Production of *Bacillus subtilis* amylases via agroresidues and their application in cassava starch hydrolysis

Abstract

Amylases are highly important for biotechnology, represent a class of industrial enzymes and occupy the world enzyme market. The aim of this work was to isolate bacteria for the production of amylase. Freshly caught *R. kanagurta* fish samples were purchased from the Vizhinjam fish port, Kerala. Bacteria isolated from the intestines of *R. kanagurta* were cultured on a culture medium containing starch (1%) and kept in an incubator at 30 °C for 48 h. After the incubation period, the growth of bacteria on starch agar plates was analysed, and the samples that were able to grow in this medium were selected for further analyses. The α-amylase enzyme-producing bacteria, which produced a stronger halo in the primary screening stage, were selected for secondary screening and enzyme activity measurement. On the basis of these biochemical and morphological characteristics, isolate A3 was identified as *Bacillus subtilis*. A high amount of amylase was produced by the *B. subtilis strain* isolated from the gut sample (120 U/ml). Among the tested carbon sources, starch had the greatest influence on amylase (158 ± 2.5 U/ml) production by *B. subtilis* (A3), whereas a minimum amount of amylase was produced by *B. subtilis* when it utilized sucrose as a carbon source (111 ± 5.1 U/ml). Among the tested nitrogen sources, ammonium sulfate had the greatest influence on amylase (152 ± 2.9 U/ml) production by *B. subtilis,* whereas the minimum amount of amylase produced by this organism was when it utilized casein as a nitrogen source (134 ± 13 U/ml). The maximum amylase yield (205 ± 1.5 U/ml) was obtained using the culture containing Mn^{2+} compared with all the other tested elements and the control.

Keywords: Gut bacteria; Amylase; Agroresidues; Solid-state fermentation; Optimization

1. INTRODUCTION

Starch-converting enzymes such as amylases are highly important for biotechnology and represent a class of industrial enzymes accounting for approximately 25% of the world's

enzyme market (Reddy et al., 2003; Rajagopalan and Krishnan, 2008). Many applications of alpha-amylase involve different industrial processes, such as bakery, clinical, medicinal, analytical chemistry, textile, food, brewing, fermentation, paper, fine chemical and detergent industries (Pandey et al., 2000; Gupta et al., 2003; Kandra, 2003). hese enzymes are used in various formulations of detergents for laundry and automatic dishwashing to degrade the residues of starchy foods, such as chocolate, custard, potato, and gravies, into smaller oligosaccharides (Olsen and Falholt, 1998; Hmidet et al., 2009; Mukherjee et al., 2009). Furthermore, these methods are extensively employed in food processing industries, such as baking, brewing, the production of digestive aids, cakes, fruit juices and syrups. The texture of bread can be improved by the addition of these enzymes. Solid-state fermentation (SSF) and submerged fermentation (SMF) can be employed for the production of alpha amylases. Low-cost media are formulated for the cheaper production of α-amylase in industry. Generally, synthetic media have been applied for the production of bacterial amylases through submerged fermentation (Haq et al., 1997; Haddaoui et al., 1999; Hamilton et al., 1999). The contents of synthetic media are very expensive; hence, they could be replaced with cheaper agricultural byproducts to minimize the cost of the medium. Solid-state fermentation refers to the growth of microorganisms on moist solid substrates with negligible free water (Pandey et al., 2001) and has been used to convert moist agricultural byproducts such as soy, wheat, cassava, rice, and sugarcane bag gases into fermented products, including industrial enzymes (Rahardjo et al., 2005). The solid substrate provides not only support but also nutrition to the microorganisms. Among the different substrates used for SSF, wheat bran has been found to produce promising yields. Other agro-substrates, *viz. Moreover,* soybean meal, rice husk, sunflower meal, cottonseed meal, rice bran and pearl millet have been used for amylase recovery under solid-state fermentation (Nandakumar et al., 1996; Mulimani et al., 2000; Baysal et al., 2003; Kunamneni et al., 2005). Compared with submerged fermentation, solid-state fermentation has several advantages, including improved productivity; easier technique; lower fixed and working capital; lower energy demand; low level of catabolite repression; end product inhibition; lower water output; improved product yield; and lack of foam formation. Solid-state fermentation has been found to be most appropriate by many researchers for the production of enzymes and other products, especially when greater recovery is needed (Tanyildizi et al., 2005; Couto and Sanromán, 2006). Since various physical and chemical parameters, such as pH, fermentation period, temperature, agitation, and moisture carbon sources, act as inducers, nitrogen sources, phosphates, surfactants and different metal ions have been found to affect the production of α-amylase in relation to SSF; therefore, the optimization of various parameters and manipulation

