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Isolation, identification of yeast strains producing bioethanol and improvement of bioethanol production on cheese whey

Biyoetanol üreten maya türlerinin izolasyonu, tanılaması ve peynir altı suyunda biyoetanol üretiminin sağlıklıştırılması

ABSTRACT: Objective: In this study, bioethanol production on cheese whey (prehydrolysed enzymatically using β galactosidase by isolated yeast strains was investigated.

Methods: The yeast strains were isolated from Algerian natural sources (soil and grape) and further were selected on the basis of high ethanol tolerance and high ethanol production on prehydrolysed cheese whey. The selected ones were identified by morphological, physiological and biochemical characteristics. Then, a molecular identification was carried out by amplification and sequencing the D1/D2 domain of 26S rDNA region. In addition, the operating parameters of fermentation such as temperature, pH and substrate concentration (mixture of glucose and galactose) were tested for efficient yeast strain.

Results: Among the selected and identified yeast strains, three strain isolates were found to be able to produce bioethanol. These strains are Hanseniaspora opuntiae Z087A0VS, Candida tropicalis Z087B0VS and Candida tropicalis Z087D0VS with an identity of 99% and 100% respectively comparing with the stocked strains in data bank. Furthermore, Hanseniaspora opuntiae presents an ethanol tolerance ethanol up to 11% whereas the two other strains of Candida tolerate up to 12%. The fermentation parameters of most efficient strain were optimized, the temperature 30°C, pH 5 and sugar concentration (glucose and galactose) of 12.5% (w/v) are considered as optimum values for Candida tropicalis Z087B0VS.

Conclusion: Candida tropicalis Z087B0VS can be considered as a good candidature for industrial bioethanol production.

KEYWORDS: Cheese whey, bioethanol, yeast, isolation, fermentation, lactose hydrolysis

ÖZET: Amaç: Bu çalışmada izole edilmiş maya türleri ile peynir altı suyunda (β galactosidaz kullanılarak enzimatik olarak prehydrolızte edilmiş) biyoetanol üretimi çalışılmıştır.

Metod: Maya türleri Cezayir’deki doğal kaynaklardan (toprak ve üzüm) izole edilmiştir. Daha sonra, prehydrozize edilmiş peynir altı suyunda etanol toleransı ve yüksek etanol üretimi açısından yeniden seçilmiştir. Seçilen türler morfolojik, fizyolojik ve biyokimyasal özellikleri açısından tanımlanmıştır. Moleküler tanımlama ise 26SrDNA’nın D1/D2 bölgesinin amplifikasyonu ve dizilmesi ile yapılmıştır. Buna ek olarak sıcaklık, pH, ve substrat konsantrasyonu (glukoz ve galaktosu karşıtı) gibi operasyon parametreleri her türe için verimliliği açısından test edilmiştir.

Bulgular: Elde edilen tanımlanmış maya türlerinden üç tür...
izolatı biyoetanol üretebilir olarak elde edilmiştir, bunlar Hanseniaspora opuntiae Z087A0VS, Candida tropicalis Z087B0VS ve Candida tropicalis Z087D0VS'dir. Bu türler veri bankalarında %99 ve %100 tanınamış türlerdir. Buna ek olarak, etanol toleransı açısından Hanseniaspora opuntiae %11'e dayanırken diğer iki Candida türü %12'ye dayanabilmiştir. En etkin tür olan Candida tropicalis Z087B0VS için fermentasyon parametreleri sıcaklık 30°C, pH 5 ve şeker konsantrasyonu (glukoz ve galaktoz) %12.5 (w/v) olarak optimize edilmiştir.

Sonuç: Candida tropicalis Z087B0VS endüstriyel biyoetanol üretimi için uygun bir tür olarak değerlendirilebilir.

Anahtar Kelimeler: Peynir altı suyu, biyoetanol, maya, izolasyon, tanılama, fermentasyon, laktoz hidrolizi

Introduction

Yeasts are unicellular eukaryote fungi. They play an important role in the production of traditional food such as bread; beer and wine. They contribute also in the development of desirable flavor during cheese ripening [1]. In addition, they are ubiquitous in environment. Yeasts are isolated from natural substrates like leaves, flowers, sweet fruits, grains, exudates of trees, insects, dung and soil [2].

