ENHANCED AMYLOLYTIC ACTIVITY OF INTRACELLULAR α-AMYLASE PRODUCED BY BACILLUS TEQUILENSIS

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ARTICLE INFO
Received 14. 6. 2016
Revised 14. 5. 2017
Accepted 15. 5. 2017
Published 1. 6. 2017

Regular article
Open Access

ABSTRACT
The amylolytic enzyme plays a very important role in industrial applications. This study aimed to screen amylase producing Bacillus sp. and to promote its amylolytic activity by mutagenesis. Samples were collected from coastal mud samples and starch hydrolyzing isolates were screened. A single isolate having the highest enzyme activity was identified as Bacillus tequilensis by 16S rRNA analysis. A starch medium was optimized and fermentation period studies revealed that the mutant strain (after 60 sec of UV exposure) had higher activity (868 U/mL/min) than the parental strain (418 U/mL/min) after 36 hours of incubation at 37°C, pH 7.0. It was also found that amylase from intracellular mutant strain had maximum activity; on the other side parental strain had maximum activity with an extracellular enzyme. Optimized temperature, pH and salt concentration revealed that the intracellular amylase from mutant strain had the maximum activity of 978 U/mL/min, 985 U/mL/min, 960 U/mL/min respectively. Varying the source of carbon in the medium had a significant impact on enzyme activity. Metalloenzymes like amylases were reported to have strong activity towards metal ions, so amylase activity was analysed by adding different metal ions in the medium and found that calcium ions strongly promoted amylase activity and Fe²⁺, Zn²⁺, Cu²⁺, Mg²⁺ inhibited the activity. SDS-PAGE results showed that the molecular weight of isolated amylase to be approximately 55.0 kDa. Our study showed the capability of mutant B. tequilensis strain to produce double the amount of intracellular amylase than the parental strain.

Keywords: Amylolytic, mutagenesis, metalloenzymes, metal ions, zymogram

INTRODUCTION
Amylases are extracellular enzymes which hydrolyze the alpha 1, 4 glycosidic linkages of starch into sugars. Based on the cleavage site, amylase was classified into three types namely α- amylase, β- amylase and γ- amylase. Among them, α- amylase is one of the significant industrial enzymes which are widely utilized for the purpose of brewing, baking, textile and detergent (Gupta et al., 2003). The biologically active enzymes are immensely present in natural resources such as plants, animals and microorganisms (Mageswari et al., 2012) of which microbial source plays a vital role since it can be produced in huge quantity meeting the demands of the market (Pandey et al., 2000). Bacillus tequilensis is a Gram-positive, motile rods, produces central endospore in unsown sporangia and its pathogenicity was undetermined (Gatson et al., 2006). Several studies on ultraviolet (UV) light have been reported to be mutagenic in a variety of organisms. The impact of UV radiations on alpha amylase producing ability of Bacillus species were studied earlier and attempts have been made for the overproduction of microbial enzyme by induced mutagenesis (Demirkan, 2011). The increase in amylase activity after exposing to UV light may be due to the changes in the promoter zone of a gene encoding for this enzyme and also the transcription of mRNA corresponding to the enzyme might have deregulated (De Nicolas-Santiago et al., 2006). The impact of ultraviolet radiations on Bacillus tequilensis producing α-amylase was studied for the first time and the comparison of mutant and wild (parental) strain for increased α-amylase activity was performed.

MATERIALS AND METHODS
Sample collection
Samples were collected from the coastal environment in Pudukkottai district, (10° 1’ 12” North, 79° 13’ 35” East) Tamil Nadu, India. Under sterile conditions, mud samples were collected and the samples were transferred to the laboratory and stored at -20°C until further processing. The samples were collected from three different spots; [I] from the top layer (PDU-1), [II] from 10cm depths (PDU-2) and [III] near the trees (PDU-3).

Isolation, screening and identification of α-amylase producer
Collected samples were used for the isolation of amylase producing bacteria. Samples were processed by taking one gram of mud sample suspended in 9.0 mL of sterile water and agitated for a min. 0.1 mL of the suspended sample was spread plated onto nutrient starch agar (2.0% starch, 0.75% peptone, 0.5% beef extract, 0.5% NaCl, 1.8% agar) and incubated at 37°C for 24 hours. To identify the amylolytic bacteria, plates were overlaid with iodine reagent (0.01 M I₂-I⁻). Based on the starch hydrolysis properties, isolates were identified by the clear zone formed around the isolates. All the isolates that produced a clear zone of hydrolysis (Amy+) were further selected and screened for amylase production. Selected positive isolates (Amy+) were sub-cultured twice for purity and pre-cultures were maintained in nutrient agar slants (-20°C). Single selected isolate B- PDU22 was further identified by 16S rRNA sequencing. DNA was isolated using Hi-media bacterial DNA mini-prep kit as per manufacturer’s protocol and amplified using universal 16S rRNA primers; 27F: 5'- AGAGTTTGATCMTGGCTCAG- 3' and 1492R: 5' - CCGTACGCTATTGACTT- 3' (Prasanth et al., 2016). Species level identification was done at www.ncbi.nlm.nih.gov/BLAST/ and BLAST nucleotide sequence similarity with 98% or above was deemed as sufficient for species identification.