of media are the most important techniques used for the production of enzymes in large quantities. Interactions among these factors have been reported to have a significant effect on enzyme production. The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature is dependent on the type of culture, i.e., mesophilic or thermophilic. The most commonly used *Bacillus* spp., such as *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus*, have been reported to produce α-amylase at temperatures of 37–°C (Sivaramakrishnan et al., 2006). pH is one of the important factors that determines the growth and morphology of microorganisms, as it is sensitive to the concentration of hydrogen ions present in the medium. Generally, *Bacillus* sp. such as *B. subtilis*, *B. licheniformis*, and *B.* amyloliquefaciens require an initial pH of 7.0 (Syu and Chen, 1997; Tanyildizi et al., 2005). To date, very little research has been performed on the isolation of α-amylase enzyme-producing bacteria that coexist with the digestive systems of *Rastrelliger kanagurta* from the west coast of India. In this study, the symbiotic bacteria of the intestine of Rastrelliger Kanagurta fish were isolated, and then, the α-amylase enzyme-producing bacteria were screened. The bacteria with the highest potential to produce α-amylase were identified biochemically, and enzyme production was optimized. Finally, some biochemical characteristics of the enzymes were investigated.

2. MATERIALS AND METHODS

2.1. Collection of *Rastrelliger kanagurta* Fish

Freshly caught *R. kanagurta* fish samples were purchased from the Vizhinjam fish port, Kerala. The mixture was transferred to the laboratory in a flask containing ice at a temperature of 2–8 °C.

2.2. Isolation of Intestinal Bacteria from *Rastrelliger kanagurta* Fish

First, the abdominal surface of each fish was cleaned with 70% alcohol. After the stomach of each fish was opened with a surgical blade, the intestines were removed under sterile conditions. After the samples were homogenized and diluted with physiological saline, they were cultured in nutrient agar culture medium and incubated at 30 °C for 48 h to isolate bacteria. After the incubation period, the plates were examined morphologically (color and appearance) under a laminar hood, and the bacterial colonies were purified.

2.3. Primary screening of α-amylase-producing bacteria

Bacteria isolated from the intestines of *R. kanagurta* were cultured on a culture medium containing starch (1%) and kept in an incubator at 30 °C for 48 h. After the incubation period, the growth of bacteria on starch agar plates was analysed, and the samples that were able to grow in this medium were selected for further analyses. After 24 h, Lugol's solution or Gram's iodine was poured onto the agar plate containing starch; then, on the basis of the diameter of the clear halo around the bacterial colony, which indicates starch hydrolysis and enzyme production by bacteria, the α-amylase enzyme-producing colonies were isolated and selected.

2.4. Secondary screening of α-amylase-producing bacteria and measurement of enzyme activity

The α-amylase enzyme-producing bacteria, which produced a stronger halo in the primary screening stage, were selected for secondary screening and enzyme activity measurement. At this stage, bacteria were cultured in nutrient broth and incubated in a shaker incubator at 30 °C and 150 rpm. After 24 h, the culture medium containing the grown bacteria was centrifuged at 12,000 rpm for 20 min, and the supernatant was used as a mixture containing the α-amylase enzyme in the next stages of the experiment. The Bernfeld method was used to measure alpha-amylase enzyme activity. For this purpose, 400 µL of phosphate buffer solution, 100 µL of α-amylase enzyme extract, and 500 µL of 1% starch were incubated at 60 °C for 20 min. Then, 1 mL of DNS solution was added to the test tube and incubated for 5 min in a boiling water bath. After cooling, 1 mL of distilled water was added. After the contents of the absorption tube were stirred, the sample was read at a wavelength of 540 nm.