Recently, yeasts are used in the production of ethanol from various agricultural and industrial wastes. Further, this product has great applications in chemical, pharmaceutical and food industries in the form of raw materials, solvent and fuels [3]. Among these industrial wastes, cheese whey represents an important source of environmental pollution. It shows a high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) which are 60000 ppm and 50000–80000 ppm respectively [4]. The most representative compound of this whey is lactose which can function as carbon substrate for growth and product formation in numerous biotechnological processes such as bioethanol production [5]. Many reports have shown the potential applications of yeast strains in bioethanol production from cheese whey but most of them focus on Kluyveromyces sp due to its ability to directly ferment lactose [6,7]. However lactose fermenting yeast strains are more sensitive to high ethanol concentrations and they are known by their relatively slow growth [8].

Besides, indirect fermentation organisms represent an interesting alternative because ethanol production yield and relative alcohol tolerance are much higher [9]. The fact that these yeasts are incapable of directly fermenting lactose, it would be necessary to hydrolyze lactose enzymatically with beta galactosidase into monosaccharides, glucose and galactose, which are readily and efficiently fermented.

In the present study, our aims consist of isolating some yeast strains producing bioethanol on prehydrolyzed cheese whey from Algerian natural source (soil and grape) and further identify them using biochemical and molecular methods. Then, to test the fermentation performance of the selected yeast strain.

Materials and Methods

Preparation and pretreatment of medium

Cheese whey, containing 6% w/v of lactose, was obtained from cheese factory of Boudouaou (Boumerdes, Algeria). It was deprotenized as follow:

The pH of cheese whey was adjusted to 4.6 with 1N HCl. Then, the cheese whey was heated at 90°C for 10 min and it was cooled down later in a room temperature. This treatment induced the precipitation of proteins which were removed further by filtration through filter paper. The obtained medium was adjusted to pH 4.5 and it was autoclaved at 121°C for 20 min [10]. The lactose hydrolyse in whey was conducted according to Dos Santos et al. [11] with some modification: In a 250 ml erlenmeyer flask 0.5 mg of beta galactosidase from Aspergillus oryzae G 5160-500 ku (Sigma), was added aseptically into100 ml of sterilized cheese whey. Then, the flask was incubated at 30°C for 24h. This pretreatment assures the conversion of all quantity of lactose to monosaccharides, glucose and galactose. The final glucose concentration was 2.5% w/v.

Collection of samples

Samples were aseptically collected from Algerian natural sources (soil and grape) using sterile bottles which were immediately transported to the laboratory and kept at 4°C.

Isolation and selection of yeast strains

10 g of soil were suspended in 90 ml of sterile physiologic water and homogenized in a vortex for 10 min whereas grape had a spontaneous fermentation for 10 days [12]. For the two sources, serial dilutions were made until 10⁻⁵. One milliliter of the obtained solution (soil and grape) was transferred into Petri dishes containing Yeast Glucose Peptone Agar medium (YGPA) with a composition (g/l) of Yeast extract 10,
Glucose 20, Peptone 5 and Agar 20. The plates were incubated at 30°C for 48 hours; chloramphenicol was added to YGPA at 0.05 mg/ml to inhibit bacteria growth. The developed yeast colonies were further purified by subculturing on Petri dishes containing the same medium.

After purification, all the isolated strains were tested for their ability to produce a high bioethanol concentration on pretreated cheese whey using an enzymatic hydrolysis with β-galactosidase. For any strain, a colony from agar slant was transferred to glass tube containing 10 ml of Yeast Glucose Peptone broth (YGP) and incubated at 30°C for 24 hours. Five milliliters of this culture were then transferred into 100 ml erlenmeyer flasks containing 20 ml of sterilized YGP broth and maintained at 30°C in a rotary shaker at 150 rpm for 18 hours [13]. After this incubation, the active cells were centrifuged at 8000 g for 10 min and then washed with sterile distilled water to be used as inoculums. The fermentation was carried out at 30°C and pH 5 with agitation of 150 rpm for 24 hours. At the end of experiment, the ethanol was quantified and the yeast isolates were selected based on their ethanol amount produced.

