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Solid State Fermentation of *Bacillus amyloliquefaciens* $S8_{ST}$ for the Production of α-amylase

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Abstract. α-Amylase is an industrially important enzyme that catalyses the hydrolysis of starch to give products like glucose and maltose. Amylase producing strain was isolated using starch agar screening medium and was identified as *Bacillus amyloliquifaciens* using 16S rRNA gene analysis. Optimization of α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$ was done using technique of solid state fermentation (SSF). Various agro industrial substrates (wheat straw, rice husk, wheat porridge, wheat bran) were used for α-amylase production but wheat bran gave highest α-amylase activity (3.65 U/mg) when moistened with 10 mL diluent (D-3) at pH 7.0, temperature 37 °C with 10% vegetative inoculum after 72 h of incubation.

Keywords: wheat bran, screening, agro-industrial substrates, enzyme characterization

Introduction

α-Amylase (E.C.3.2.1.1) is an important enzyme that catalyses the hydrolysis of α-1,4 glycosidic linkage of starch to yield products, maltose, glucose and maltotriose units. Amylase comprises a well characterized group of proteins. They are all slightly acidic and water soluble proteins (Hussain *et al*., 2013; Bhargav *et al*., 2008).

α-Amylase is used in different industries (textile, paper detergent, biofuel, food and in medicine) for hundreds of years (Bhargav *et al*., 2008). It is used in food industry in baking for the preparation of cakes and in brewing. α-amylase is also used to improve colour, taste and aroma of the bread and to enhance the quality of bread (Couto and Sanroman, 2006; Kim *et al*., 2006). α-Amylase also plays a vital role in detergent industry. It is used to remove tough strains of starch and used to improve the detergency of laundry bleaches (Hmidet *et al*., 2009 and 2008).

α-Amylase is most commonly found in plants, animals and micro-organisms. In humans, amylase is secreted by both the pancreas and salivary glands. Among micro-organisms, α-amylase is produced by bacteria, yeast and fungi. Different researchers have used different fungi (*Aspergillus oryzae, Aspergillus niger, Mucor miehei* and *Thermomyces lanuginosus)* for α-amylase production (Kathiresan and Manivannan, 2006; Kunamneni *et al*., 2005). Different strains of yeast (*Aureobasidium pullulans*, *Candida rugosa, Candida famata*, *Candida kefyr* and *Saccharomyces cerevisiae)* have been used for α-amylase production (Aruna *et al*., 2014). Different bacteria (*Bacillus amyloliquefaciens, Bacillus brevis, Bacillus megaterium, Bacillus subtilis, Bacillus licheniformis, Bacillus circulans* and *Klebsiella pneumoniae*) have been reported by different researchers for amylase production (Hussain *et al*., 2013; Serin *et al*., 2012; Irfan *et al*., 2011; Tanyildizi *et al*., 2007). However, *Bacillus subtilis* is most commonly used for α-amylase production (Irfan *et al*., 2011).

Micro-organisms have substantial potential of α-amylase production due to efficient production strategies. Nevertheless, bacterial sources have various applications on industrial scale because they are easy to handle and rapid growth rates lead towards short fermentation cycles. Bacterial culture can be well managed for solid state fermentation, they have strong capacity to secrete proteins into extracellular environment, they need less space and serve as a most cost effective source (Konsoula *et al*., 2007). In addition, bacteria give consistent production of α-amylase, process optimization is easy and can be manipulated by genetic engineering or other means to obtain enzymes with desired characteristics (Asgher *et al*., 2007; Konsoula *et al*., 2007; Ellaiah *et al*., 2002).

Isolation of efficient amylase producing bacterial strains is important. Micro-organisms can be isolated from many sources, the most significant and efficient strains are typically isolated from substrate rich environment

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(Sanders, 2012). Different methods are used for identification of micro-organisms like PCR ribo-typing, 16S rRNA gene sequencing and Internally Transcribed Spacer (ITS), rDNA region RFLP (Brunk *et al*., 1996). 16S rRNA is the constituent of the small sub unit of a prokaryotic ribosome (30S). 16S rRNA gene sequence provides information for identification upto species level for different bacteria. 16S rRNA gene analysis is used in phylogenetic relationship between different species of *Achaea* and bacterial species and is the most common house keeping genetic marker with conserved and variable regions (Brunk *et al*., 1996).

