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Production of the Food Enzyme Acetolactate Decarboxylase (ALDC) from *Bacillus subtilis* ICA 56 Using Agro-Industrial Residues as Feedstock

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Abstract: During the beer brewing process, some compounds are formed in the primary fermentation step and may affect the final quality of beer. These compounds, called off flavors, such as diacetyl, are produced during fermentation and are related to a buttery taste. The use of acetolactate decarboxylase (ALDC) in the traditional beer brewing process may significantly increase productivity since it allows for a faster decrease in the adverse flavor caused by diacetyl. However, production costs directly impact its application. For this reason, we analyzed the effect of different cultivation media on ALDC production by *Bacillus subtilis* ICA 56 and process economics. Different carbon and nitrogen sources, including agro-industrial residues, were evaluated. The best result was obtained using sugarcane molasses and corn steep solids (CSS), allowing a 74% reduction in ALDC production cost and an enzyme activity of 4.43 ± 0.12 U·mL^{-1}. The enzymatic extract was then characterized, showing an optimum temperature at 40 °C and stability at different pH levels, being able to maintain more than 80% of its catalytic capacity between pH values of 3.6 and 7.0, with higher enzymatic activity at pH 6.0 (50 mM MES Buffer), reaching an ALDC activity of 5.30 ± 0.06 U·mL^{-1}.

Keywords: beer fermentation; cost analysis; diacetyl; sugarcane molasses; corn steep solids

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1. Introduction

The most important step in beer production is fermentation. During this process, different compounds are formed, such as ethanol and carbon dioxide, and others in smaller amounts, one of them being diacetyl [1], a product of yeast metabolism. Diacetyl is produced in greater amounts in primary fermentation, and it is pointed out as an undesirable flavor compound. Diacetyl is a compound that most of the time is undesirable for beer. It is an off flavor, found naturally in various vegetable oils and beverages such as beer and wine, and confers to beer a "buttery" taste. Thus, in large breweries, it is an important quality parameter that needs to be removed from the process [2,3]. In large breweries, there are periods when beer demand increases considerably, so in order to meet the demand, it is necessary that the processes can be completed as quickly as possible. For diacetyl elimination, α-acetolactate decarboxylase (ALDC), which is an enzyme that converts α-acetolactate directly into flavorless acetoin, can be used without producing diacetyl [2,4,5]. This will make the stage of maturation of the beer faster, thus being able to accelerate the other processes and increase the brewing production. In addition, acetoin is a platform chemical, and the bio-based product, which can be obtained by the enzymatic route, is considered petrol-independent [2,6].

ALDC can be widely obtained from several microorganisms, such as *Bacillus subtilis* [2,6], *Bacillus brevis* [7], *Brevibacillus brevis* [8], *Enterobacter aerogenes* [9,10], *Enterobacter cloacae* [11], *Klebsiella pneumonia* [12], *Acetobacter aceti* subsp. *xylinum* [13], *Streptococ-*

cus thermophiles [14], and *Lactococcus lactis* subsp. *lactis* [15]. Due to its ability to catalyze α-acetolactate into acetoin, ALDC can be used in the food and cosmetic industries [16]. Furthermore, ALDC can also catalyze the decarboxylation of racemic acyclic and cyclic α-hydroxy-β-ketoacids, which may contribute to synthetic organic and medicinal chemistries [14].

ALDC, like other enzymes, has excellent functional properties, such as specificity, activity, and selectivity, and can catalyze complex biochemical processes under simple environmental conditions, such as mild conditions of temperature, neutral aqueous conditions, and absence of substrate functional group protection [1]. They can be excellent catalysts in the chemical and food industries, for example. On the other hand, the costs of production sometimes disfavor their use in industrial processes. The cost of medium can considerably impact the overall process economics since it requires costly nitrogen sources from microbes (such as yeast extract) and expensive components (such as glucose). Therefore, giving insight into the influence of medium formulation on overall economics would help in defining a feasible process for ALDC production at a large scale.

High added-value products can be obtained from reusing agro-industrial residues, using the concept of circular economy, for instance, in the formulation of culture media, providing nutrients for microbial growth or product formation [17]. Enzymes [17–19], biopolymers [20], biosurfactants [21], and microbial oils produced by oleaginous microorganisms [22] have been widely reported as examples of products achieved when using an alternative culture medium prepared by agricultural wastes and food processing by-products. Since the use of these residues may decrease the production costs associated with raw materials, they are often reported as a way to achieve high yields using inexpensive raw materials, with low capital costs [23].

In general, the culture media used on the laboratory scale are not economically viable for large-scale application. In this way, the search for cheaper sources of carbon and nitrogen is of great importance to the success of scale-up. Nevertheless, to the best of our knowledge, the cost implications of different media on ALDC production have not been considered nor reported. Therefore, in this work, a preliminary cost analysis of the culture medium was conducted to provide a convenient guide for the selection of a cultivation strategy. For that, different culture medium compositions were evaluated in order to optimize the production process of the ALDC from *Bacillus subtitlis* ICA 56. Six carbon sources and five nitrogen sources were tested, including two agro-industrial residues, namely sugarcane molasses and corn steep solids (CSS). Molasses, a dark brown and viscous liquid, is a by-product of sugar refineries. It is rich in sugars (mainly sucrose, glucose, and fructose) and contains amino acids, vitamins, and inorganic salts (such as $SO4^{2-}$, Ca^{2+}, Na^{+}) [24,25]. CSS (spray-dried corn steep liquor at 95% of dry matter) is an industrial by-product of corn wet-milling processes and abounding in nutrients such as carbohydrates, amino acids, polypeptides, inorganic salts, and vitamins [26,27].

2. Materials and Methods

2.1. Microorganism

Bacillus subtilis ICA56 was previously isolated from a Brazilian mangrove and belongs to the bacterial collection of the Laboratory of Microbial Ecology and Biotechnology (LEMBIOTECH, Fortaleza, Brazil) of the Department of Biology of the Federal University of Ceará. Its 16S rRNA gene sequence can be accessed in the GenBank (KM235112). The strain was maintained on ATGE medium slant (15.0 $g{\cdot}L^{-1}$ agar, 5.0 $g{\cdot}L^{-1}$ triptone, 5.0 $g{\cdot}L^{-1}$ glucose, 2.5 $g{\cdot}L^{-1}$ yeast extract) at 4 °C [28].

