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Research Article

Mutational Effects of Thermo-Stable α-Amylase Producing *Bacillus* Species

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Enzymes have the great importance in different industries like pharmaceutical, leather, bakery, alcohol production, detergent, textile and paper industry. Amylases are important hydrolase enzymes which have been widely used since many decades. In the present study α-amylase producing *Bacillus* specie was isolated by potato baiting technique and characterization was done by cultural, morphological and biochemical tests. Thermostable α-amylase producing strain was selected by determining the optimal enzyme activity through the DNS reagent. Glucose concentration per ml determined by the Benedict reagent. Mutation was carried out by the UV irradiation, ethidium bromide. UV light exposure for different range of time (15-120 minutes) was used. Enzyme thermostability at 10-110°C was estimated. α-amylase production at different ranges of pH like 4-9 was also determined. Wild and mutant strain comparison on the basis of temperature, pH and incubation time were done. Mutant strain produce 2 times higher enzyme production as compare the wild strain. Mutation created by the UV light and ethidium bromide produce the highly thermostable enzyme. Mutant bacteria produce the maximum enzyme at 8.0 pH while parent strain produce at neutral pH. It was significantly noticed that ethidium bromide mutation indicate that chemical agent was best for the higher enzyme

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production, higher stability up to 110°C at 72 hours of fermentation time ($p<0.05$). Submerged fermentation for -amylase production was used instead of solid state fermentation.

INTRODUCTION

Enzymes play vital role in human life which are essential because they are protein in nature to carry out many reactions. Amylases, lipases and proteases are most widely used enzymes. Amylase is one of enzyme which is used to disintegrate the carbohydrate. Eukaryotes like animals, plants and prokaryotes are source of production for amylase enzyme. It has great importance according to their utilization in their biotechnology and industrial field to help in preparation of textile, edible items like bread and other baking things. Their production rate is 30% over total enzymes in over all the world that show their importance (Nanganuru *et al*., 2012). α-amylase produced by microbes, meet the industrial criteria as compare with the amylase produced from the plants and animals. Now-a-days amylase enzyme is produced by prokaryotes because they have genetic variation and biochemical assortment properties. Prokaryotes are ubiquities and easy to use that's why it is used in industry by replacing the eukaryotes. Use of prokaryotes have great advantage because enzymes are produce in great magnitude, it helps to overcome economical problem and get desired enzyme. Species of *Bacillus* are used because they have ability to grow at different rate and using different kind of substrate to get enzyme in specific culture. Bacillus species like *Bacillus subtilis and Bacillus licheniformis* produce highly active α-amylase for starch hydrolysis and yields oligosaccharide (Sivaramakrishnan *et al*., 2006). *Halophilic* bacteria such as *Halomonas meridian* and *Haloarcula hispanica* produce α-amylase (Kathiresan and Manivannan, 2006). *Aspergillus oryzae, Aspergillus niger, Aspergillus awamori, Penicillium chrysogenum* and *Penicillium brunneum* are important fungal sources for the amylase production. Modified organisms are also being used in industry for production of amylase (Erdal and Taskin, 2010). Thermo-stable enzymes are main target for industry because at high temperature, contamination of microorganism is zero or minimum, help to decrease the stickiness and intensify the substrate solubility. This reaction takes place fast at high temperature as compared to low temperature. Hydrolysis is important step in bioenergy and bio-refinery fields for production of biodiesel from lipase and production of xylanase from pulp industry respectively which carry out at high temperature (Joo *et al*., 2011). Due to its unique properties like thermotolerant, thermophilic, alkaline and acidophilic, amylases are used in all industries. Thermostable α-amylase secreted by *B. licheniformis* is more stable as compare to *B. amyloliquefaciens* and *B. stearothermophilus* despite strong similarities among these three thermostable α-amylases of *B. licheniformis* used in liquefaction processes. It is characterization of α-amylase that work efficiently for liquefication and gelatination at high temperature like 80-90°C and 100-110°C respectively. It needs calcium ion for optimal activity (Declerck *et al.,* 2002, Reddy *et al.,* 2003). Different methods by which microorganisms can be manipulated at a genetic level in order to improve and optimize the production of α-amylase. The microbes can be mutated by physical and chemical agents. Techniques for mutation, chemicals like nitrosomonas, ethyl methyl sulfonate, N-methyl-N′-nitro-N-nitrosoguanidine, nitrous acid and UV light exposure were found to be appropriate mutagens for the α-amylase improved production by *Bacillus* species and gained mutants have a higher production capacity for amylase. Culture method and supply of nutrients play important role in the production of α-amylase enzyme. Thermal stability, pH and selected microorganism also affect the

