

Other details in Fig 4 showed that organic and inorganic components from CWP have a positive effect (10%) on bioethanol production when compared to lactose alone. This could be due to the competitive inhibition of Zn^{2+} or inhibition effects of metal ions (present in CWP) on glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) [21–23]. G6PD is a key enzyme that initiates the reactions of the pentose phosphate pathway, catalyzing the conversion of glucose-6-phosphate to 6-phospho-gluconate in the presence of nicotinamide adenine dinucleotide phosphate (oxide form, $NADP^+$) [21]. It is found in various organisms including the genus Kluyveromyces [24]. This inhibition may cause important physiological changes, e.g. increasing production of pyruvate, which plays important role in the alcoholic fermentation.

5. CONCLUSIONS

This study evidenced that K. marxianus CCT 7585 was capable to decrease the lactose content in CWP solution, which certainly will provide low COD and BOD rates. Satisfactory bioethanol yields were also observed (≈ 4% v/v), which has encoraged us to investigate new approaches to be potentially applied to increase ethanol yields such as yeast fusants, mutants and co-culture techniques. Hydrolysis of CWP can be suggested as an inductor process to increase bioethanol yield (increases of around 6%). Despite the ethanol production from K. marxianus CCT 7585 using CWP solution cannot replace currently available strategies for ethanol production from sugar cane bagasse (traditional brazilian perspective), it can complement them.