2.2. Strain and Inoculum Preparation

Stock cultures of *B. subtilis* ICA56 cells were maintained in TGE medium (5.0 $g{\cdot}L^{-1}$ triptone, 5.0 $g{\cdot}L^{-1}$ glucose, 2.5 $g{\cdot}L^{-1}$ yeast extract) containing 20% glycerol as a cryoprotectant. The samples were maintained at −80 °C to ensure their stability. The

inoculum was performed by inserting culture loops in culture medium incubated at 37 °C for 6 h.

The effect of inoculum age on enzyme production was investigated. Based on the cell growth curve, the inoculum times tested were 6 h, 8 h, and 12 h, which represent different points of the exponential phase.

2.3. Culture Medium and Culture Condition in Shake Flasks

A synthetic medium composed of monosodium phosphate (1 $g \cdot L^{-1}$), potassium phosphate dibasic (1 $g \cdot L^{-1}$), ammonium sulfate (2.5 $g \cdot L^{-1}$), sodium chloride (0.25 $g \cdot L^{-1}$), magnesium sulfate (0.20 $g \cdot L^{-1}$), ferric chloride (0.04 $g \cdot L^{-1}$), manganese sulphate (0.0025 $g \cdot L^{-1}$), glucose (10 $g \cdot L^{-1}$), and yeast extract (3 $g \cdot L^{-1}$) was used as a standard culture medium [29].

ALDC enzyme production was conducted in 500 mL Erlenmeyer flasks. For that, 250 mL of culture medium was inoculated (10% v/v) and incubated in an orbital shaker (Tecnal-TE240, Piracicaba, Brazil) at 37 °C, 150 rpm, for 14 h.

2.4. Extraction of the Enzyme α-Acetolactate Decarboxylase

α-acetolactate decarboxylase is an intracellular enzyme and has to be released from the cells. The cell pellets obtained from the centrifugation were washed with 10 mL of 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES) buffer pH 6.0. A tip ultrasound (Q500 Sonicators, Sonicator Qsonic, Newtown, CT, USA) was used to break the cells for 10 min with amplitude of 40%, pulses of 10 s on and 20 s off, and potency of 500 W. The procedure was performed in an ice bath. After breaking, it was centrifuged again at 5000 rpm, 4 °C, for 20 min, and the supernatant (crude ALDC extract) was then characterized (enzyme activity, protein concentration, and molecular weight analysis).

2.5. Enzyme Activity

In order to determine the catalytic activity, a solution was prepared containing 0.2 mL of substrate solution (0.3 mL of 1M NaOH, 0.3 mL of H_2O, 4.3 mL of 50 mM MES buffer pH 6.0, 10 µL of ethyl 2-acetoxy-2-methylacetoacetate) and 0.2 mL of enzyme extract. The mixture was incubated at 30 °C for 20 min. The product acetoin reacts with a solution of 1-naphthol and creatine, acquiring a color that can be quantified in a spectrophotometer (Biochrom Libra S22, Cambridge, UK) at 522 nm by the colorimetric method based on the adapted methodology of Stormer [30]. One unit of ALDC activity (U) was defined as the amount of enzyme capable of producing 1 µmol of acetoin per minute at pH 6.0 and 30 °C.

2.6. Protein Quantification

Protein concentration was determined by the Bradford method using bovine serum albumin as reference [31].

2.7. Electrophoresis SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel)

SDS-PAGE was carried out in 12% polyacrylamide gel in an electrophoresis unit (Mini-PROTEAN tetra-cell, Biorad), according to Laemmli [32]. In the procedure, 10 µL of enzyme extract was re-suspended in 100 µL of rupture buffer (4% SDS and 10% mercaptoethanol), boiled for 10 min at 100 °C, and a 15 µL sample of the mixture was used in the experiments. Protein bands were detected by the Coomassie brilliant blue method using low-molecular-weight marker proteins (LMW-SDS Marker—GE Healthcare LifeSciences) as standard.

2.8. Effect of Temperature and pH on ALDC Activity

The effect of temperature on ALDC activity was determined at a pH of 6.0 at temperatures ranging from 10 to 50 °C. The crude extract containing the enzyme was incubated at the desired temperature and the residual activity was determined using a standard assay.

Similarly, the pH effect on ALDC activity was determined at 30 °C and pH values between 3.6 and 7.5. The crude extract containing the enzyme was incubated at the desired pH and the residual activity was determined using a standard assay. The following buffer

systems were used at a concentration of 50 mM: sodium acetate buffer, pH 3.6–5.0; MES buffer, pH 5.5–6.5, potassium phosphate buffer, pH 6.0–7.5; and citrate phosphate buffer, pH 4.0–7.0.

2.9. Effect of Metal Ions on ALDC Activity

The effect of different metal ions on ALDC activity was investigated using common bivalence metal ions, including Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+}. The reaction media was supplemented with the desired ion at a final concentration of 1 mM and incubated at 4 °C for an hour. Afterwards, ALDC activity was determined, and the relative activity was calculated as a ratio of observed activity in the presence and absence of the ion.

2.10. ALDC Thermal Stability

The thermal stability of ALDC was performed at 30 °C in 50 mM MES buffer at pH 6.0. Samples were collected periodically, and the enzymatic activity was determined. Relative activities were calculated as a percentage of the initial activity.

2.11. Determination of Kinetics Parameters

The steady-state kinetics of ALDC were also analyzed by the colorimetric procedure. The reaction mixture comprised 0.2 mL ALDC extract and 0.2 mL substrate solution (4.3 mL 50 mM MES buffer (pH 6.0), 0.3 mL 1 M NaOH, 0.3 mL H_2O, and 10 µL ethyl-2-acetoxy-2-methylacetoacetate at different concentrations (0.1–25 mM)). The incubation was carried out at 30 °C for 20 min. K_m and V_{max} were obtained by non-linear fitting of the Michaelis–Menten equation to the experimental data using Origin software (OriginLab Corporation®, Northampton, MA, USA).