amylase production (Haq *et al*., 2009). Enzyme production and bacterial growth completely depends upon the carbon and nitrogen availability. Starch and other sugars are used as the carbon source while ammonium salts and organic compounds are used as nitrogen source for growth. Divalent ions like Ca^{2+}, $Mg^{2+,}$ Mn^{2+} and Zn^{2+} are needed for the amylase activity that's why regarded as a metal ion-dependent enzyme. Now-a-days bacterial cultures are improving through mutation and selection techniques. Different chemicals or nitrosamines have been commonly used for mutation. Although, the yield of α-amylase produced by the bacterial strains can be increased with culture optimization and strain screening technique. Mutated strain of *Bacillus amyloliquefaciens* exhibited an activity of 102.78±2.22 U/mL/min which was 1.4 times higher than the parent strain. Genetic mutation, higher yield of α-amylase, inducible expression, faster production, and easier purification take place by recombination of enzyme (Prakash and Jaiswal, 2009). Genetic manipulations and media engineering increase the production of enzyme secretion which is the strategy of bacteria in survival of environment. The present study involves the isolation of α-amylase producing *Bacillus* species through potato baiting technique and strain of *Bacillus* (*Bacillus lichniformis*) conformation by various biochemical tests. Alpha-amylase activity was determined through the DNS method. Mutation for strain improvement carried through chemical ethidium bromide and UV light for increased α-amylase production. A series of trials were carried out to study the effect of various pH and temperature range on the growth and the production of α-amylases by *Bacillus lichniformis* and its mutant strains. The shake flask technique was carried out for the screening and selection of hyper-producer strain of α-amylase. High enzyme activity producing mutant strain was selected, purified and characterized. The optimization of the cultural conditions and nutritional requirements for enzyme production was carried out in a 1 L flask. The specific objectives of this study were to isolate, identify and characterize thermo-stable α-amylase enzyme producing *Bacillus* species and evaluate mutational effect on enzyme production.

MATERIALS AND METHODS

Collection of samples: A total of 100 soil samples were collected through Potato Baiting Technique. Potato pieces were buried about four inches deep under the soil. After 8 days, these pieces of potato were brought out from the dig and taken to the lab in a plastic bag.

Isolation and identification of microorganisms: Shortly, ten grams of decaying potato pieces were mixed with 90 mL sterile distilled water were in beaker. 0.1 mL above solution was streaked on starch agar media plates and incubated at 37ºC for 24 hours. After incubation, starch hydrolysis colonies were observed. Identification of pure culture isolates was done by morphological (Gram staining, Spore staining), cultural characteristics (creamy color of colonies) and different bio-chemical tests (IMVICK) as detailed by Leslie *et al*., 1998.

Amylase production of isolates: Fifty milliliter of inoculum medium containing nutrient broth, pH 7.0 was transferred to a conical flask and cotton plugged. It was sterilized in an autoclave at 15 Lbs pressure (121°C) for fifteen minutes. After cooling to room temperature, a loop full of bacteria was aseptically transferred to it. Then flask was incubated at 37°C for 24 hours.

Extraction of α-amylase: For extraction a centrifugation machine was used. The amylase producing media was poured into centrifuge tubes.

Centrifugation was performed for 10 minutes at 10000 rpm. transfer the supernatant, that is extract of enzyme.

Gram iodine test: Gram iodine test was used to measure activity of α-amylase.

Characterization of α-amylase: Effect of temperature and pH was used to characterize alpha amylase enzyme.

Culture improvement by UV irradiation: Total amount of bacterial culture was taken and poured into nutrient broth medium and centrifugation was carried out aseptically at 8000 rpm for fifteen minutes. The supernatant was discarded and pellet was re-suspended in 50 mL of phosphate buffer saline water and ten-fold diluted. The diluted samples were poured into petri-plates at 180°C temperature for 2 hours. These plates were kept under ultraviolet lamp (15 W, 2537 A) with a distance of 30 cm and was irradiated for 15-120 minutes. After that 1 ml of sample were transferred to the petri plates containing nutrient starch agar medium. The plates were placed in a cooled incubator at 37°C for 24-48 hours.