**Ethanol tolerance test**

The yeast isolates were examined for their tolerance to high alcohol concentrations according to the method of Osho [14]. These yeast strains were transferred into 10 ml of YGP broth containing different concentrations of ethanol (0%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 15% and 20% (v/v)). All the cultures were conducted with same initial cellular concentration at 3x10^6 cells /ml and further incubated at 30°C for 48 hours. After this incubation period, the cell concentration was determined by measurement of the optical density at 600 nm which was correlated with the cell count. The increase in optical density in tubes was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts [15].

**Physiological and biochemical identification**

The selected yeast strains were morphologically, physiologically and biochemically characterized according to standard methods as described by Kurtzman and Fell [16].

**Molecular identification**

The molecular characterization of these strains was realized according to White et al. [17] DNA was extracted from yeast strains isolate by using QIA quick Genomic Extraction Kit. Then, the polymerase chain reaction (PCR) was performed for the 26S rDNA D1/D2 domain using the following primers: LROR 5’ACCCGCTGAACCTGAAGC3’ and LR6 5’CGCCAGTTCTGCTTACC3’. It was conducted in a final volume of 50 µl containing 10 Mm Tris HCl, 50 mM KCl, 1.5 mM MgCl2, dNTP at concentration of 0.2 mM, 1.25 IU of Taq polymerase, each primer at concentration of 0.2 mM and 0.2 µl of the DNA. This reaction was carried out in DNA thermal cycler (Biometra, Germany) at an initial 5 min denaturation step at 94°C followed by 35 cycles of denaturation step at 94°C for 1min, annealing step at 56°C for 1 min and extension step at 72°C for 2 min followed by a final extension step at 72°C for 10 min. After 35 cycles, the PCR products and their restriction fragments were visualized by 1.5% agarose gel electrophoresis. Moreover, the sequencing results were aligned with those deposited in the gene bank of the National Center for Biotechnology and Information (NCBI), using the basic local alignment search tool (BLAST) and the identification of species was determined based on the best score. The phylogenetic tree construction was done by the neighbor joining method using mega 6 logiciel [18].

**Analytical methods**

Cell growth was determined by optical density measurement at 600 nm using UV visible spectrophotometer Varian 50 Tablet and cell numeration on Thoma hemacytometer [19]. A standard curve of absorbance versus cell population was established. One unit of absorbance was equivalent to 2.43 x10^7 cells /ml. The yeast viability was determined by methylene blue staining method [20]. The ethanol content was expressed as % (v/v). It was directly measured from the samples of the fermentation by ebulliometer Dujardin Salleron (Paris, France) using 50 ml of sample in each analysis. The temperature was read at the lowest constant boiling point and the blank reading was taken using distilled water. The zero of the scale was adjusted to the boiling point of water and the percentage of alcohol % (v/v) was read from the scale [21,22]. The glucose released during the lactose hydrolysis was measured by GOD-POD (glucose oxidase-peroxidase) method. Before analysis; a standards calibration curve was established between glucose concentration and absorbance at 505 nm using spectrophotometer [23]. The initial lactose content of cheese whey was determined through phenol-sulfuric acid method [24]. The degree of hydrolysis achieved after 24 h was determined based on the amount of lactose and D-Glucose in the sample.
Effect of temperature, pH and substrate concentration on bioethanol production

The effect of temperature, pH and substrate concentration was investigated by cultivating the best isolated yeast strain producing ethanol at different temperatures (25°C to 40°C), pH (3 to 7) and various glucose and galactose concentrations (5% to 25% w/v).

Results and Discussion

Selection of yeast isolates producing ethanol

In this study, 20 different yeast strains were isolated from natural Algerian sources (grape and soil) using YPG broth containing 0.05 mg/ml of chloramphenicol. Among them, three yeast isolates were found to be bioethanol producing on pretreated cheese whey. They were designated as S₁ isolated from grape, S₂ and S₃ isolated from soil. According to the Fig. 1, all these strain isolates produce a bioethanol but at different amounts. The strain isolate S₂ presents a high ethanol production (2.3% v/v) comparing with the others S₁ and S₃ with ethanol content 1.6% and 1.77% respectively.