2.12. Optimization of Culture Medium

To evaluate the carbon source, six different sources were tested, replacing glucose with sucrose, lactose, fructose, glycerol, molasses, and an equimolar mixture of fructose and glucose. The nitrogen source was also evaluated. For that, yeast extract was replaced with urea, ammonium sulfate, sodium nitrate, malt powder, and corn steep solids (CSS). ALDC enzyme was produced in 500 mL Erlenmeyer flasks. Inoculum (10% v/v) was transferred to 250 mL of culture medium and experiments were carried out in a rotary shaker at 37 °C, 150 rpm, for 14 h. At the end of the assay, cultures were centrifuged at 5000 rpm, 4 °C, for 30 min. The supernatant was discarded, and the cell pellets were stored at −20 °C for further evaluation. All alternative nutrients in the formulation of the culture medium are present on Table 1.

Table 1. Carbon and nitrogen sources in culture medium for standard and alternative culture media used to produce ALDC by *B. subtilis* ICA 56. The remaining media components were kept constant. Experiments were carried out in a rotary shaker at 37 °C, 150 rpm, for 14 h.

Experiment	Medium	Carbon Source (10 g·L^{-1})	Nitrogen Source (3 g·L^{-1})
01	Standard	Glucose	Yeast extract
02	02	Fructose	Yeast extract
03	03	Fructose + Glucose	Yeast extract
04	04	Sucrose	Yeast extract
05	05	Lactose	Yeast extract
06	06	Glycerol	Yeast extract
07	07	Molasses	Yeast extract
08	08	Glucose	Corn steep solids
09	09	Glucose	Ammonium sulfate
10	10	Glucose	Sodium nitrate
11	11	Glucose	Malt powder
12	12	Glucose	Urea
13	Optimized	Molasses	Corn steep solids

2.13. Medium Costs

The culture medium cost (MC) was estimated as proposed by de Freitas et al. from Equation (1) [17]. The cost was based on the amount of components necessary to formulate 1 L of medium. The production cost (PC) of ALDC was defined in Equation (2) in order to compare the different culture mediums analyzed [17]:

$$MC = \sum_{i=1}^{n} C_i P_i, \quad (1)$$

$$PC = \frac{MC}{PP}, \quad (2)$$

where MC is the culture medium cost (US¢·L^{-1}), C_i is the concentration of component i in the medium (kg·L^{-1}), P_i is the purchase cost of component i in the medium (US¢·kg^{-1}), PC is the production cost of ALDC in dollar cents per unit (US¢·U^{-1}), and PP is ALDC activity in units per liter of culture medium (U·L^{-1}). The e-commerce platform Alibaba was used to estimate the prices of the medium components (Table 2).

Table 2. Concentrations and costs in dollar cents (US¢) of each medium component used in the different shake flask cultures studied, namely standard medium (experiment 1), medium 2 (experiment 2), medium 3 (experiment 3), medium 4 (experiment 4), medium 5 (experiment 5), medium 6 (experiment 6), medium 7 (experiment 7), medium 8 (experiment 8), medium 9 (experiment 9), medium 10 (experiment 10), medium 11 (experiment 11), medium 12 (experiment 12), and optimized medium (experiment 13).

Component	Cost (US¢·kg^{-1})	Concentrations for Each Experiment												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
KH_2PO_4 (g L^{-1})	120	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Na_2HPO_4 (g L^{-1})	200	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$(NH_4)_2SO_4$ (g L^{-1})	13	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	5.5	2.5	2.5	2.5	2.5
NaCl (g L^{-1})	6	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
$MgSO_4$ $7H_2O$ (g L^{-1})	30	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
$FeCl_3$ $6H_2O$ (g L^{-1})	12	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
$MnSO_4$ H_2O (mg L^{-1})	50	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Glucose (g L^{-1})	70	10	-	5	-	-	-	-	-	-	-	-	-	-
Fructose (g L^{-1})	170	-	10	5	-	-	-	-	-	-	-	-	-	-
Sucrose (g L^{-1})	25.0	-	-	-	10	-	-	-	-	-	-	-	-	-
Lactose (g L^{-1})	200	-	-	-	-	10	-	-	-	-	-	-	-	-
Glycerol (g L^{-1})	100	-	-	-	-	-	10	-	-	-	-	-	-	-
Molasses (g L^{-1})	9.8	-	-	-	-	-	-	10	-	-	-	-	-	10
Yeast extract (g L^{-1})	170	3	3	3	3	3	3	3	-	-	-	-	-	-
CSS (g L^{-1})	0.5	-	-	-	-	-	-	-	3	-	-	-	-	3
$NaNO_3$ (g L^{-1})	43.0	-	-	-	-	-	-	-	-	-	3	-	-	-
Malt powder (g L^{-1})	150	-	-	-	-	-	-	-	-	-	-	3	-	-
Urea (g L^{-1})	48.0	-	-	-	-	-	-	-	-	-	-	-	3	-

3. Results and Discussion

3.1. Optimization of ALDC Production

3.1.1. Effect of Inoculum Age on ALDC Activity

Inoculum exerts an effect on the subsequent performance of the fermentation process, and it is known that its age and density are directly related to the duration of the lag phase, specific growth rate, biomass yield, sporulation, and quality of the final product, and, therefore, the production costs [33–35]. Figure 1 shows the influence of inoculum age on ALDC activity, produced in the standard medium (Figure 1).

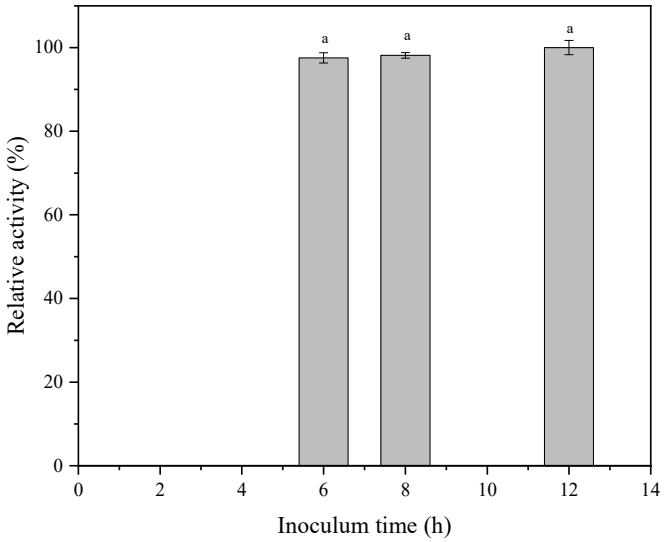

Figure 1. Effects of the inoculum age (6, 8, and 12 h) on ALDC activity from *Bacillus subtilis* ICA 56. Fermentation was conducted with an inoculation of 10% (*v*/*v*) in 250 mL at 37 °C, 150 rpm, for 14 h. The Tukey test was performed to evaluate statistical differences, considering standard deviations and a p value < 0.05 as significant. The averages followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability.