Inoculum and crude enzyme preparation
Inoculum preparation was done by inoculating overnight grown culture from starch agar plates into 50 mL of starch broth and incubated at 37°C for 24 hours.
Exponential phase was achieved that contained 3.0X10^4 CFU/mL and suitable volume from this suspension was used for the tests. To prepare crude enzymes, inoculated overnight grown cultures in starch broth were centrifuged at 10,000 rpm for 15 min and the supernatant was used as a crude enzyme for the enzyme assay for determination of activity.

**Enzyme assay**

The enzyme activity was assayed by using the DNS method. Briefly, a reaction mixture contained 0.5 mL crude enzyme solution and 0.5 mL soluble starch (1 g/100 mL, prepared using 0.01M phosphate buffer) that was incubated for 5 min and reducing sugar was measured. The reaction mixture without crude enzyme served as a control (Miller, 1959). One unit of amylase activity is defined as the amount of enzyme that produces one micromole of reducing sugar in one minute at a constant temperature using soluble starch as substrate. Lowry’s protein estimation method was used for total protein content determination (Lowry et al., 1951).

**Mutagenic studies**

The grown overnight bacterial culture was centrifuged at 10,000 rpm for 15 min and the bacterial cells were resuspended in 50 millilitres of peptone water and further diluted to 10^5 times and dilutions were plated, that served as a control. Ten millilitres from the diluted culture was transferred to another sterile petri plate. Mutagenesis by UV rays was studied by exposing the petri plates at different time intervals (30s, 60s, 90s and 120s) under the UV lamp. After exposing to pre-determined time intervals, 0.5 mL of bacterial culture was plated onto a starch agar plate and incubated at 37°C for 24-48 hrs and further used for enzyme assay. The intracellular and extracellular activity of amylase was studied for both parental and mutant strains. To extract the intracellular amylase, cells were sonicated and centrifuged to collect the intracellular crude enzyme.

**Optimization of physicochemical and nutritional parameters**

Parameters that can influence the amylase production were optimized independently and individually. So, optimization conditions were used subsequently for all the experiments both for the parental and mutant strain. For temperature optimization, the basal medium was inoculated and incubated at 35, 40, 45, 50, 55°C under standard assay conditions and for every 12 hours samples were withdrawn to study the effect of the incubation period. For pH optimization, the culture medium was prepared with variable pH 5.0 to 9.0 and assayed for standard assay conditions. The growth medium was supplemented with varying concentrations of starch (1% to 4%) and also with different carbon sources including maltose, sucrose, lactose, glucose and fructose for enzyme production. To analyse the effect of salt concentration on enzyme production varying concentrations of sodium chloride (1% to 6%) was used in the growth medium.

**Effect of metal ions on enzyme activity**

Metal ions have a strong influence on enzyme production and activity. To investigate the role of metal ions towards amylase activity various metal ions, FeSO₄, KCl, CuSO₄, ZnSO₄, CaCl₂, MgSO₄ were used at 25 mM concentration. Different metal ions were added to the medium and enzyme activity was assayed in standard assay conditions (Demirkan, 2011).

**SDS-PAGE** and **zymogram analysis**

The molecular weight of the enzyme was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The prepared enzyme from both parental and mutant strain was loaded along with protein marker in 12% gel and protein stain. Zymogram was done with native-PAGE by incubating in 1% starch for 1 hour and staining with iodine solution.

**Kinetic properties of α-amylase and statistical analysis**

To assess the kinetic properties, hydrolysis by DNS method with different concentrations of starch was used. Kᵥ and Vᵥmax were calculated using the Michaelis-Menten equation. All the experiments were performed in triplicates and the resulting values were presented as the mean of three independent observations. GraphPad Prism 5.0 software was used for calculating mean-standard deviation for each experimental result.