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Figures

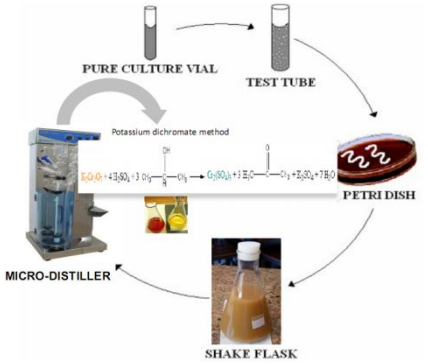

Figure 1

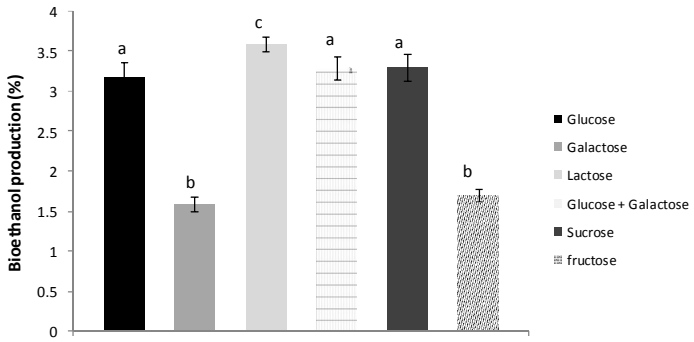

Figure 2

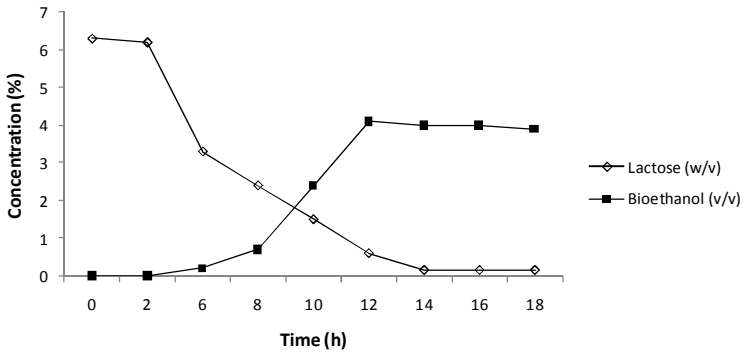

Figure 3

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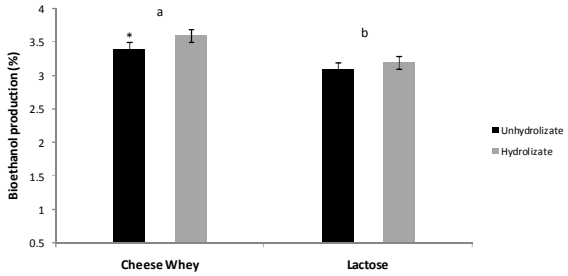
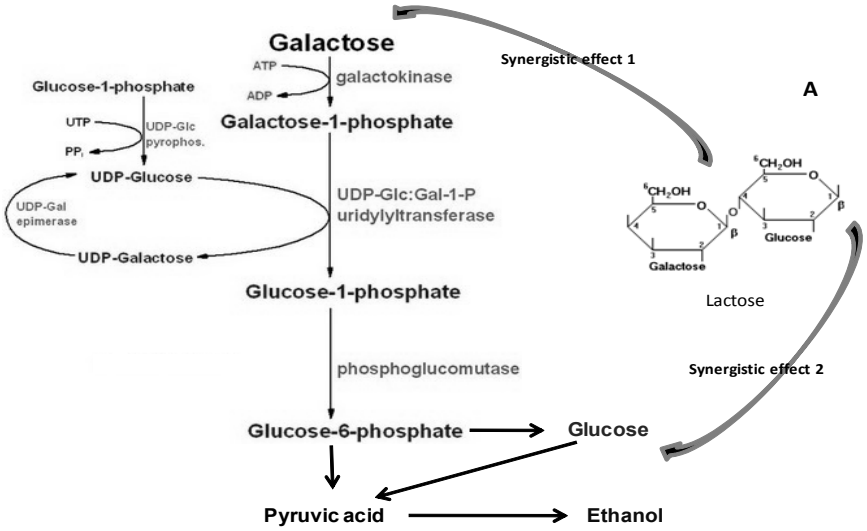