According to Figure 1, enzyme activity is almost constant, showing that varying the inoculum in the logarithmic growth phase has no significant influence on enzyme activity. For that reason, the best condition for the inoculum age is 6 h, once it reduces the total fermentation time with productivity of ALDC activity around 4.70 $U \cdot mL^{-1}$. Similarly, the statistical Tukey test suggests that there is no significant difference between these cultivation times.

3.1.2. Analysis of Culture Medium

The composition and/or concentrations of nutrients in a culture medium are important factors in the production of enzymes, especially the sources of carbon and nitrogen. Therefore, exploratory experiments were conducted in shake flasks to test the influence of carbon and nitrogen sources on biomass and ALDC production from *Bacillus subtilis* ICA 56. The literature mentions that *B. subtilis* is capable of using numerous carbohydrates as a source of carbon and energy [36]. Figure 2 shows the cell growth profile of *B. subtilis* ICA 56 using different carbon sources. Maximum specific growth rate (μ_{max}), lag and log phases, biomass concentration, and ALDC activity are presented in Table 3. As can be seen, the carbon source influences the lag phase, i.e., the period when the cells are adjusting to the environment. This time is longer for culture medium using lactose and glycerol and shorter when molasses is used as a carbon source. It should be mentioned that biomass growth was not affected substantially by the carbon source. All carbon sources tested were suitable for cell growth, reaching a biomass concentration between 7 and 11 $g \cdot L^{-1}$, but on the other hand, caused remarkable changes in ALDC activity (Table 3). This result could be due to different media compositions leading to alterations in the intracellular metabolic activities that may lead to different fermentation products [37].

The best carbon source for ADLC production was obtained when *B. subtilis* ICA 56 was cultivated using molasses, accomplishing an ALDC activity of 23.94 ± 0.92 $U \cdot g^{-1}$ (Table 3). The composition of molasses may explain this result. Besides the high fermentable sugar content (mainly sucrose, fructose, and glucose), molasses also has trace elements and inorganic salts, such as minerals (Ca, Mg, Na, and K), phosphate, and nitrogen compounds (crude proteins) [24,25]. This directly influences fermentation, due to changes in the carbon/nitrogen ratio, as well as the presence of additional nutrients to support microbial growth and product formation. Some studies outlined molasses as a favorable

carbon source for *Bacillus subtilis* growth and enzyme production, corroborating the results obtained in this work [38–40].

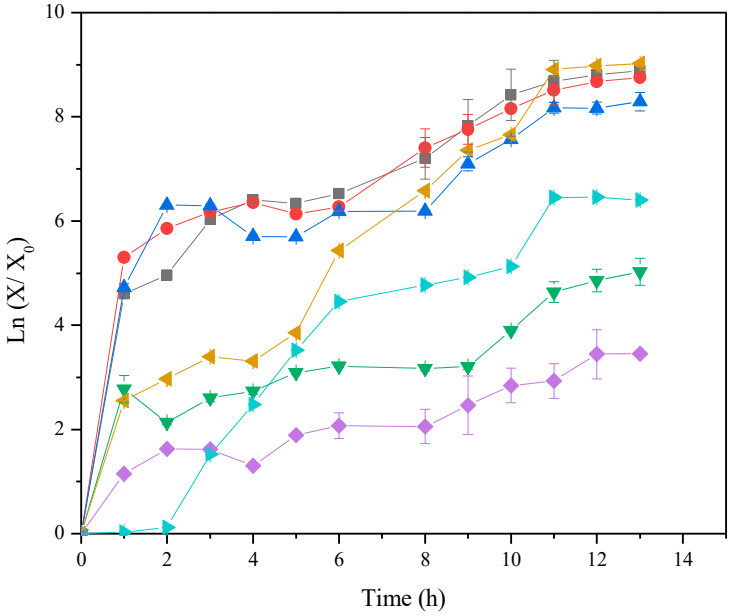

Figure 2. Effect of different carbon sources on cellular growth of *B. subtilis* ICA 56. Culture medium using: 10 g·L^{-1} sucrose (■), 10 g·L^{-1} glucose (●), 10 g·L^{-1} fructose (▲), 5 g·L^{-1} glucose + 5 g·L^{-1} fructose (◀), 10 g·L^{-1} lactose (▼), 10 g·L^{-1} glycerol (♦), and 10 g·L^{-1} molasses (▶). Culture medium incubated at 37 °C and 150 rpm.

Table 3. Growth parameters for *Bacillus subtilis* ICA 56 and activity of ALDC produced using different carbon sources. Culture medium incubated at 37 °C and 150 rpm.

Culture Medium	Parameters					
	Carbon Source	Lag Phase (h)	Log Phase (h)	μ_{max} (h^{-1})	Biomass (g·L^{-1})	ALDC Activity (U·g^{-1})
Standard	Glucose	0–3	3–12	0.30 ± 0.02	9.19 ± 0.21	17.36 ± 0.19
02	Fructose	0–4	4–12	0.31 ± 0.06	9.64 ± 0.19	19.50 ± 0.37
03	Fructose + Glucose	0–3	3–10	0.65 ± 0.02	8.29 ± 0.11	20.69 ± 0.45
04	Sucrose	0–3	3–11	0.39 ± 0.03	10.99 ± 0.41	16.67 ± 0.41
05	Lactose	0–8	8–12	0.25 ± 0.02	9.44 ± 0.35	0.04 ± 0.03
06	Glycerol	0–8	8–12	0.20 ± 0.06	8.62 ± 0.17	1.16 ± 0.18
07	Molasses	0–2	2–10	0.57 ± 0.03	7.99 ± 0.13	23.94 ± 0.92