Ethanol tolerance

The Fig. 2 represents the ethanol tolerance of our yeast isolates. It has shown that all the three stains have the ability to resist up to 11% (v/v). Above this value, only strains S₂ and S₃ are able to tolerate ethanol at concentration of 12%. According to Birch and Walker [25], the ethanol concentration reduces cell viability and increases cell death. This can be explained by Hu et al. [26] who reported that ethanol influences cell metabolism and a macromolecular biosynthesis by inducing the generation of heat stock-like proteins, lowering the rate of RNA and protein accumulation, enhancing the frequency of small mutations, altering metabolism, denaturing proteins and intracellular glycolytic enzymes and reducing their activity.

Our finding data are in agreement with other research studies. As indicated by Tikka et al. [27], the range of ethanol tolerance obtained for their yeasts was 7–12%. Matapathi et al. [28] found resistant yeasts in lower levels (5 to 7.5%). In another study, Ingram and Buttke [29] were able to isolate yeasts tolerating ethanol in the range of 13 to 20%. To give an explication about this difference in ethanol tolerance, Stanley et al. [30] signaled that the outcomes of genome-wide screens for ethanol tolerance commonly report genes associated with vacuole function and amino acid biosynthesis as being important for ethanol tolerance. The importance of the former may be related to the need for vacuole based functions such as homeostasis of intracellular pH, maintenance of ion concentration and protein degradation, at a time when ethanol stress has disturbed electrochemical gradients and initiated considerable protein turnover in the cell.

Identification of yeast isolates

The primary identification of the yeast isolates is carried out on the basis of morphological characteristics of colonies on solid media (Table 1). As a result, the colonies are entire, creamy and smooth with absence of reddish diffus-
ing pigments. They have also a butyrous texture showing pseudophae formation especially for the two strains isolates S$_2$ and S$_3$. Moreover, The microscopic observation reveals that all strain isolates present ovoid cell shape with budding asexual reproduction (Fig. 3).

Subsequently, these strain isolates are identified by physiological characteristics using several biochemical tests including fermentation of different sugars, assimilation of carbon and nitrogen compound (Table 2). According to these findings, we can notice that the assimilation of sugars are variable from strain to another, it appears clearly that our isolates are capable to ferment glucose in their growth. Besides, all strain isolates show a negative result with lactose. The galactose is well assimilated and fermented by all the strains except S$_1$. In addition, the isolates S$_2$ and S$_3$ are able to ferment xylose which was not assimilated by the strain S$_1$. Concerning the nitrogen assimilation test, all the isolate strains have positive results with ethylamine, L lysine, cadaverine, putrescine except proline which is not assimilated by the isolate S$_1$.

Comparing all these results to the characteristics cited by Kultzman and Fell. [16] and Gadaga et al. [31], our selected isolate yeast strains are identified as belonging to two different genus; *Candida* and *Hanseniaspora*. The strain isolates S$_2$ and S$_3$ are able to ferment xylose during their growth which is a characteristic of *Candida* species. On contrary, the other isolated strain (S$_1$) has a negative xylose test. Kim et al. [32] cited that these strains lack xylose reductase and xylitol dehydrogenase gene responsible of xylose fermentation. As shown in Tables 1 and 2, the isolates S$_2$ and S$_3$ closely resemble to *Candida tropicalis*. Several researches signaled that this strain isolate is pathogen. Ellis et al. [33] cited that *Candida tropicalis* is considered like agent of candidiasis. However, many previous studies used this microorganism in biotechnology. Kim et al. [34] reported that *Candida tropicalis* has been used extensively for xylitol production from xylose. Jeffries [35] signaled also the use of *Candida tropicalis* ATCC 1369 under aerobic conditions for ethanol production from xylose. Another research showed an ethanol production via fermentation of rice straw by *Candida tropicalis* ATCC 13803 [36].