2.5. Substrate

In the present study, orange peel, apple peel, pomegranate peel, and mango peel were used as substrates for solid-state fermentation. The substrates were allowed to stand for 2 days under sunny conditions. The dried residues were then ground to powder form (2 mm mesh) and stored in polythene bags at room temperature (30±2 °C) until use as substrates for amylase production.

2.6. Preparation of Inoculum

For the preparation of inoculum, a volume of 50 ml of nutrient broth was inoculated with a loop full of cells from a 24 h-old culture and kept at 37 °C in a rotary shaker (150 rpm). After 18 h of

incubation, 0.5 ml of this nutrient broth culture of *B. subtilis* was used as the inoculum for solid-state fermentation.

2.7. Solid-state fermentation

The fermentation media in the flasks (orange peel, apple peel, pomegranate peel, and mango peel) were autoclaved at 121 °C for 30 minutes and cooled to approximately 30 °C. The flasks were inoculated with 10% inoculum of *B. subtilis*, and the contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum. The flasks were incubated at 37 °C for 48 h under static conditions. All the experiments were run in triplicate (Gangadharan et al., 2006).

2.8. Extraction of enzyme

After fermentation, the fermented matter in each flask was extracted by the addition of double distilled water, and the total extraction volume was 50 ml. The entire mixture was mixed thoroughly at 30 °C for 1 h in a rotary shaker at 180 rpm and filtered through Whatman filter paper no. 1. The suspensions were then centrifuged at 8000 rpm at 4 °C for 10 minutes. The supernatant was carefully collected and used as a crude enzyme extract for the estimation of total protein content and alpha-amylase activity.

2.9. Identification of Microbes Producing Extra-Cellular Enzymes

The selected bacterial strains were characterized on the basis of their colony morphology, biochemical characteristics and 16S rDNA gene sequence.

2.10. Optimization of various parameters for enzyme production

The physical and nutritional parameters are extremely important for determining the yield and characteristics of enzyme production. In the present study, *B. subtilis* was used to optimize the production of amylase. Different carbon sources, nitrogen sources and ion sources were tested to achieve the maximum yield of amylase. The experiments were conducted in 100 ml Erlenmeyer flasks containing the appropriate media for the production of amylase, which were sterilized at 15 lbs pressure for 15 minutes. After sterilization of the broth by autoclaving, the flasks were cooled, and the strains were inoculated and incubated differently for different parameters, as described in the following paragraphs, by taking one parameter at a time.

2.11. Effects of Different Carbon Sources on Enzyme Production

To test the effects of different carbon sources on enzyme production by *B. subtilis*, five different carbon sources were screened. They were glucose, xylose, maltose, starch and sucrose. They were supplied individually in addition to orange peel at a concentration of 1%

in conical flasks. The flasks were subsequently sterilized at 121 °C for 15 minutes and allowed to cool (37 °C). Then, 0.5 ml of each inoculum was added to each flask and incubated at 30 °C for 2 days under static conditions. Enzyme production was subsequently estimated in the culture supernatant via the spectrometric assay method.

2.12. Effects of different nitrogen sources on enzyme production

The effects of different nitrogen sources on enzyme production by *B. subtilis* were determined. Four different nitrogen sources, namely, yeast extract, casein, beef extract, and ammonium sulfate, were individually tested. These compounds were added individually in addition to the orange peel for amylase production at a concentration of 1% in conical flasks. The flasks were sterilized at 121 °C for 15 min and allowed to cool at room temperature. Then, 0.5 ml of inoculum was added to an Erlenmeyer flask and incubated at 30 °C for 2 days under static conditions. Finally, enzyme production in the culture supernatant was determined via a spectrophotometric assay.

2.13. Effects of Different Ionic Sources on Enzyme Production

Trace elements, which include metal ions, also impact the production of enzymes, which was studied by varying the concentrations of metal ions, such as Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+} ions. Metallic cofactors are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with essential nutrient sources can inhibit or enhance enzyme activity. A total of 5 mM of various metal ions, namely, Mg^{2+}, Mn^{2+}, Ca^{2+}, and $Zn^{2+,}$ were added to the production medium (orange peel), and the activity of the enzyme was measured under standard assay conditions.