Production of α-amylase has been carried out through different methods like submerged fermentation (SmF) and solid state fermentation (SSF) (Perez-Roses and Guerra, 2009). SSF has various benefits over SmF as it is simple technique which has higher volumetric productivity and has low capital investment. In addition, raw material and processing is cheap with low risk of contamination (Couto and Sanroman, 2006).

Major factors affecting microbial production of enzymes in SSF include solid substrate selection and also selection of micro-organism, moisture level, inoculum size and incubation time period. Different agro industrial substrates that are used for SSF include agricultural raw materials, waste products, industrial wastes and synthetic materials. Commonly used substrates for α-amylase production include rice husk, wheat bran (Negi and Banerjee, 2010), waste of dates, sugarcane baggase (Acourene and Ammouche, 2012; Perez-Roses and Guerra, 2009), corncob leaf, rye straw, oil cakes, wheat straw (Bhargav *et al*., 2008) coffee pulp and banana peel (Khan and Yadav, 2011).

Materials and Methods

Isolation and screening of α-amylase producing bacteria. Bacteria producing α-amylase were isolated from diverse samples (feed, soil, flour and compost) by serial dilution method on starch agar media (pH 7.0). The plates were incubated at 37 °C for 24 h following inoculation. After 24 h, the plates were flooded with iodine. Starch hydrolysis zone around bacterial colonies confirmed amylase producers.

Identification of isolate. ***Morphological characterization*.** Bacterial isolate was studied for colony characteristics (shape, colour, form, elevation and margin). Microscopic study was carried out to examine the cell morphology and Gram nature of bacteria.

***Biochemical characterization*.** Biochemical characterization was done using catalase test, oxidase test and Remel RapID ONE test strips after manufacturer's instructions.

***Molecular characterization*.** Isolation of DNA was done by method of Kronstad *et al*. (1983). 16S rRNA gene was amplified by using the primer 27F and 1492R.

For PCR reaction, 1 µL (50 mg) DNA was used, it contained 10 µM Primers (1 µL), dNTPs (2.5 µL), I X PCR buffer (5 µL), 25 mM magnesium chloride (2.5 µL) and *Taq* polymerase (1 µL) in 50 µL reaction combination. This reaction mixture was incubated at 94 °C for 5 min followed by 30 cycles comprising of denaturation step at 94 °C for 30 sec, annealing step for 30 sec at 52 °C and extension step for 60 sec at 72 °C. After 30 cycles, the final extension step was done for 10 min at 72 °C. After PCR completion, the bands of DNA were observed using agarose gel electrophoresis (0.8%) and visualized under UV light system. 16S rRNA gene was sequenced from 1st base sequence laboratories Singapore commercially.

Phylogenetic analysis. To observe highest similarities and relationship of 16S rRNA gene sequences, phylogenetic tree was constructed using BLAST tools (Altschul *et al*., 1990). Phylogenetic tree was constructed using neighbor joining technique (Saitou and Nei, 1987) and MEGA X (Kumar *et al*., 2018). Bootstrap examination of neighbor joining data on basis of 1000 replicates was used to check the stability of relationship.

Nucleotide sequence accession number. 16S rRNA gene sequence has been deposited in Genbank (MK72907).

Solid state fermentation. Diluent (10 mL) was added to 10 g solid substrate in 250 mL flask. After sterilization flasks were cooled to room temperature and then added bacterial inoculum (1 mL; 2.5×10^5 cells) to each flask carefully and flasks were incubated at 37 °C for 72 h. After incubation time period, about 50 mL phosphate buffer was added in each flask and placed in water bath for 1 h at 150 rpm (37 °C). Enzyme was filtered with muslin cloth and the filtrate was centrifuged at 6000 rpm for 10 min. Extract was used for protein and amylase activity analysis.

α-Amylase assay. The activity of reducing sugar and α-amylase was estimated by using DNS method following protocol of Rick and Stegbauer (1974). One unit of α-amylase is defined as the quantity of enzyme

required to release one μmol of maltose per min under the assay conditions.

Protein estimation. Protein was assessed using Bradford method (1976).

Diluents. Following diluents (g/L; pH: 7.0) were evaluated for α-amylase production. All the diluents were sterilized at 121 °C, 15 psi for 15 min.