Statistical analyses: Two-way analysis of variance (ANOVA) was applied. Significance has been presented in the form of probability (<p>) values.

RESULTS AND DISCUSSIONS

production using physical and chemical mutational agents. In this way, production of α-amylase by isolated bacterial species can be made high cost effective. The parameters for mutant strain were studied with reference to wild strain.

Isolation of α-amylase producing bacteria: *Bacillus licheniformis* was obtained from the soil through the potato baiting technique. Another study was carried out by Sadiq *et al.,* 2014 for the isolation of *Bacillus lechniformis* through potato baiting technique. Through Potato baiting isolated bacteria was confirmed according the earlier work. Morphological and biochemical tests confirmed that bacteria was *Bacillus lechniformis.* Gram staining, spore staining, citrate utilization, catalase, VP, starch hydrolysis, growth on dextrin, arginine and rhamnose agar confirmed that isolated bacteria was *Bacillus lichniformis* (Sadiq *et al*., 2014). Different isolates of parental strain *Bacillus licheniformis* were screened on the basis of starch hydrolyzing zone. Large starch hydrolyzing zone indicate that bacteria have ability to produce higher yield of α-amylase (Vaseekaran *et al*., 2010). Fermentation was carried out in the 1 L flask.

α-amylase production: Conventionally submerged fermentation used for the economical production of industrially important enzyme. Due to variety of facilities like easiness to control over pH, temperature, moisture and agitation speed submerged fermentation used. Polysaccharases, nucleic acid hydorlysing and proteases enzymes secreted by the *Bacllus* spp. Commercial production of amylase from *Bacillus licheniformis, Bacillus subtilis,* and *Bacillus caldolyticus* began in the late 1940s with the introduction of submerged culture or fermentation (Sivaramakrishnan *et al*., 2006). Submerged fermentation gave better product recovery as compare the solid state fermentation. From Submerged fermentation studies, it is revealed that *Bacillus subtilis* and *Bacillus licheniformis* are candidate of higher production of *bacillial* α-amylases (Raul *et al*., 2014).

Mutation induced by physical and chemical agents: Among the isolates, 3 isolates gave very high enzyme production. One isolate have the ability to produce large starch hydrolyzing zone, so it selected for the further studies. Previously ethyl methanesulfonate was directly used for the mutation

which cause higher enzyme production (Jin *et al*., 1998; Haq *et al*., 2009). Physical agent UV light exposure and chemical agent ethidium bromide were used for the mutation to check hyper-production of glucoamylase. Both mutagenic agents were applied on petri plates having bacterial growth (Ghani *et al*., 2013). The treatments of nitrous acid (NA) and Ethyl Methane Sulphonate (EMS) were used for improvement of *Bacillus subtillus* for the higher β-amylase production (Ali *et al.,* 2014). Another study, mutation created by the UV light and nitrosoguanidine (NTG) increases the production as compare the parent strain of *B. subtilis*. Mutation created in *Geobacillus stearothermophilus* by cloning the α-amylase gene in *E.coli* for the higher production of α-amylase (kebede, 2009). Similarly random mutagenesis was employed by exposing the bacterial strain with the chemical ethidum bromide and physical agent UV light exposure for increasing the yield of α-amylase. For fermentation process lactose 10.0, bactopeptone 14.0, yeast extract 6.0, KCl 1.0, $MnSO_4$ 0.001, $CaCl_2$ 0.25, $MgCl_2$ 0.2, $FeSO_4$ 0.0005, pH 7.0 medium was used. Similar medium was used earlier (Kelly *et al*., 1997, Haq *et al*., 2009). This medium gave maximum nutrition for the α-amylase production.