**RESULTS AND DISCUSSION**

Isolation, screening and identification of amylase producing bacterial cultures from different temperatures. The resulting values were presented as the mean of three independent observations. GraphPad Prism 5.0 software was used for calculating mean-standard deviation for each experimental result.
increase in temperature gradually decreases the activity of amylase (Fig. 3, 4). The temperature was also found to have a profound effect on Bacillus sp. in amylase production (Raud et al., 2014). An incubation temperature of 37°C was optimum for most Bacillus sp. to produce maximum activity of amylase though some thermostable amylase can sustain temperatures up to 45 – 90°C (Sodhi et al., 2005; Asgher et al., 2007).

Effect of different pH

Optimization with different pH (5.0 – 9.0) revealed that both extracellular and intracellular amylase were not stable at stronger acidic as well as alkaline conditions. Also, pH of the medium had a strong influence on enzyme activity (Fig. 5, 6). At neutral pH 7.0 extracellular amylase of parental strain had an activity of 538 U/mL/min and intracellular amylase from the mutant strain (985 U/mL/min) had maximum activities. Bacillus sp. used for industrial amylase production was found to have optimum pH of 6.0 to 7.0 (Haq et al., 2010). Variation in pH also indicates the initiation and end of enzyme synthesis (Friedrich et al., 1989). The incubation period of 36 hours was found to have maximum enzyme activity (Fig. 2) and increase in incubation time decreases the enzyme activity that may be due to an interaction of synthesized enzyme with other components in the medium (Ramesh and Lonsane, 1987).

Effect of different salt concentration on enzyme production

Enzyme activity was found to be decreasing as salt concentration (NaCl) increases from 1%. Though bacterial growth was observed at higher salt concentrations up to 7% enzyme activity was inhibited with an increase in salt concentration (Fig 7). Accordingly, the parental strain had maximum activity at 1% (510 U/mL/min) as same as the mutant strain (960 U/mL/min).

Effect of different carbon sources

The starch broth was found to give maximum enzyme activity/production. Any increase or decrease in starch concentration (1% to 4%) had a lesser effect on enzyme activity on both parental and mutant strains (510 U/mL/min and 960 U/mL/min) whereas 2% starch had maximum activity after 36 hours of incubation (Fig. 8). Supplemented with other carbon sources had affected the enzyme activity in that case glucose and fructose gave lesser enzyme activity than maltose, sucrose and lactose (maltose > lactose > sucrose > glucose > fructose). Some reports found maltose to be a good inducer of amylase activity (Goto et al., 1988; Narang and Satyanarayana, 2001). In some cases, glucose was found to repress amylase activity and also glucose was reported to be an inducer (Normurodova et al., 2007). Varying starch concentration was also reported influencing the enzyme activity (Tiwari et al., 2014).
Effect of different metal ions

For the study of metal ions, 25 mM each of FeSO₄, KCl, ZnSO₄, CaCl₂, CuSO₄, MgSO₄ were used in the medium at optimum temperature, pH and incubation period. Out of which calcium ions was found to be best for amylase production. Other ions Fe²⁺, Zn²⁺, Cu²⁺, Mg²⁺ had slightly decreased the enzyme activity compared to Ca²⁺ ions (Fig.9). It was found that calcium ions increase the activity of amylase compared to control (without ions). The catalytic activity of amylolytic enzymes can be affected by mono- and divalent metal ions but this metalloenzyme has up to six Ca²⁺ atoms at its active site so that can be activated by calcium ions (Asgher et al., 2007; Normurodova et al., 2007).

Stability of amylase and kinetic properties

Stability of enzyme was particularly important for its application in industries. Amylase produced from B. tequilensis was found to be active and stable at 45°C and pH 7.0 that correlates with its higher production rate (Fig.3,4,5,6). Protein content and amylase activity were found to be strongly influenced by the temperature and pH, although the temperature was controlled by incubation conditions and pH in the medium. Amylase from Bacillus spp. was also reported being stable at acidic as well as alkaline conditions (Demirkan et al., 2005; Demirkan, 2011). Michaelis-Menten kinetic parameters were calculated to be 1.27 mg/mL and 1.97 mg/mL for Kₘ and 121 U/mL and 187 U/mL for Vₘₐₓ respectively.

SDS-PAGE analysis and zymography

The molecular weight was determined to be approximately 55.0 kDa (Fig.10) and a single band in SDS-PAGE confirmed the purity of amylase produced from B. tequilensis. Alpha-amylase from Bacillus spp. was found to have a molecular weight ranging from 50-60 kDa. A similar study using B. tequilensis reported molecular weight to be 67 kDa for synthesized amylase (Tiwari et al., 2014). Zymogram results also indicated the presence of an amylolytic enzyme that hydrolysis the starch. The clear zone indicates the presence of an enzyme (protein) in zymography (Fig.11).

REFERENCES