D-1: $MgSO_4.7H_2O$ (1.0); NaCl (1.0); NH_4NO_3 (10); KH_2PO_4 (2.0) (Gangadharan *et al*., 2006).
D-2: Peptone (10); yeast extract (4); Starch (20); NaCl (0.5); $CaCl_2$ (0.2) $MgSO_4$ (0.5) (Haq *et al*., 2012).
D-3: Yeast extract (30); Peptone (20); Starch (10); $CaCl_2$ (2.0); $MgSO_4$ (0.5); $FeSO_4$ (0.1); $(NH_4)_2SO_4$ (2.0) (Haq *et al*., 2012).
D-4: Starch soluble (10); Nutrient Broth (10); NaCl (5); $CaCl_2$ (2); Lactose (5.0) (Haq *et al*., 2012).
D-5: Glucose (50); $MgSO_4$ (1.0); $(NH_4)_2SO_2$ (1.0); KH_2PO_4 (1.0) and yeast extract (10) (Abate *et al*., 1999).
D-6: Nutrient Broth (1.6); Glucose (6); (NH_4) SO_4 (0.45); $(NH_4)_2HPO_4$ (0.2); $MgSO_4$ (0.10); NaCl (2.0); $CaCl_2$ (0.02) (Haq *et al*., 2012).
D-7: Distilled water.

Statistical analysis. Significant differences among the replicates were calculated using computer software SPSS by Duncan's multiple series range test and expressed as probability (P) values. The results were calculated by using one-way ANOVA at the significance level 0.05.

Table 1. Morphological and biochemical characteristics of isolate

Morphological characterization	
Colony Characteristics	
Color	Cream
shape	circular
form	gelatinous
elevation	convex
margin	entire
Biochemical Characterization	
Gram's Reaction	+
Catalase	+
Oxidase	+
Urea (URE)	-
Arginine (ADH)	-
Ornithine (ODC)	-
Sugar aldehyde (KSF)	-
Sorbitol (SBL)	+
Fatty acid ester (LIP)	-
Lysine (LDC)	-
Aliphatic thiol (TET)	+
Nitrophenyl 1-D- glactsidase (ONPG)	-
p-nitrophenol D- glucuronide (GUR)	-
p-nitrophenyl-D-xyloside (BXYL)	-
Glutamyl-nephthylamide (GGT)	-
p-nitrophenyl-N-acetyl-D-glucosaminide (NAG)	-
Malonate (MAL)	-
p-nitrophenyl-D-glucoside (BGLU)	-
Proline-naphthylamide (PRO)	-
Tryptophan (IND)	-
Pyrrolidonyl-naphthylamide (PYR)	+
Adonitol (ADON)	+

+ = positive; - = negative

Results and Discussion

Isolation and screening of amylase producing strain. Isolation of fifty different of types of amylase producing bacterial strains was done from different samples (soil, feedstock, flour, compost) using plate screening method. Isolate $S8_{TS}$ that gave largest zone of hydrolysis was chosen for optimization studies (data not given).

Identification and characterization of isolate $S8_{TS}$. In colony characterization, colour, shape, margin, elevation and form were observed. The isolate formed cream coloured, circular and gelatinous colonies. The colonies were convex with entire margin whereas the cells were gram positive rods. Isolate $S8_{TS}$ was catalase and oxidase positive (Table 1), Genomic DNA was isolated from the isolate $S8_{TS}$ and 16S rRNA gene was amplified using following primers.

27F 5′-TTCCGGTTGATCCTGCCGGA-3′
1492R 5′- GGTTACCTTGTTACGACTT-3′

16S rRNA gene fragment (1.5 Kb) was successfully amplified for isolate $S8_{TS}$ (Fig. 1). 16S rRNA gene sequence analysis showed that the isolate was related to *Bacillus amyoliquefaciens* (98% similarity). A phylogenetic tree displayed similar relationship (Fig. 2).