Furthermore, the isolate S$_1$ seems to be more closely to *Hanseniaspora* sp, it is incapable to ferment galactose while the other isolates have positive results. According to Yun et al. [37], the ability of yeast cells to assimilate galactose indicates the expression of Gal genes. Our results confirm previous observations of Lee et al. [38] who indicated that this strain is the most frequently isolated native species from undamaged grapes and may account for over

| Strain 1 (S$_1$) | Strain 2 (S$_2$) | Strain 3 (S$_3$) |
|-----------------|-----------------|-----------------|
| Colony margin   | Entire          | Entire          | Entire          |
| Colony colour   | Cream           | Cream           | Cream           |
| Colony appearance | Smooth          | Smooth          | Smooth          |
| Colony texture  | Butyrous        | Butyrous        | Butyrous        |
| Filaments       | No Hyphae       | Pseudohyphae    | Pseudohyphae    |
| Asexual reproduction | Budding (bipolar budding) | Budding (multilateral budding) | Budding (multilateral budding) |
| Cell shape      | Lemon and oval  | Oval            | Oval            |
| Ascospores shape| –               | –               | –               |
| Sexual reproduction | –              | –               | –               |

(-): Negative test.

Figure 3: Microscopic characteristics of isolated yeast strains cultivated on YPG broth at 30°C after 24 h (G X100). (a) (S$_1$), (b) (S$_2$), (c) (S$_3$).
50% of the total yeast flora from fruits.

These results are confirmed by molecular identification. After PCR amplification and sequencing, the obtained sequences are compared by Blast algorithm with those stocked in database of National Center for Biotechnology and Information (NCBI). The phylogenetic analysis of strain isolates confirms that the isolate S1 is belonged to Hanseniaspora opuntiae with a similarity of 99% whereas the strains S2 and S3 are identified as Candida tropicalis with a similarity of 100%. They are coded respectively Z087A0VS, Z087B0VS and Z087D0VS. All their sequences are presented in Fig. 4.

**Effect of operating parameters on bioethanol production**

In our study, Candida tropicalis Z087B0VS (S2) is very interesting because we are looking for strains which have efficiency yield of bioethanol production from both glucose and galactose. For this reason, the fermentation parameters were optimized only for this strain isolate.

**Effect of temperature**

On the basis of temperature profile (Fig. 5), the optimum temperature for ethanol production appears to be 30°C for our strain isolates with maximum ethanol values of 2.33%. Michelle [39] suggested that the yeasts in this case are under minimal stress and are not inhibited by the produced ethanol. In addition, at 35°C and 40°C, the ethanol yield decreases with an increase of fermentation temperature. According to Phisalaphong et al. [40], the increase in temperature accelerates the inhibition effect of the ethanol on the cell activities by lowering both cell and

| Table 2: Biochemical characteristics of yeast strain isolates. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Fermentation                   | S1             | S2             | S3             | S1             | S2             | S3             | S1             | S2             | S3             |
| D-Glucose                      | +              | +              | +              | Raffinose      | –              | –              | –              | –              | –              |
| D-Galactose                    | –              | +              | +              | Melezitose     | –              | +              | +              | –              | +              |
| Maltose                        | –              | +              | +              | Inulin         | –              | –              | –              | –              | –              |
| Me a-D-Glucoside               | –              | –              | –              |                |                |                |                |                |                |
| Sucrose                        | –              | +              | +              | Glycerol       | –              | w              | –              | –              | –              |
| A,A-Trehalose                  | –              | +              | +              | Erythritol     | –              | –              | –              | –              | –              |
| Melibiose                      | –              | –              | –              | Ribitol        | –              | +              | +              | –              | –              |
| Lactose                        | –              | –              | –              | Xylitol        | –              | –              | –              | –              | –              |
| Cellobiose                     | –              | –              | –              | L-Arabinitol   | –              | –              | –              | –              | –              |
| Melezitose                     | –              | –              | –              | Raffinose      | –              | –              | –              | –              | –              |
| Raffinose                      | –              | –              | –              | Melezitose     | –              | +              | +              | –              | –              |
| Inulin                         | –              | –              | –              | Galactitol     | –              | –              | –              | –              | –              |
| **Carbon assimilation**         |                |                |                |                |                |                |                |                |                |
| D-Glucose                      | +              | +              | +              | D-Glucono-1,5-lactone | +    | w              | –              | –              | –              |
| D-Galactose                    | –              | +              | +              | 2-Keto-D-Glucurate | +    | +              | +              | +              | +              |
| L-Sorbose                      | –              | +              | +              | 5-Keto-D-Glucurate | –    | –              | –              | –              | –              |
| D-Glucosamine                  | –              | w              | –              | D-Glucurate    | +              | w              | +              | –              | –              |
| D-Ribose                       | –              | w              | –              | D-Glucuronate  | –              | –              | –              | –              | –              |
| D-Xylose                       | –              | +              | +              | D-Galacturonate | –              | –              | –              | –              | –              |
| L-Arabinose                    | –              | w              | –              | Succinate      | –              | +              | +              | –              | –              |
| D-Arabinose                    | –              | –              | –              |                |                |                |                |                |                |
| L-Rhamnose                     | –              | w              | –              | Nitrate        | –              | –              | –              | –              | –              |
| Sucrose                        | –              | +              | +              | Nitrite        | –              | –              | –              | –              | –              |
| Maltose                        | –              | +              | +              | Ethylamine     | +              | +              | +              | –              | –              |
| A,A-Trehalose                  | –              | +              | +              | L-Lysine       | +              | +              | +              | –              | –              |
| Me a-D-Glucoside               | –              | +              | +              | Cadaverine     | +              | +              | +              | –              | –              |
| Cellobiose                     | +              | +              | +              | Creatine       | –              | –              | –              | –              | –              |
| Salicin                        | +              | –              | –              | Creatinine     | –              | –              | –              | –              | –              |
| Arbutin                        | w              | –              | –              | Glucosamine    | –              | –              | –              | –              | –              |
| Melibiose                      | –              | –              | –              | Imidazole      | –              | –              | –              | –              | –              |
| Lactose                        | –              | –              | –              | D-Tryptophan   | –              | –              | –              | –              | –              |

