NEW ISOLATED ALPHA AMYLASE-PRODUCING BACTERIA STRAINS FOR DIFFERENT INDUSTRIAL APPLICATIONS

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ABSTRACT

Bacterial α-amylases are highly demanded in the industrial enzyme market specially for their applications in numerous manufacture processes. Furthermore, the substantial search for new source of amylolytic bacteria with novel characteristics aims to improve the industrial-scale production of α-amylases. For this reason, the aim of this study is the screening and isolation of potential α-amylase-producing bacterial strain from soil. Amylolytic bacteria were isolated form soil samples using the serial dilution method. Then, a qualitative and quantitative screening was conducted to select the strain with the best enzyme activity. The influence of different carbon sources on the production of α-amylase was also determined, testing substrates such soluble starch, cassava bran and soluble starch from cassava. After qualitative screening five of the twenty-seven isolated strains were chosen to produce α-amylases under submerged fermentation. Strain AMI-25 demonstrated the best enzyme activity at 24 hours of incubation at 37°C. The amylolytic activity of AMI-25 was evaluated by utilizing a base medium with different carbon sources. AMI-25 displayed the highest α-amylase activity with soluble starch (18.31 U/mL) followed by soluble starch from cassava (13.91 U/mL) and cassava bran (12.68 U/mL), which are comparable with the highest reported results. Great perspectives are expected for high-titers α-amylases production by the isolated strains.

Keywords: Alpha amylase. Isolation. Screening. Carbon source.

1 INTRODUCTION

Industries are especially interested in amylases enzymes due to their wide applications in diverse manufacturing processes. Amylases contribute to approximately 25% of the world enzyme market, particularly in the biotechnology sector. Nowadays, alpha-amylases have increased in demand for industrial applications for starch hydrolysis in diverse industries such as food, paper, brewing, fuels, detergent, textile, and more[1]. α-Amylases can be obtained from diverse source like plants, animals, and microorganisms. However, microbial sources are preferred because of their plasticity, rapid growth, biochemical diversity, and stability. Microbial α-amylases have largely replaced chemical methods for starch hydrolysis in starch processing industry[2, 3]. Bacterial source of α-amylases, such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus stearothermophilus,* have demonstrated their capacity to produce a significant amount of enzymes for industrial process, especially under harsh conditions (e.g., high temperatures, acidity, or alkalinity). The enzyme market is particularly focused on researching thermostable α-amylases because many starch processing steps like saccharification, gelatinization, and liquefaction, require high temperatures, necessitating an enzyme with stability over a wide temperature range[4]. Additionally, their production is cost-effective due to the extensive availability of substrates from inexpensive sources like agro-industrial wastes, domestic wastes, or textile wastes[5]. Despite the importance of amylolytic bacteria, the main aim of the study was to isolate and select α-amylase-producing bacterial strain from soil in order to determinate its enzyme activity under submerged fermentation using different carbon sources.

2 MATERIAL & METHODS

Isolation potential amylolytic bacteria strains

Soil samples were collected from a farm in the State of Paraná, Brazil. Bacterial strains were isolated from these soil samples using the serial dilution method. First, one gram of the samples was dissolved in 9 mL of sterilized saline solution (0.85% NaCl). The diluted samples were heated at 80°C for 15 minutes followed by cooling at 4°C. After the thermal shock the samples were diluted from 10^{-1} to 10^{-5}. A volume of 0.1 mL of the two last dilutions were spread on trypticase soy Agar and starch Agar plates (soluble starch 10 g/L, beef extract 3 g/L, agar 12 g/L)[6]. The plates were incubated at 30°C for 24 hours. Colonies with different morphology on starch Agar plates were purified by subculturing and streaking on starch Agar. Pure bacteria strains were stored in vials with starch (1%) Agar medium.

Qualitative strain screening for amylase production

All the purified bacterial strains, which grew up in starch Agar plates were tested for α-amylase production. Strains were cultured in duplicated in 10 mL of α-amylase production medium (soluble starch 25 g/L, KH_2PO_4 0.2 g/L, $(NH_4)_2SO_4$ 5.6 g/L, urea 1 g/L, peptone 2 g/L, $CaCl_2.2H_2O$ 0.3 g/L and $MgSO_4.7H_2O$ 0.3 g/L, pH 7) [7] and incubated for 24h hours at 37°C. After incubation, the cultures were collected, and the cells were removed via centrifugation at 10000 rpm for 10 minutes. For qualitative determination the well-cut diffusion method [8] was employed using a starch Agar plate with four wells. A volume of 100 µL of the supernatant was added to each well, respectively. The fourth well was inoculated with sterile medium as a negative control, then the plates

were incubated for 24 hours at 37°C. Afterwards, the plates were flooded with 1% of Gram's iodine and kept undisturbed for 15 minutes. The excess solution was decanted to observe the formation of a halo and the diameter was measured.

Quantitative strain screening for amylase production

Five strains with the highest enzyme activity were selected for further α-amylase production to identify the strain with the best enzyme activity. Production of α-amylase was carried out in α-amylase production medium using 250 mL Erlenmeyer's with 99 mL of medium, in duplicate. Fermentation was conducted at 37°C and 120 rpm for 120 hours with samples taken at regular intervals. After sample collection, the cells were removed via centrifugation at 10000 rpm for 10 minutes. Alpha-amylase activity was determined by incubating 20 µL of the supernatant with 180 µL of starch solution (1% w/v) prepared in phosphate buffer (pH 7) at 37°C for 10 minutes. The amount of maltose released was assayed using the 3,5- Dinitrosalicylic acid (DNSA) method proposed by Miller[9]. The absorbance of the samples was measured at 540 nm, and D (+) - maltose was used to create a standard curve. One unit of α-amylase activity was defined as the amount of enzyme that liberates 1 µmol of maltose per minute [10].