Table 4 shows the relation between the values of enzymatic activity and the amount of protein obtained using different culture media. It is possible to notice that the sources of carbon and nitrogen directly influence the amount of protein produced, as well as the ALDC activity. Various nitrogen substitutes were tested, and variation was seen in ALDC activity of all these cases (Table 5). This could be as a consequence of differences in the protein composition of each of the alternative sources, as well as the low availability (solubility) of these proteins present in these sources [37]. Of the six types of nitrogen source evaluated (Experiments 1, 8, 9, 10, 11, 12), only yeast extract (Exp. 1) and corn steep solids (Exp. 2) were suitable for the production of the ALDC enzyme, reaching catalytic activity around 4.0 U·mL^{-1}. CSS contains a high sugar concentration and other nutrients (nitrogen, amino acids, trace elements, and vitamins) that support bacterial growth and product formation [26], which may be the reason for the efficient fermentation performance observed using CSS. It has been successfully used in the production of enzymes [17,41,42].

The considerable number of proteins obtained using malt as a nitrogen source is probably due to the fact that malt itself already consists of some enzymes, such as α-amylase and β-amylase [43,44].

Table 4. Shake flask production of ALDC from *Bacillus subtilis* ICA 56 using different culture media (medium composition is shown in Table 2). The Tukey test was performed to evaluate statistical differences, considering standard deviations and a p value < 0.05 as significant. The averages followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability.

Experiment	Medium	At (U·mL^{-1})	Pr (mg·mL^{-1})	Sp Act (U·mg^{-1})
01	Standard	3.99 ± 0.04 ^{d}	5.57 ± 0.05	0.72 ± 0.10
02	02	4.70 ± 0.07 ^{a}	6.48 ± 0.18	0.73 ± 0.25
03	03	4.29 ± 0.05 ^{c}	5.68 ± 0.03	0.76 ± 0.08
04	04	4.58 ± 0.17 ^{a,b}	4.77 ± 0.06	0.96 ± 0.23
05	05	0.01 ± 0.01 ^{g}	0.76 ± 0.01	0.01 ± 0.01
06	06	0.25 ± 0.03 ^{g}	2.88 ± 0.02	0.09 ± 0.05
07	07	4.78 ± 0.12 ^{a}	3.23 ± 0.02	1.48 ± 0.14
08	08	4.03 ± 0.04 ^{d}	3.87 ± 0.02	1.04 ± 0.06
09	09	0.10 ± 0.04 ^{g}	0.17 ± 0.08	0.60 ± 0.11
10	10	0.00 ± 0.01 ^{g}	0.12 ± 0.11	0.00 ± 0.13
11	11	1.46 ± 0.01 ^{e}	2.24 ± 0.09	0.65 ± 0.10
12	12	0.58 ± 0.01 ^{f}	1.50 ± 0.20	0.39 ± 0.21
13	Optimized	4.43 ± 0.12 ^{b,c}	3.22 ± 0.01	1.37 ± 0.13

At: ALDC activity; Pr: protein concentration; Sp act: specific activity.

Table 5. Culture medium cost (MC), ALDC activity per liter of culture medium (PP), and production cost of ALDC (PC) for each culture medium used for enzyme production by *Bacillus subtilis* ICA 56. MC is expressed in dollar cents per liter (US¢·L^{-1}) and PC in dollar cents per unit of enzyme produced (¢·U^{-1}). Media composition is specified in Table 2.

Experiment	Medium	MC (US¢·L^{-1})	PP (U·L^{-1})	PC (US¢·U^{-1})
01	Standard	1.57	0.1596	9.84
02	02	2.22	0.1880	11.81
03	03	1.90	0.1716	11.07
04	04	1.12	0.1832	6.11
05	05	2.87	0.0000	*
06	06	1.87	0.0100	187.00
07	07	0.79	0.1912	4.13
08	08	1.07	0.1612	6.64
09	09	1.10	0.004	275.00
10	10	1.19	0.0000	*
11	11	1.21	0.0580	20.86
12	12	1.51	0.0232	65.09
13	Optimized	0.46	0.1772	2.60

* Culture medium was not able to produce ALDC from *Bacillus subtilis* ICA 56.

Regarding the carbon sources tested, lactose (Exp. 5) and glycerol (Exp. 6) cannot be used as carbon sources since the ALDC has no activity when *B. subtilis* ICA 56 was cultivated using the alternative carbon source. Some studies point out that lactose as a carbon source may promote the formation of other enzymes from *Bacillus subtilis* [45,46]. The use of glycerol as a carbon source was reported for the production of biosurfactants from *Bacillus subtilis* ICA 56 [28,47]. This may have resulted in a low production of ALDC from *Bacillus subtilis* ICA 56 when these alternative nutrients were used.

The best conditions of ALDC production were obtained using molasses as a carbon source and yeast extract (Exp. 7) or CSS (Exp. 13) as a nitrogen source, obtaining enzymatic activity of approximately 4.78 U·mL^{-1} and 4.43 U·mL^{-1}, respectively. Tukey's test shows that the best conditions differ from each other, as well as from the standard condition.

3.2. Characterization of ALDC Enzyme Obtained from B. subtilis ICA 56

3.2.1. SDS-PAGE Analysis

The production of the target protein was confirmed by gel electrophoresis. Figure 3 shows the SDS-PAGE analysis for enzymatic extract obtained from *B. subtilis* ICA 56 using the standard and optimized culture media. Electrophoretic profiles confirm the production of ALDC by the presence of a band at molecular weight of around 30 kDa. This result is in accordance with other studies found in the literature. Jia et al. reported a molecular weight of 28.80 kDa of ALDC from *Bacillus subtilis* IPE5-4 [48]. Similarly, Marlow et al. described ALDC from *Bacillus brevis* with molecular weight of 29.96 kDa [7], and Wu et al. characterized ALDC from *Klebsiella pneumonia* with molecular weight of 29.96 kDa [12].