Optimization of α-amylase production. *Selection of suitable substrate*. Different solid substrates (Wheat bran, Rice husk, Wheat straw, Wheat porridge) were tested for α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$, wheat straw gave lowest production (1.6 U/mg) of α-amylase, while wheat bran gave maximum production (2.78 U/mg) of α-amylase (Fig. 3). The reason might be that wheat bran comprises of suitable

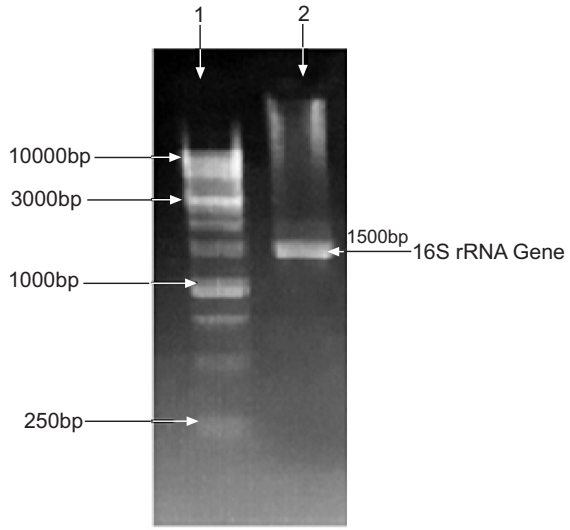

Fig. 1. 16S rRNA gene amplification of isolate $S8_{TS}$, Lane 1, Ladder 1 kb, Lane 2, 16S rRNA gene (1500 bp).

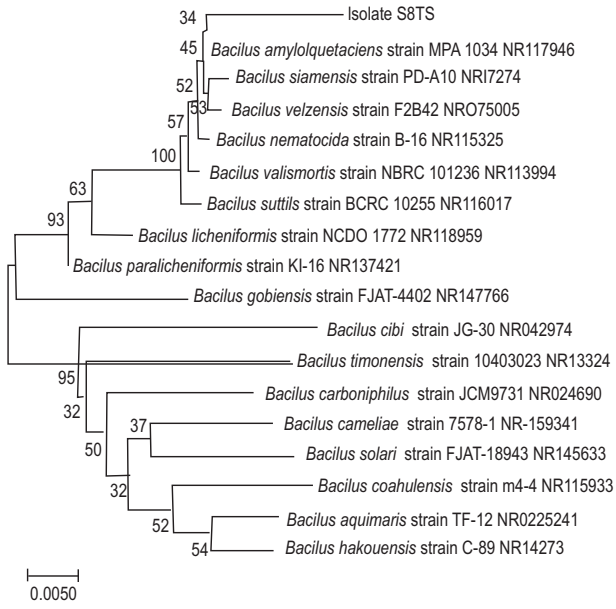

Fig. 2. Phylogenetic tree displaying evolutionary association between isolate and some *Bacillus* reference strains.

amount of fibre, cellulose, fats and proteins that are required for microbial growth and enzyme production (Haq *et al*., 2012; Rajagopalan and Krishnan, 2010). Almanaa *et al*. (2020) obtained enhanced α-amylase production with *Bacillus subtilis* D19 using wheat bran.

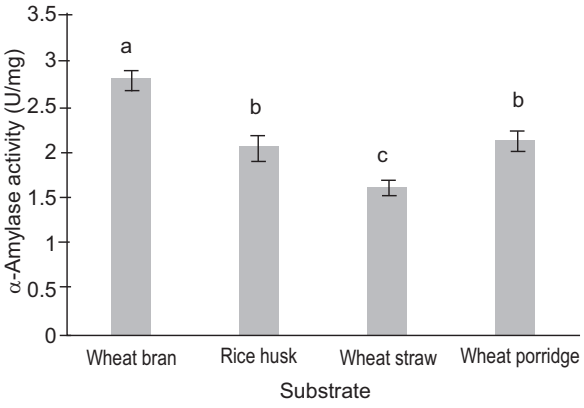

Fig. 3. Effect of solid substrates on α-amylase production by isolate $S8_{TS}$.
± indicate standard deviation among three replicates. Values followed by the different alphabet [a, b, c, d] differ significantly from one another ($P \leq 0.05$) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another, substrate value followed by letter "a" gave best α-amylase activity. Diluent D-3; diluent volume 10 mL; time period 48 h; temperature 37 °C; initial pH 7.0; inoculum size 10%.

Bacillus sp. KR-8104 also gave maximum amylase production with wheat bran (Hashemi *et al*., 2020).