3. RESULTS

3.1. Isolation of bacteria from the fish gut

The fish gut samples were serially diluted, plated on nutrient agar media and incubated for 24 h. After 24 h, five different bacteria were observed at dilutions of 10^{-}-9. The isolated bacteria were subjected to amylase screening.

3.2. Screening of amylase-producing bacteria from the fish gut

Extracellular amylase production by bacterial strains isolated from the fish gut sample was screened. Initial screening of bacteria for enzyme production revealed that four of the strains isolated from the gut produced translucent zones on starch agar plates (Table 1).

Table 1. Qualitative extracellular enzyme activity of five bacterial strains isolated from the fish gut.

S.no	Sample	Bacterial strains	Amylase activity (mm)
1	Fish gut	A1	10
2		A2	12
3		A3	15
4		A4	-
5		A5	12

Among the isolates, strain A3, which was isolated from the fish gut sample, displayed very high translucent zones on starch agar plates.

3.3. Identification of Microbes Producing Extra-Cellular Enzymes

The results of the morphological and biochemical tests indicated that the selected isolate (A3) was gram-positive, rod shaped, motile, spore-forming, negative for indole production, methyl red, positive for the Voges-Proskauer test, citrate utilization test, catalase test, negative for the nitrate reduction test, urease test, oxidase test, and H_2S production test. On the basis of these biochemical and morphological characteristics, isolate A3 was identified as *Bacillus subtilis* (Table 2).

Table 2. Morphological and Biochemical Characterization of the Most Promising (In Terms of Enzyme-Producing Ability) Bacterial Strain for Amylase

S.no	Morphological and Biochemical test	A3
1	Gram staining	+ rod
2	Motility	+
3	Spore staining	+
4	Indole production test	-
5	Methyl red test	-
6	Voges Proskauer test	+
7	Citrate utilization test	+

8	Nitrate reduction test	-
9	Urease test	-
10	Oxidase test	-
11	Catalase test	+
12	H_2S production test	-

+: positive result, -: negative result

3.4. Quantitative Extracellular Enzyme Production by *Bacillus subtilis*

The quantitative production of amylase by strain A3 isolated from the fish gut sample was evaluated. The isolated strains were cultured in nutrient broth medium and incubated for 24 h. After 24 h, enzyme production was determined in the culture supernatant. A high amount of amylase was produced by the *B. subtilis strain* isolated from the gut sample (120 U/ml) (Table 3). The other tested bacterial isolates produced the lowest quantity of amylase. Hence, *B. subtilis* was selected for optimization studies.

Table 3. Amylase production by bacterial strains isolated from fish gut samples.

S.no	Sample	Bacterial strains	Amylase activity (U/ml)
1	Fish gut	A1	29
2		A2	41
3		A3	120
4		A4	-
5		A5	27

3.5. Effects of Different Carbon Sources on Enzyme Production

Raj and Hemashenpagam (2012) carried out SSF of wheat bran and rice bran by using *Bacillus* sp. and reported the maximum enzyme activity of α-amylase using a wheat bran substrate. Kalaiarasi and Parvatham (2013) reported wheat bran as the best substrate for the production of α-amylase among different substrates, such as wheat bran, millet bran, black gram bran, and green gram bran, and oil cakes, such as coconut, sesame, cottonseed, and groundnut. The results of the ability of *B. subtilis* to produce amylase by utilizing carbon sources are given in Table 4. Among the tested carbon sources, starch had the greatest

influence on amylase (158 ± 2.5 U/ml) production by *B. subtilis* (A3), whereas a minimum amount of amylase was produced by *B. subtilis* when it utilized sucrose as a carbon source (111 ± 5.1 U/ml). *Bacillus* species are considered to be the most important source of alpha amylase and have been used for enzyme production via SSF. A similar finding was obtained by Gangadharan et al. (2006), who reported that the highest enzyme yield was achieved by the addition of starch, followed by maltose. The maximum alpha amylase yield was also reported by Ashraf et al. (2005) when starch was added to the medium at the 1% level.