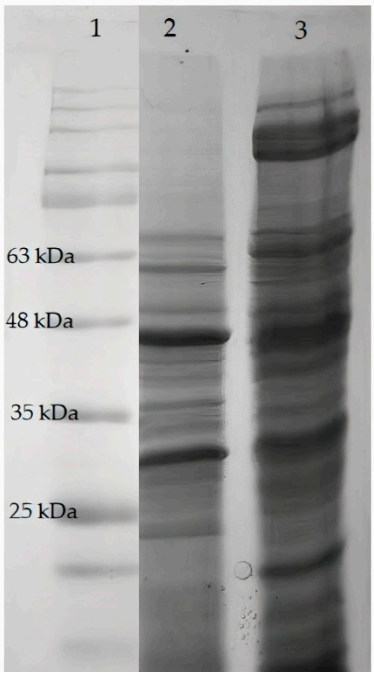

Figure 3. Electrophoretic profile of enzymatic extract produced. (1) Low-molecular-weight marker; enzymatic extract produced using (2) standard medium and (3) optimized medium.

3.2.2. Effect of Temperature and pH on ALDC Activity

ALDC activity was assayed at temperatures ranging from 10 to 50 °C at a constant pH of 6.0 in order to determine the effect of temperature, as shown in Figure 4. From 30 to 45 °C, the enzyme exhibited relative activities higher than 70% and optimal activity (100%) at 40 °C. This optimum temperature value is similar to other studies reported in the literature for ALDC from other microorganisms [49–51].

The effect of pH on ALDC activity was carried out 30 °C and pH ranging from 3.60 to 7.5, as shown in Figure 5. The highest activities ALDC (5.30 ± 0.06 U·mL^{-1}) were observed at pH 6.0. These results are in good agreement with other studies reported for ALDC from other microorganisms [14,52,53]. At the pH ranging from 3.6 to 7.0, ALDC exhibited more than 80% of relative activity. After that point, the activity of the enzyme dropped, showing low tolerance to alkaline conditions. This enzyme stability is similar to the comportment obtained by Ji et al. for ALDC from *Enterobacter aerogens*, in which the enzyme remained stable when varying the pH between 4.5 and 8.0 (10 mM Tris-HCl buffer) [9].

The results (Figure 5) show that pH and the nature of the buffer affect the enzyme performance. Buffer ions influence ALDC activity, being able to shift the optimal pH of the enzyme. These results may be rationalized by recognizing that buffer ions, besides fixing pH, also interact specifically with the amino acids of the catalytic triad, thus interfering with the catalytic mechanism. The literature reveals some studies indicating that buffers can alter biochemical properties [54–57]. Salis et al. reported that the catalytic activity is

strongly affected by surface pH that determines the ionization state of two amino acids of the lipase from *C. rugosa* (CRL) catalytic triad [58]. Kornecki et al. disclosed that the stability of β-galactosidase from *Aspergillus oryzae* immobilized on glutaraldehyde strongly depends on the buffer, and sodium phosphate shows a strong destabilization effect [59].

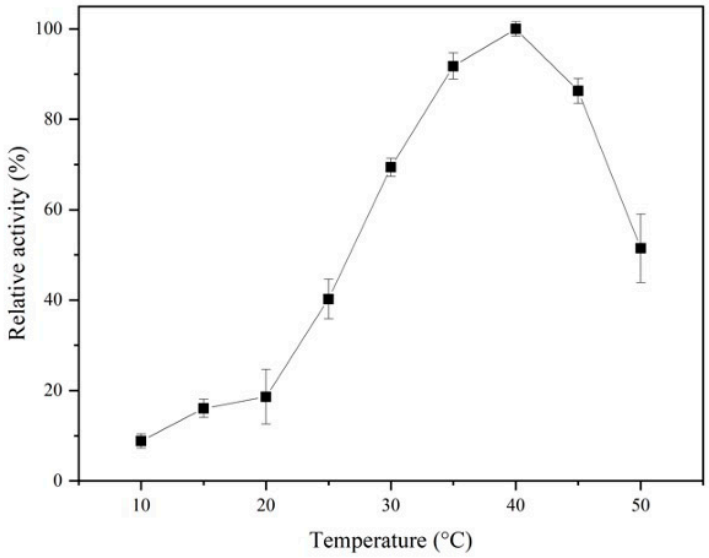

Figure 4. Effect of the temperature on ALDC activity at pH 6.0 (50 mM MES buffer). Results correspond to the average of triplicate experiments ± standard deviation. Relative activity is expressed as a ratio of the maximum activity.

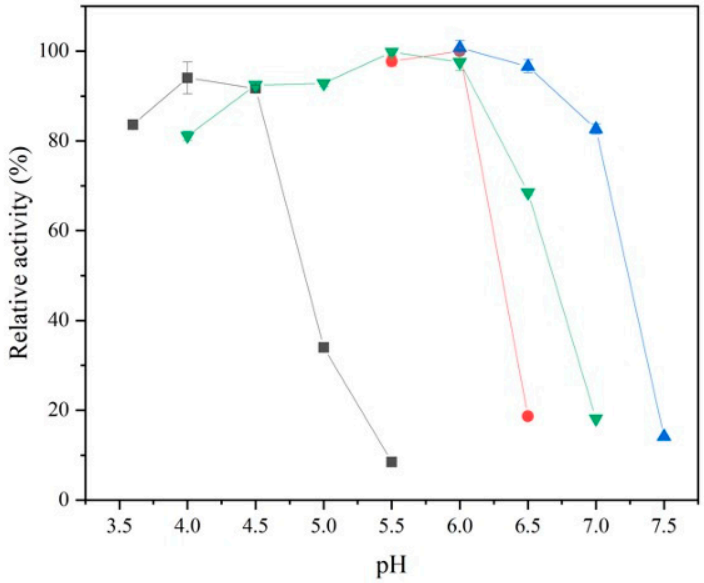

Figure 5. Effect of pH on ALDC activity at 30 °C and 50 mM sodium acetate buffer. Effect of the pH on lipolytic activity of CRL at 40 °C and (■) 50 mM sodium acetate buffer, (▼) 50 mM citrate phosphate buffer, (●) 50 mM MES buffer, or (▲) 50 mM potassium phosphate buffer. Results correspond to the average of triplicate experiments ± standard deviation. Relative activity is expressed as a ratio of the maximum activity.