***Effect of diverse types of diluents*.** Different types of diluents (D-1-D-7) were tested for α-amylase production. Diluent D-1 gave lowest yield (1.5 U/mg) of α-amylase while D-3 gave the best production (2.82 U/mg) of α-amylase (Fig. 4). Diluent D-3 showed maximum enzyme production because it contained $CaCl_2$. Calcium ions are necessary for the maintenance of activity, structure and constancy of amylase and also enhance enzyme production (Saha *et al*., 2014). Tested different types of diluents for α-amylase production and reported the same diluent best for amylase production Haq *et al*. (2012).

***Effect of diluent volume*.** Effect of diverse volume of diluent D-3 (5, 10, 15, 20 and 25 mL) was tested on α-amylase production. The lowest production (1.53 U/mg) was obtained with 5 mL diluent, while highest productivity of α-amylase was observed (2.73 U/mg) with 10 mL diluent (Fig. 5). Further increase in diluent volume reduced the production of amylase because higher moisture level decreases the pore size of the substrate.

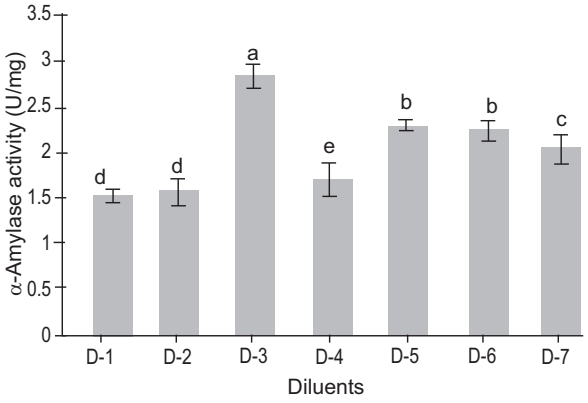

Fig. 4. Effect of different diluents on α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$.
± indicate standard deviation among three replicates. Values followed by the different alphabet $^{(a, b, c, d, e)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. Diluent value followed by letter "a" gave best α-amylase activity. Solid-substrate with wheat bran, pH 7; inoculum size 10%; temperature 37 °C; diluent volume 10 mL; time period 48 h.

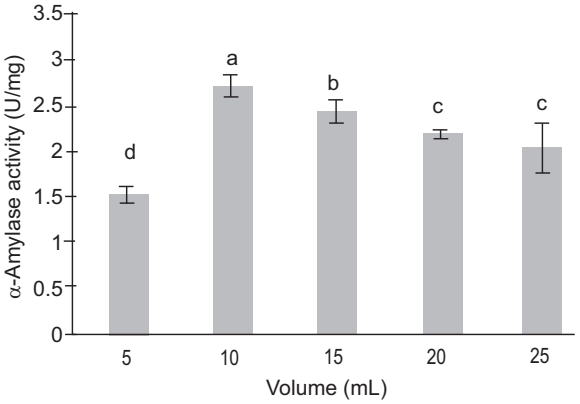

Fig. 5. Effect of different diluent volume on α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$.
± indicate standard deviation among three replicates. Values followed by the different alphabet $^{(a, b, c, d)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. Volume value followed by letter "a" gave best α-amylase activity. Solid substrate wheat bran, pH 7; temperature 37 °C; inoculum size 10%; time period 48 h; diluent D-3.

So, the oxygen transfer rate also decreases (Saxena and Singh, 2011). This is in agreement to Haq *et al*. (2012) who also reported maximum production of α-amylase with 10 mL diluent.

***Effect of pH*.** Effect of pH (6.0, 6.5, 7.0, 7.5 and 8.0) was examined on α-amylase production. pH 5.5 gave lowest production (1.63 U/mg) of α-amylase. Maximum α-amylase units were observed at pH 7.0 (2.92 U/mg) (Fig. 6). Further increase in pH resulted in decreased amylase production due to fact that alkaline pH inhibit bacterial growth and also effect the secretion of α-amylase. The initial pH also affects the membrane and cell activities (Irfan *et al*., 2011). Zambare *et al*. (2008) isolated bacterial strain *Enterobector* NACASA2 from soil collected from garden for α-amylase production that gave maximal amylase activity at pH 7. In contrast, Raplong *et al*. (2014) isolated bacterial species *B. cereus* and this strain gave highest amylase activity at pH 6.5 and Almanaa *et al*. (2020) obtained maximum amylase activity at pH 9 using *B. subtilis* D19.