Table 4. Effects of different carbon sources on amylase production by *B. subtilis*

Carbon Source (%)	Enzyme Activity (U/ml)
Maltose	154 ± 17.1
Xylose	129 ± 12.3
Glucose	140 ± 3.3
Starch	158 ± 2.5
Sucrose	111 ± 5.1
Control	125± 10.1

3.6. Effects of different nitrogen sources on amylase production

The effects of nitrogen sources on the ability of *B. subtilis* to produce amylase are shown in Table 5. Among the tested nitrogen sources, ammonium sulfate had the greatest influence on amylase (152 ± 2.9 U/ml) production by *B. subtilis*, whereas the minimum amount of amylase produced by this organism was when it utilized casein as a nitrogen source (134 ± 13 U/ml).

Table 5. Effects of different nitrogen sources on amylase production by *B. subtilis*.

Nitrogen Source (%)	Enzyme Activity (U/ml)
Casein	134 ± 13
Yeast extract	138 ± 17.1
Ammonium sulfate	152 ± 2.9
Control	134± 5.1

3.7. Effects of Different Ion Sources on Amylase Production

The results revealed the effects of different divalent ions on enzymatic activity. The maximum amylase yield (205 ± 1.5 U/ml) was obtained using the culture containing Mn^{2+} compared with all the other tested elements and the control. However, supplementation with Zn^{2+} critically affected enzyme production (142 ± 0.4 U/ml) (Table 6). The present study revealed that Mn^{2+} was suitable for the maximum production of α-amylase. In a similar study, Gangadharan et al. (2006) reported an increase in amylase enzyme yield when ammonium chloride was supplemented but a decrease in enzyme yield when ammonium sulfate and sodium nitrate were used as additional supplements in the medium. Aiyer (2004) reported the lowest enzyme production by *Bacillus licheniformis* SPT 27 when ammonium sulfate was used in the medium.

Table 6. Effects of different Ion sources on Amylase production by *B. subtilis*

Ion Source (%)	Enzyme Activity (U/ml)
Ca^{2+}	134 ± 1.8
Mg^{2+}	151 ± 1.0
Zn^{2+}	142 ± 0.4
Mn^{2+}	205 ± 1.5
Control	147 ± 2.8

4. CONCLUSION

The α-amylase enzyme-producing bacteria, which produced a stronger halo in the primary screening stage, were selected for secondary screening and enzyme activity measurement. In the present study, orange peel, apple peel, pomegranate peel, and mango peel were used as substrates for solid-state fermentation. The quantitative production of amylase by strain A3 isolated from the fish gut sample was evaluated. Among the tested carbon sources, starch had the greatest influence on amylase (158 ± 2.5 U/ml) production by *B. subtilis* (A3), whereas a minimum amount of amylase was produced by *B. subtilis* when it utilized sucrose as a carbon source (111 ± 5.1 U/ml). Among the tested nitrogen sources, ammonium sulfate had the greatest influence on amylase (152 ± 2.9 U/ml) production by *B. subtilis,* whereas the minimum amount of amylase produced by this organism was when it utilized casein as a nitrogen source (134 ± 13 U/ml). The results revealed the effects of different divalent ions on enzymatic activity. The maximum amylase yield (205 ± 1.5 U/ml) was obtained using the culture containing Mn^{2+} compared with all the other tested elements

and the control. The enzyme activity reached a maximum at pH 9.0, after which the enzyme activity decreased. The enzyme activity reached a maximum at 40 °C. The enzyme activity reached a maximum at a 2% substrate concentration when amlylase was incubated for 30 min. The efficiency of crude amylase in cassava starch hydrolysis was evaluated. The addition of amylase improved the starch yield at increasing concentrations when the mixture was treated with cassava starch. The highest reducing sugar yield of 128±0.1 mg/g starch in biomass was obtained at 0.1 µg enzyme. The present research revealed the potential of amylases in cassava starch hydrolysis.

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