3.2.3. Effect of Metal Ions on ALDC Activity

As many decarboxylases require divalent metal ions as a cofactor, the influence of several metal ions on the ALDC activity of *B. subtilis* ICA56 was examined (Figure 6). As indicated in Figure 6, Fe^{2+}, Ca^{2+}, Mg^{2+}, and Cu^{2+} all had an active effect on ALDC activity, while Ba^{2+}, Mn^{2+}, and Zn^{2+} slightly decreased the activity of ALDC. Similar behavior of the

effect of metal ions on the activity was reported for ALDC from *Lactococcus lactis* DX, which was activated by Fe^{2+}, Mg^{2+}, Ba^{2+}, and Ca^{2+} [49]. Ji et al. found that Ba^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} increased the activity of ALDC from *Bacillus subtilis* [6]. Zheng et al. indicated that the presence of Mg^{2+}, Mn^{2+}, Zn^{2+}, or $Ca2^{2+}$ was able to activate ALDC from *Streptococcus thermophilus* [14].

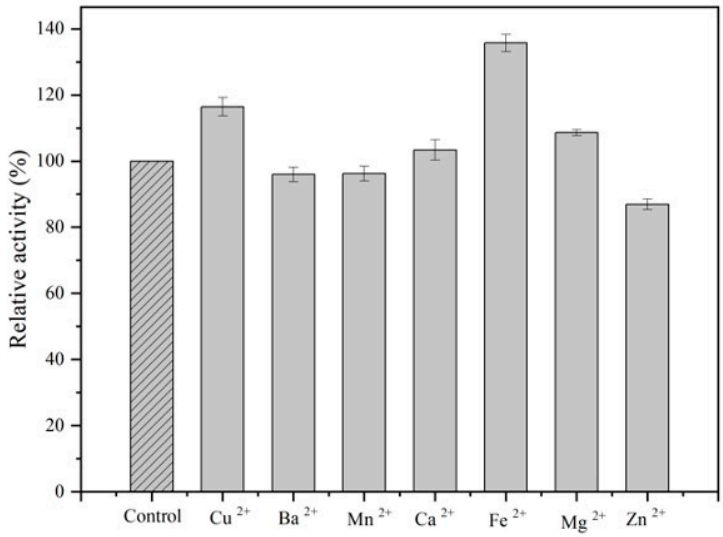

Figure 6. Effect of metal ions on ALDC activity at 30 °C and pH 6.0. Results correspond to the average of triplicate experiments ± standard deviation. Relative activity is expressed as a ratio of the control activity.

3.2.4. ALDC Thermal Stability

The results of thermal stability at 30 °C are shown in Figure 7. The enzyme is thermally stable at 30 °C, being able to maintain more than 77% of its catalytic activity after 35 h. This result indicates that the enzymatic extract is stable at the low temperatures used in the beer process (10–15 °C).

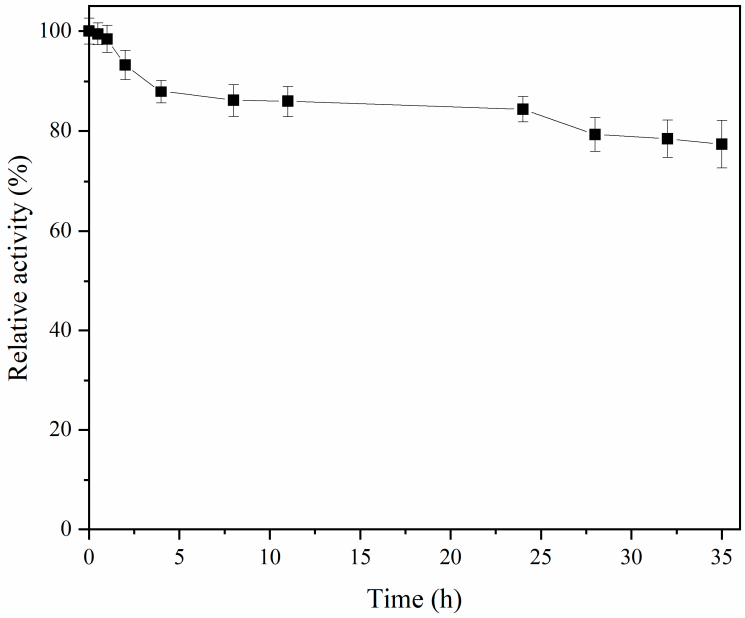

Figure 7. Thermal deactivation profile for ALDC at 30 °C in 50 mM MES buffer at pH 6.0.

3.2.5. Kinetic Parameters

The effects of substrate concentration on the reaction rate were realized at 30 °C and are shown in Figure 8. As can be seen, the reaction rate increased with the in-

crease in substrate concentration until 5 mM. For higher substrate concentrations, the reaction rate was almost constant. K_m and V_{max} were found to be 0.62 ± 0.16 mM and 5.30 ± 0.33 μmol·mg^{-1}·mim^{-1}, respectively. These results are similar to other studies reported in the literature. Choi et al. described a K_m of ALDC from *Bacillus subtilis* as 0.60 ± 0.30 mM [10]. Marlow et al. pointed out a K_m = 1.01 ± 0.47 mM to ALDC from *Bacillus Brevis* and a K_m = 0.25 ± 0.08 mM to ALDC from *Bacillus subtilis* R142K [7]. O'Sullivan et al. showed a K_m of 1.3 mM for ALDC from *Leuconostoc lactis* NCW1 [60].