***Effect of temperature*.** Incubation temperature also plays a vital role in metabolic actions of microbes. Optimum temperature is obligatory for appropriate bacterial growth as well as for enzyme production. Range of temperature (30, 37, 40 and 45 °C) was tested for α-amylase production. Lowest activity of α-amylase (0.987 U/mg) was observed at 30 °C. The most suitable temperature which gave the maximum α-amylase production (2.80 U/mg) was 37 °C (Fig. 7). Increase in cultivation temperature caused reduced enzyme production as the increase in temperature above the optimum level decreases the moisture level in the substrate and also effects the metabolic activities of the micro-organisms so the growth of micro-organisms might be retarded and low yield of amylase was obtained. Low temperature also attained lower enzyme yield because low temperature is not suitable for growth of micro-organism and less growth resulted in lower enzyme yield. Raul *et al*. (2014) and Akcan *et al*. (2011) also observed that optimal α-amylase production occurred at temperature 37 °C. Saha *et al*. (2014) using *Bacillus amyloliquefaciens* (MTCC1270) strain also found highest amylase production at temperature 37 °C. Hashemi *et al*. (2020) also reported maximal enzyme production at 37 °C with *Bacillus* sp.

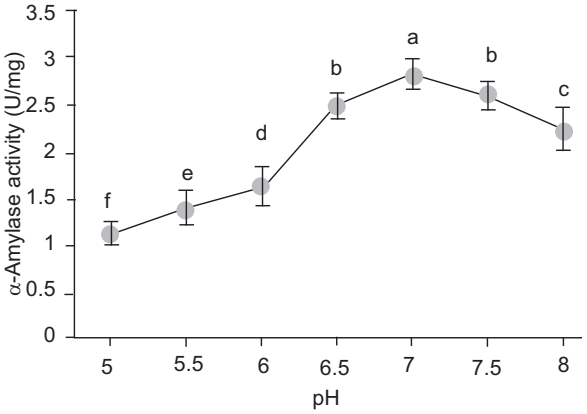

Fig. 6. Effect of pH on α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$.
± indicate standard deviation among three replicates. Values followed by the different alphabet $^{(a, b, c, d, e, f)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. pH value followed by letter "a" gave best α-amylase activity. Diluent volume 10 mL; time period 48 h; diluent D-3; inoculum size 10%; temperature 37 °C; solid substrate wheat bran.

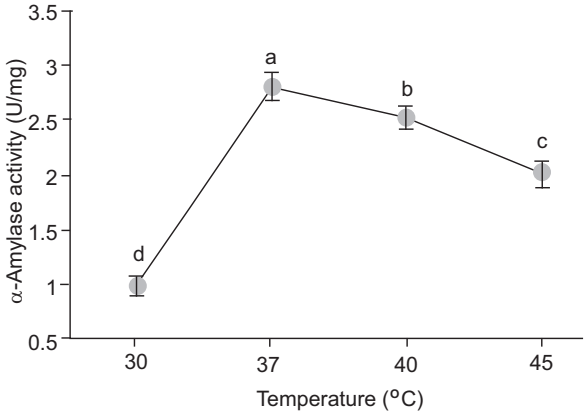

Fig. 7. Effect of different temperature on α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$. ± indicate standard deviation among replicates. Values followed by the different alphabet $^{(a, b, c, d)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. Temperature value followed by letter "a" gave best α-amylase activity. pH 7; solid substrate wheat bran; diluent volume 10 mL; inoculum size 10%; Time period 48 h; diluent D-3.

Effect of time period. Time period also effect the production of α-amylase. Effect of different time was observed for amylase production. α-amylase production was maximum (3.14 U/mg) after 72 h, but amylase production decreased with further increase in time period. Lowest production (0.97 U/mg) was observed after 24 h (Fig. 8). It might be due to reason that increase in time period retarded the microbial growth and reduced the yield of enzymes (Kanimozhi *et al*., 2014). Alva *et al*. (2007) also observed highest amylase activity after incubation period of 72 h. Alghabpoor (2012) isolated *Bacillus* sp that also gave the highest amylase production after 72 h. In contrast, Hashmi *et al*. (2020) obtained maximum α-amylase production after 48 h of incubation.