Figure.4



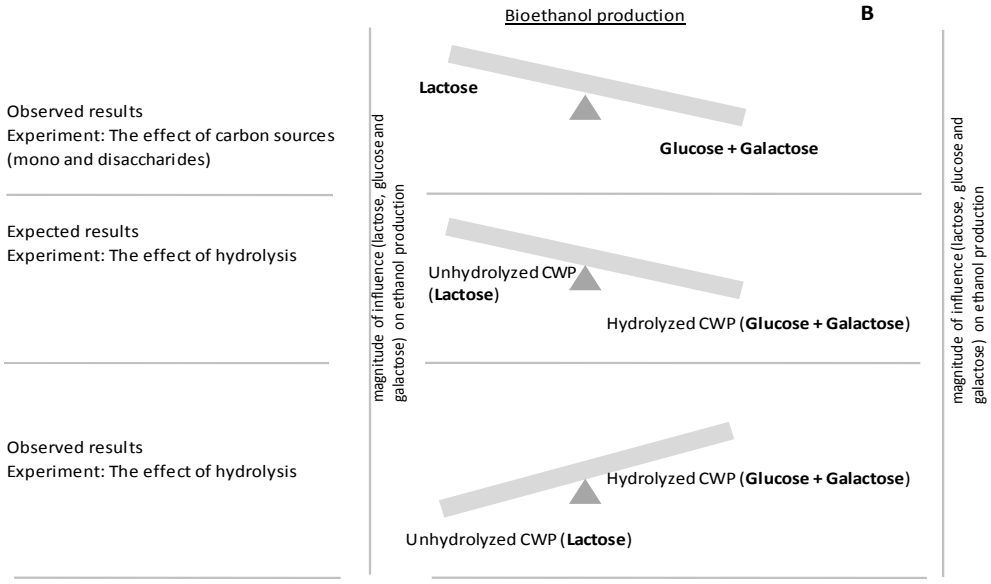


Figure 5

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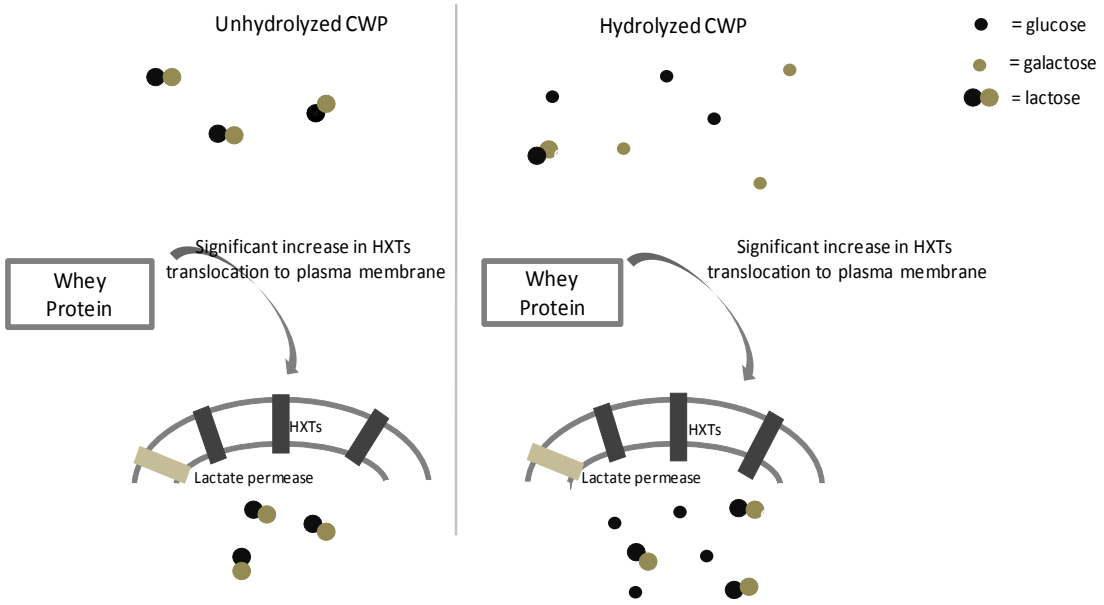

Figure 6

Figure captions

Figure 1- Preparation of k. marxianus CCT 7585 culture and ethanol quantification method.

Figure 2- Effect of different carbon sources (mono and disaccharides) on bioethanol production under shake-flask culture conditions. Data are mean ± SEM values of triplicate. ^{b,c} $p < 0.05$ (same letters are not significantly different at the 0.05 level according to statistical analyses as compared to glucose group).

Figure 3- Time course on bioethanol production and lactose consumption in CWP solution under shake-flask culture conditions.

Figure 4- Effect of hydrolysis on bioethanol production. CWP solution and lactose medium was used for this experiment under shake-flask culture conditions. Data are mean ± SEM values of triplicate. ^{b} $p < 0.05$ compared to CWP group. * $p<0.05$ variation within groups.

Figure 5 – (A) Galactose metabolism (B) Observed and expected results from the experiments: "Effect of different carbon sources (mono and disaccharides) on bioethanol production under shake-flask culture conditions " and "Effect of hydrolysis on bioethanol production ". According to [25].

Figure 6 – Schematic diagram of whey protein effect on HTXs translocation. According to [20].

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Microorganism	Substrate	Ethanol yield	Reference
K. fragilis	CWP	80.95 Kg m^{-3}	[26]
K. marxianus	CWP	3.8% (v/v)	[9]
K. marxianus IMB3	CWP	22.5 $g.L^{-1}$(packed – columm bioreactor)	[27]
K. marxianus DSMZ 7239	CWP	3.7 % (v/v)	[18]

Table 1- Bioethanol production from fermentative process by native yeast strains of K. marxianus.

Substrate	Microorganism/ Culture conditions/ Extraction conditions	Product obtained	Reference
CWP	K. marxianus TY-22 Saccharomyces cerevisiae AY-5	Ethanol	[7]
CWP	Mesophilic /Termophilic dark fermentations	Hydrogen gas	[28]
CWP	Wetland systems	Macro-sized nutrients	[29]
CWP	K. fragilis	Ethanol	[26]

Table 2- Application of CWP for obtaining new products.

Mono and disaccharides utilized in this study	Wastes/Byproduct	Reference
Glucose	Waste copier paper (hydrolyzed), exhausted coffee waste	[30,31]
Galactose	Solid wastes obtained from enzymatic extraction of soybean oil, Nonforage byproduct feeds (dicotyledonous)	[32,33]
Lactose	Cheese whey	[34]
Sucrose	Sugarcane molasses, waste sweet potato	[35,36]
Fructose	Pineapple waste (Pineapple crown extract), orange peel wastes	[13,14]

Table 3 – Wastes containing predominantly the following mono and disaccharides.

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CONFLICT OF INTEREST

The authors declare that there is no duality of interest associated with this study.

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