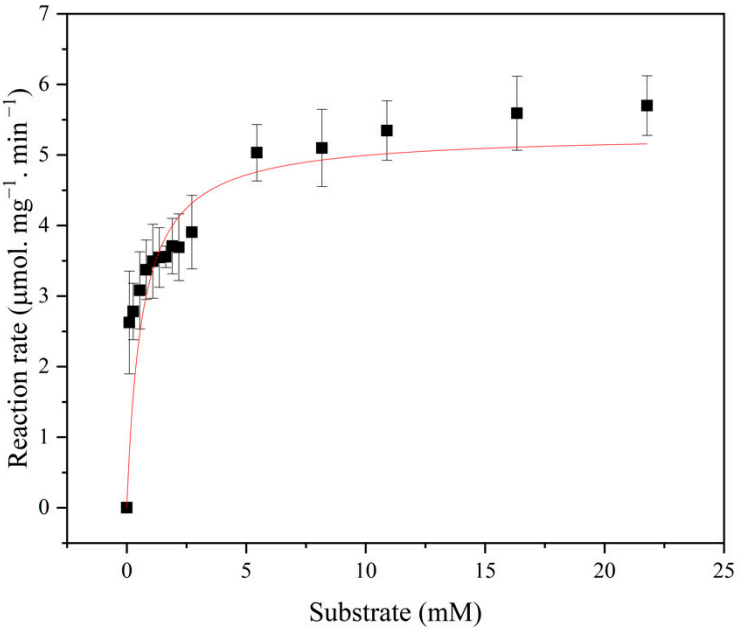

Figure 8. Effect of substrate concentration on reaction rate. Ethyl-2-acetoxy-2-methylacetoacetate was used as substrate, with concentrations ranging from 0.1 to 25 mM. Enzyme activities were measured at 30 °C and pH 6. K_m and V_{max} were obtained by fitting the experimental data to the Michaelis–Menten equation. The error bars represent standard deviations from three replicates.

3.3. Preliminary Economic Analysis of ALDC Production

One of the challenges in developing an industrially feasible process for enzyme production is the design of culture medium, because medium cost can considerably impact the overall process economics [61].

The cost of the culture medium is one of the main components of the total production cost, with carbon and nitrogen sources being the major contributors. These components represent more than 75% of the price of the standard medium for ALDC production (Figure 9a). Table 5 presents the comparison between the values of ALDC production, the cost of culture medium, and the cost of enzyme production for the different culture media analyzed in this work.

It is possible to affirm that the composition of the culture medium affects the production of enzymes and consequently the production cost. The impact of the nitrogen source on enzyme production is evident (Exp. 1, 8, 9, 10, 11, and 12). The substitution of yeast extract for other sources of nitrogen significantly reduces the production of ALDC, making it highly expensive or impracticable. On the other hand, the use of CSS as a nitrogen source was able to maintain the same enzyme activity (0.16 U·L^{-1}), and as it is a low-value by-product, it provided a 32.59% reduction in the cost of the culture medium. Regarding the carbon source, the highest production was obtained using fructose (Exp. 2) or molasses (Exp. 7); however, the cost of the culture medium with fructose increases by more than 40% (2.22 US¢·L^{-1}), while the use of molasses reduces it by half (0.79 US¢·L^{-1}).

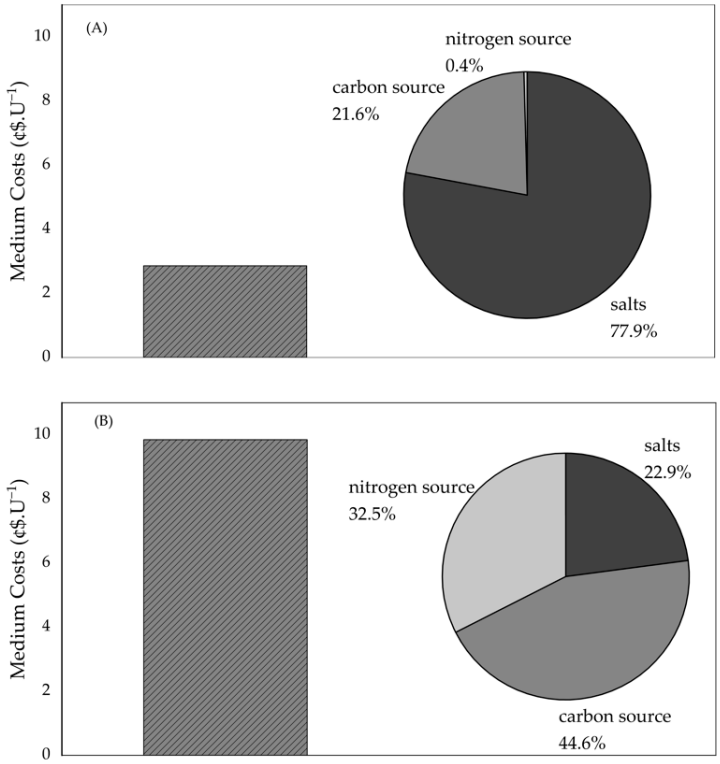

Figure 9. Medium costs and cost distribution among the different components of the culture medium: (**A**) standard medium (Exp. 1) and (**B**) optimized medium (Exp. 13).

When both glucose and yeast extract (Exp. 1) were replaced by molasses and CSS (Exp. 13), which are abundant and low-value by-products, it was possible not only to increase enzyme activity but also to reduce the cost of ALDC production by almost 74% (0.46 US¢·L^{-1}). Figure 9 shows the distribution of the cost of the culture medium for the standard medium (glucose and yeast extract) and the optimized medium (molasses and CSS).

Nitrogen and carbon sources constitute the largest portions of the cost of the standard culture medium. These components represent around 77%, while the other components are less important (Figure 9a). The carbon source expense was reduced by seven times, from 0.70 to 0.10 ¢$·$Kg^{-1}$, when glucose was replaced with molasses. Considering the nitrogen source, the use of CSS (0.02 ¢$·$Kg^{-1}$) drastically reduced the cost, being 255 times cheaper than yeast extract (Figure 3b). As a result, the cost of the culture medium decreased from almost 10 US¢·U^{-1} to US2.6 ¢·U^{-1} using the optimized medium, in which the carbon and nitrogen sources played less important roles.

4. Conclusions

The reuse of agro-industrial residues, especially molasses and CSS, is an alternative for the production of enzymes by submerged fermentation. α-acetolactate decarboxylase was produced by *Bacillus subtilis* ICA 56 in a medium containing 10 g·L^{-1} of molasses and 3 g·L^{-1} of CSS as carbon and nitrogen sources, respectively. The use of the alternative low-cost raw material favored ALDC production and allowed reducing the cost of the medium from almost 10 US¢·U^{-1} to US2.6 ¢·U^{-1}, which represents a reduction of 74%, thus highlighting its potential in industrial enzyme production. Therefore, it has been shown that sugarcane molasses and CSS can be used for the industrially scalable ALDC production from *Bacillus subtilis* ICA 56. Finally, this work stablished a cost-effective fermentation process for the production of α-acetolactate decarboxylase (ALDC) using a non-pathogenic microbe, *Bacillus subtilis* ICA 56.

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