Effect of inoculum size. Inoculum size displays an important role for α-amylase production. The enzyme showed less production (1.5 U/mg) at inoculum size (1%) and showed highest production (3.50 U/mg) at inoculum size (10%) (Fig. 9). The reason might be that the inoculum level (1%) contained less number of viable cells in the production medium and they require more

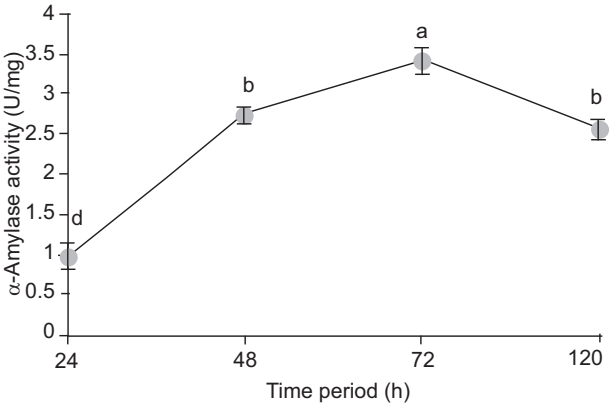

Fig. 8. Effect of time period on α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$.
± indicate standard deviation among replicates. Values followed by the different alphabet $^{(a, b, c, d)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. Time period value followed by letter "a" gave best α-amylase activity. pH 7; diluent volume 10 mL; Temperature 37 °C; inoculum size 10%; solid substrate wheat bran; Diluent D-3.

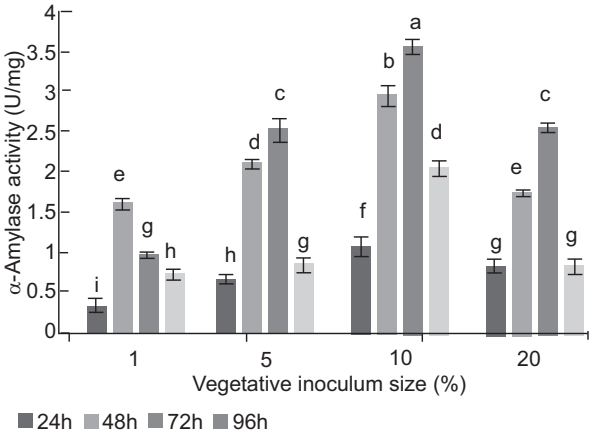

Fig. 9. Effect of inoculum on α-amylase production by *Bacillus amyloliquefaciens* $S8_{TS}$. ± indicate standard deviation among replicates. Values followed by the different alphabet $^{(a\text{-}i)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. Vegetative Inoculum size value followed by letter "a" gave best α-amylase activity. Time period 72 h; pH 7; Temperature 37 °C; diluent D-3; solid substrate wheat bran; diluent volume 10 mL.

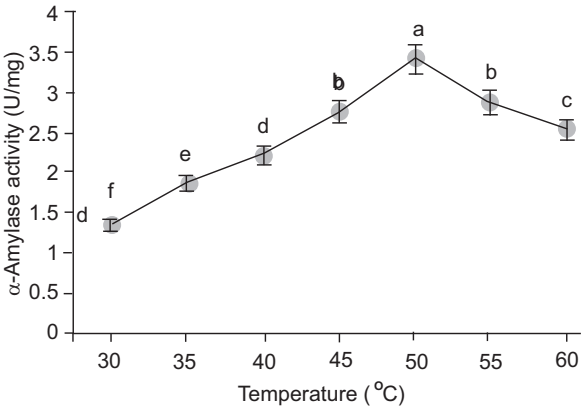

Fig. 10. Effect of different incubation temperature on α-amylase activity by *Bacillus amyloliquefaciens* $S8_{TS}$. ± indicate standard deviation among replicates. Values followed by the different alphabet $^{(a,b,c,d,e,f)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. Temperature value followed by letter "a" gave best α-amylase activity.