Incubation time effect on α-amylase production: Mostly, higher yield of amylase was obtained at 12 to 20 hours of fermentation or 24 hours as in a batch process when employing *Bacillus licheniformis* (Bozic *et al*., 2011). Different fermentation hour's comparison was done for the estimation of high yield of α-amylase. Research revealed that α-amylase production was maximum after 48 hours (Raul *et al.,* 2014). Fermentation time was estimated from 24-96 hours. It was noticed that higher α-amylase production after 72 hours of incubation. The maximum enzyme activity (102.9±1.92 U/ml/min) was obtained in fermentation flasks containing lactose and 72 hours after the inoculation (Haq *et al*., 2009). Similar work was carried out by Suman *et al*., 2010, analyzed the effect of incubation time and noticed that enzyme production was higher at 24 hours while growth of bacteria was higher after 72 hours of fermentation. The depletion of nutrients, accumulation of harmful by-products and proteolysis of α-amylase and microorganism cause the decrease production (Chamber *et al.,* 1999). In this study, different incubation time for fermentation was estimated from 24-96 hours. α-amylase production was maximum after 72 hours incubation. α-amylase production was decreases further increase the incubation period from 72-96 hours. Lactose was used in fermentation media. In fermentation medium lactose provide the carbon which cause the higher enzyme production (Sivaramakrishnan *et al*., 2006).

Effect of pH on production: It was concluded that 6.5 was the best pH for the maximum enzyme production by the mutated *Bacillus lichniforims* $EMS\text{-}200_{40}$. At 6.5 pH highest α-amylase activity (112.5±4.94 U/ml/min) was obtained which showed might be α-amylase stable at this pH (Haq *et al*., 2009). Soil isolated Bacillus thermolactics CU-48 mutated by the ethidium bromide. Mutated strain produced the 101% more activity of α-amylase as compare the wild strain at the 8.0 pH. Wild strain showed maximum production at the 7.0 pH. It showed that mutation shift the bacterial enzyme production from neutral to alkaline pH (Khodayari *et al*., 2014). The effect of different range of pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) on α-amylase production was also estimated for the wild and mutant strains EB200 (ethidum bromide) and UV300. Maximum enzyme production was obtained at 8.0 pH of ethidum bromide and UV mutated strains. It might be due to enzyme produced by the strain was quite stable at this pH. UV300 also gave highest enzyme production at alkaline pH, similar behavior as the ethidium bromide mutated *EB*200 strain. In contrast to the present research findings, achieved the optimal α-amylase production at a pH range of 8.0-9.5. It concluded that the

mutation shifts the higher enzyme production form 7.0 pH to 8.0 pH. All the other pH gave comparatively less enzyme production.

Effect of temperature on α-amylase stability: Temperature is very crucial for the microbe's. It has direct effect on the microbial growth activities. Therefore, temperature range gives the idea whether the microorganisms are of thermophilic, mesophilic, or psychrophilic nature (Sadiq *et al*., 2014). The effect of different temperatures (30, 40, 50, 60, 70, 80, 90, 100 and 110ºC) on α-amylase activity by the mutant strain of Bacillus licheniformis EB200 and UV300 was investigated. Earlier thermo-stable amylase production from *Bacillus steraothermophilus*, *Bacillus caldolytics*, and *Bacillus amyloliqufacies* was reported. Enzyme thermo-stability also estimated for the wild, UV and ethidum bromide mutated strains. Enzyme stability estimated by different ranges of temperature from 10-110ºC.

Results showed that wild strain enzyme was stable up to 60ºC. But enzyme stability of UV and ethidum bromide mutated strains were increased up to 80ºC but after 80ºC stability decrease in a very minute concentration. This showed that the α-amylase of mutated strains *EB*200 and *UV*300 was quite stable up to 110ºC. The maximum α-amylase activity of mutant strain was observed at 80ºC. Any change in pH and temperature values other than the optimal decreased the α-amylase production and activity. The present work is in accordance with the findings reported earlier (Shahhoseini *et al*., 2005; Haq *et al*., 2009; Vaseekaran *et al.,* 2010). Mutation had increased the 19% residual activity of N52Y while the wild-type did not show residual activity after incubation. The resistance to heat inactivation, half-life (t1/2), and the transition temperature (Tm) were used for the investigation of thermostability of N52Y mutant strain (Joo *et al*., 2011).

UV light exposure and nitrous acid are most commonly used mutagens (Azin and Noroozi, 2011). Effect of UV, ethyl methano sulphonate and nitrous acid were determined. It was concluded that mutating strain of ethyl methano sulphonate had better enzyme production (Haq *et al.,* 2013). Similarly, in this research ethidium bromide came out as a strong candidate for the higher enzyme production as compare the UV light exposure. *EB* 200 mutated strain was indulge in higher production at 8.0 pH, 72 hours of incubation and α-amylase was stable at 110°C. α-amylase from *B. licheniformis* is preferred by the industry due to thermostability and general perspective.

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