(+): Positive growth reaction; (-): Negative growth reaction; (w): Weak growth reaction.
ethanol yields. The increasing of temperature is mainly due to the death of yeast cells [41].

Our results are agreed with Jamai et al. [42] who found a temperature of 30°C as optimum for bioethanol production by Candida tropicalis whereas Sripiromrak [43] reported a temperature of 40°C for Candida tropicalisNCYC405.

Effect of pH

pH plays an important role in bioethanol production. As shown in Fig. 6, it clearly appears that pH 5 represents an optimum value for Candida tropicalis Z087B0VS (S) which have maximum ethanol concentrations of 2.19%. Our results are in concordance with Narendranath and Power [44], Who reported that the yeast is an acidophilic organism which grows better under acidic conditions. The optimal pH range for yeast growth can vary from 4 to 6. Fakruddin et al. [45] found as optimum pH 6 for Candida tropicalis. Moreover, as indicated in Fig. 6, any increase or decrease of pH up to the optimum for all strain isolates leads to a decrease of ethanol yield. More acidic and basic conditions retard the yeast metabolic pathways and hence the cells growth due to a lower ethanol yield [46].

Effect of substrate concentration

The concentration of sugar (mixture in equivalent mass of glucose and galactose) is tested in the range of 5 to 25% (w/v) and the results are illustrated in Fig. 7. We observe that the strain Candida tropicalis Z087B0VS (S) expresses the highest sugar tolerance at 12.5% with 6.4% of ethanol.

As indicated in Fig. 7, it appears that ethanol yield increases from 5 to 12.5% of reducing sugar concentration.
but above this value it decreases slightly with an increase of sugar concentration. Bisson and Fraenkel [47] reported that high substrate concentration negatively hampers ethanol productivity leading to a lower titer due to the repression of glycolytic enzymes. High concentrations of glucose in the medium inhibit the yeast cells growth and the ethanol production. This inhibitory effect is attributed to high osmotic pressure [14].

Conclusion

Twenty yeast strains were isolated from two natural Algerian sources (soil and grape). Among them, three strains were selected for their ability to produce ethanol from both glucose and galactose. Based on the phenotypic and genotypic results, we conclude that these isolated yeast strains are found to be Hanseniaspora opuntiae Z087A0VS, Candida tropicalis Z087B0VS and Candida tropicalis Z087D0VS with an identity of 99% and 100% respectively comparing with stocked strains in data bank. Moreover, the analysis of the fermentation characteristics under different substrate concentrations and environmental conditions, showed that the temperature of 30°C, pH 5 and sugars concentration of 12.5% w/v (mixture of glucose and galactose) are optimum for Candida tropicalis Z087B0VS. Therefore, this selected strain might be a good candidate for bioethanol production on lactose hydrolyzed cheese whey.

Conflict of Interest: The authors have no conflict of interest.

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