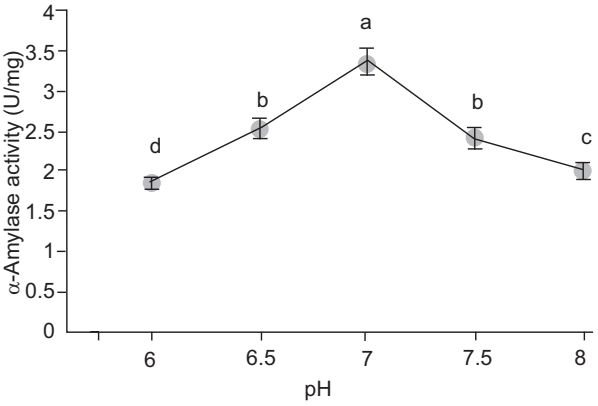

Fig. 11. Effect of different incubation pH on α-amylase activity by *Bacillus amyloliquefaciens* $S8_{TS}$. ± indicate standard deviation among replicates. Values followed by the different alphabet $^{(a,b,c,d)}$ differ significantly from one another (P≤0.05) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. pH value followed by letter "a" gave best α-amylase activity.

time for growing. 10% inoculum medium contain large amount of viable cells that showed maximum growth and increased enzyme production. Further, higher inoculum size may also decrease down the production of enzyme because of large number of viable cells and less nutrient availability or the accumulation of toxic metabolites was rapid (Zhang *et al*., 2007). Alghabpoor (2012) isolate *Bacillus* strain for α-amylase production using SSF and observed extreme productivity of α-amylase at inoculum size 10%. In contrast, Almanaa *et al*. (2020) obtained maximum α-amylase activity with 5% inoculum.

Characterization of crude enzyme. Effect of different incubation temperature (30-60 °C) was tested on the activity of α-amylase. The low activity (1.36 U/mg) of α-amylase was observed at temperature 30 °C and maximum production of α-amylase (3.41 U/mg) was observed at 50 °C (Fig. 10). Further increase in the incubation temperature caused a decline in amylase activity because gradual increase in temperature might have degraded the enzyme structure. Yusra *et al*. (2018) and Abd-Elhalem *et al.* (2015) isolated bacterial strain *Bacillus licheniformis* from soil sample. They also observed highest amylase activity at incubation temperature 50 °C.

Concerning pH, isolate $S8_{TS}$ gave maximum enzyme yield (2.42 U/mg) at pH 7 (Fig. 11). Methew *et al.* (2015); Kumar *et al*. (2014); Onofre *et al*. (2012);

Unakal and Kaliwal (2008) tested different ranges of pH and incubation time for amylase activity but the suitable pH for enzyme activity was found to be 7.0. Isolate $S8_{TS}$ showed highest amylase action (3.65 U/mg) after 15 min of incubation (Fig. 12). Bezoic *et al*. (2011) observed highest amylase activity after 15 min of incubation at 50 °C and pH 6.5.

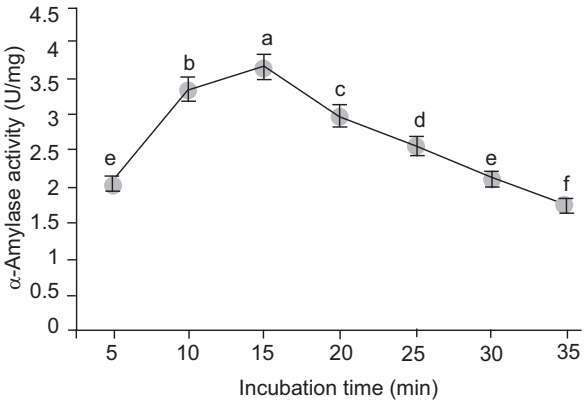

Fig. 12. Effect of different incubation time on the activity of α-amylase by *Bacillus amyloliquefaciens* $S8_{TS}$.
± indicate standard deviation among replicates. Values followed by the different alphabet $^{(a,b,c,d,e,f)}$ differ significantly from one another ($P \leq 0.05$) by Duncan's multiple range test, while values followed by the same alphabet do not differ significantly from one another. Incubation time value followed by letter "a" gave best α-amylase activity.

Conclusion

Isolate $S8_{TS}$ identified as *Bacillus amyloliquefaciens* gave maximum α-amylase production (3.65 U/mg) at pH 7, fermentation time period 72 h, temperature 37 °C, Diluent D-3 and inoculum size 10% with wheat bran as solid substrate.

Conflict of Interest. The authors declare no conflict of interest.

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