Effect of carbon source in α-amylase activity

Production of α-amylase was carried out in 150 mL Erlenmeyer's flasks with 50 mL of basal α-amylase production medium. Soluble starch, cassava bran and soluble starch from cassava were used as substrates (2.5% w/v). An inoculum of 1% ($1.7x10^9$ CFU/mL of 24-hour culture) was employed, and fermentation was conducted with agitation of 120 rpm, for 24 hours at 37°C. After fermentation, α-amylase activity was determined.

3 RESULTS & DISCUSSION

Isolation of potential amylolytic bacteria strains

The primary sources of amylolytic microorganisms are soil and hot springs. Autochthonous bacteria need to produce a variety of enzymes to assimilate all necessary nutrients for survival [11]. For this reason, soil samples were used for the isolation of alpha-amylase-producing bacteria. Based on different colony morphologies, a total of twenty-seven strains from starch Agar plates were chosen as potential amylase producers. Gram's staining method[12] were used to classify the bacterial strains into two groups: Gram-positives strains (54%) and Gram-negative (46%). This corresponds to the fact that amylase-producing isolates predominantly consist of Gram-positive bacteria, which are regarded as typical inhabitants of soil environments [8].

Qualitative strain screening for α-amylase production

For the qualitative determination of α-amylase production, five (18.5%) of the isolated bacterial strains reveled α-amylase activity with the presence of clear zones (halo) after the addition of Gram's iodine solution. The results of the measurement of the halos formed by the α-amylase activity of produced crude enzyme are presented in Table 1. Strain AMI-2 showed the highest α-amylase activity, followed by AMI-10. The halos (Fig. 1) confirmed that the isolated bacterial strains have the capacity to hydrolyze the starch. This polysaccharide forms a deep blue complex with iodine solution due to the presence of helical amylose, which fills the helical nucleus with iodine[9], while monosaccharides and disaccharides will remain colorless because of the absence of helical structure of these molecules.

Table 1 Diameter of halo of potential alpha amylase producing bacterial strains

Strain code	Diameter of average halo (mm)
AMI-2	8 ± 1
AMI-10	7.33 ± 0.58
AMI-13	7 ± 1
AMI-22	5.67 ± 1.53
AMI-25	6.33 ± 0.58

a b c d e

Figure 1 halo of potential alpha amylase producing bacterial strains: (a) AMI-2, (b) AMI-10, (c) AMI-13, (d) AMI-22, (c) AMI-25

Quantitative strain screening for α-amylase production

After the primary screening of bacterial strains with α-amylase activity, enzyme production was carried out under submerged fermentation to identify the best strain and its productivity at peak α-amylase activity. Only two of the five pre-selected bacterial strains exhibited α-amylase activity, reaching their highest activity at 24 hours of incubation. Strain AMI-25 had the highest enzyme activity with 18.31 U/mL, followed by strain AMI-13 with 17.61 mL. These results can be compared with α-amylase activity from *Bacillus atrophaeus* NRC1 which reached 20.68 U/mL after 24 hours of incubation[14]. Additionally, others studies reported that isolated strains *Bacillus cereus* spH1 reached an enzyme activity of 8.5 U/mL [15], a thermos-tolerant *Bacillus licheniformis* WF67 produced 6.63 U/mL [16] and *Bacillus licheniformis* HULUB1 and *Bacillus subtilis* SUNGB2 achieved activities of 18.1 U/mL and 22.14 U/mL, respectively [1].

Effect of carbon source on α-amylase activity

Three different carbon sources were tested for α-amylase production. Strain AMI-25 showed the maximum enzyme activity (18.31 U/mL) using soluble starch as the carbon source, outperforming soluble starch from cassava (13.91 U/mL). *Bacillus licheniformis* ATCC 9945a was employed in α-amylase production reaching 5.2 U/mL at 24 hours of incubation using starch[17]. In another work, using 3% of starch, *Bacillus tequilensis* and *Bacillus wiedmannii* reached amylase activities of 5.5 mL and 5.4 U/mL, respectively[18]. The biosynthesis of amylases using agro-industrial waste and by-products as starchy materials could solve the address industrial waste management issues and help to obtain low-cost media[19]. In this study, enzyme production using cassava bran showed an α-amylase activity of 12.28 U/mL, indicating it as a potential alternative low-cost substrate. For comparison, *Bacillus* sp. using cassava waste as substrate had a maximum enzyme production of 3.9 U/mL at 60°C[20].

4 CONCLUSION

In the present study, strain AMI-25 was selected over five bacterial strains with α-amylase activity. This bacterial strain, AMI-25, was successfully isolated and showed better amylase production at pH 7 after 24 hours of incubation using soluble starch as carbon source. Additionally, the strain demonstrated good potential to produce α-amylase using low-cost substrate such as cassava bran. Future studies will explore the optimization of α-amylase production and process scale-up the process to develop an industrial application. The produced enzyme will be formulated and tested in different applications.

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