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Process Optimization for Enhanced Production of α-amylase by submerged (SmF) and Solid State Fermentation (SSF) Using *Bacillus pumilus* VITMDS2.

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ABSTRACT

The production of α-amylase was carried out from both submerged fermentation and solid state fermentation by newly isolated *Bacillus pumilus* VITMDS2 Different parameters were optimized and studied separately involving incubation period, temperature, inoculum size and pH for obtain maximum enzyme activity. The enzyme was partial purified through acetone precipitation and gel-permeation chromatography. The extracellular α-amylase was purified 1.92 fold (SmF) and 5.9 fold (SSF) by Sephadex G-100 chromatography and its molecular mass was found to be around 38 kDa (SmF), 42 kDa (SSF) as revealed by SDS-PAGE. The produced α-amylase was analyzed for functional groups using Fourier Transform Infra-Red Spectroscopy (FTIR) and for retention time using High performance liquid chromatography (HPLC). In solid state fermentation maximal amount of α-amylase production (0.956 U/mg) was obtained with inoculum size 9 %, pH 6.5 at 37˚C for 24 h. In Submerged fermentation highest amylase production (0.75 U/mg) was obtained with inoculums size 2 %, pH 7.0 at 30˚C for 72 h. Solid state Fermentation have been established as a superior technique, economically valuable than the submerged fermentation for the production of enzyme.
Keywords: α-amylase, *Bacillus pumilus*, submerged fermentation, solid state fermentation, HPLC

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INTRODUCTION

Amylases are produced by a variety of living organisms ranging from bacteria to plants, humans and animals. The hydrolysis of starch with amylase results first in the production of short-chain–polymers called dextrin then the disaccharide maltose, and finally glucose. Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non-reducing end successively resulting in short end products [1].

The α-amylase (α-1,4 glucan-glucanohydrolase EC 3. 2.1. 1) is widely distributed in nature. This extracellular enzyme hydrolyses α-1,4glucosidic linkages randomly throughout the starch molecule in an endo-fashion producing oligosaccharides and monosaccharides including maltose, glucose and alpha limit dextrin [2, 3, 4]. Different fungal and bacterial strains have been extensively used for the enzyme production [5]. It has so far been reported that fungi and yeast, such as *Aspergillus* sp. [6] and *Rhizopus* sp. [7] and *Cryptococcus* sp. [8] as well as bacteria such as *B. subtilis*, *B.stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* [9] were good producers of raw starch digesting amylases. The ability of bacterial strains to secrete large amounts of extracellular protein has made them well suited for the industrial enzyme production. However, *Bacillus* sp is the organism of choice because of its ubiquitous nature, non fastidious nutritional requirements, ability of liquefaction of starch due to it high temperature stability and high productivity of α- amylase [10, 11]. The production of α-amylase by submerged fermentation (SmF) and solid state fermentation (SSF) has been investigated and depend on a variety of physicochemical factors [12, 13, 14, 15]. However, comparative studies between SmF and SSF claim higher yields and other advantages for products obtained by SSF, such as lower energy requirement and less water output, better product recovery and lack of foam build up, besides it is reported to be the most appropriate process for developing countries. Various physical and chemical factors have been known to affect the production of α- amylase such as temperature, pH, period of incubation, inoculums percentage, moisture and agitation with regards to SSF and SmF, respectively. Interactions of these parameters are reported to have a significant influence on the production of the enzyme. Traditionally the purification of amylases from fermentation media has been done in several steps which include centrifugation of the culture (a step of extraction may be required for solid media), selective precipitation of the enzyme by ammoniurn sulfate or organic solvents such as acetone, ethanol in the cold. Then the crude enzyme is subjected to chromatography (usually affinity or ion exchange chromatography) and gel Permeation chromatography [16].

α- amylases are one of most important and widely used enzymes whose spectrum of application has widen in many sectors such as clinical, medicinal and analytical chemistry. Beside their use in starch saccharification they also find applications in food, baking, brewing, detergent, textile and paper industries. These are important enzymes used in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar constituents. Increasing utility and consumption of α- amylase in different industries has placed a greater stress on increasing indigenous enzyme production and search of more rapid processes [17,18,19].The purpose of this research is to isolate a bacterial strain from

soil sample capable of producing α-amylase. Also, an attempt was made to increase the productivity of enzyme by optimizing the process parameters such as appropriate pH, temperature, incubation period, inoculum size, productivity and enzyme activity in solid state fermentation (SSF) and submerged fermentation (SmF)

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from: yeast extract, peptone, starch (HiMedia Laboratories, Mumbai, India); Sephadex G-100 (Sigma). All other chemicals used were of analytical grade.

Isolation of amylase producing *Bacillus* sp

Soil sample was collected at 3 to 4 cm depth from local area (kitchen waste dumping area of hotels) from Vellore market, Tamil Nadu with the help of sterile spatula and transferred to lab in to sterile autoclavable bags for microbiological analysis. For the isolation of *Bacillus sp*, 1g of soil samples were weighed and serially diluted upto 10^{-6} for the enumeration of *Bacillus sp* on Nutrient agar. The plates were incubated at 37º C for 24 h. Based on distinct morphology in color, size, elevation, shape bacterial colonies were randomly picked from plates and subjected for initial identification by gram's reaction Pure culture was maintained on Nutrient agar and stored at -20º C in Nutrient broth with 20% (W/V) glycerol for further use.

Screening of α-amylase producing bacteria

The starch hydrolysis tests for selected bacterial isolates were screened for amylase production on starch agar plate [20]. Pure bacterial isolates were streaked on the starch agar plate and incubated at 37°C for 24 h. After incubation 1% iodine solution was flooded on the starch agar plate observed for a clear zone around the bacterial growth indicated hydrolysis of starch (positive result) and were considered as amylase producing bacterial strains.

Biochemical characterization

The strain was tested for Catalase test, Oxidase test, Urease test, Indole test, Methyl red test, Simmons citrate test, Starch hydrolysis test, Voges Proskauer test, Nitrate reduction test, Gelatin hydrolysis test and Sugar fermentation test. Characteristics of the isolate were compared with data from *Bergey's Manual of Systematic Bacteriology* [21].

Molecular identification

16S r RNA gene sequence of bacterial strains was amplified using forward primer as 5' - CWG RCC TAN CAC ATG SAA GTC - 3' and reverse primer as 5' - GRC GGW GTG TAC NAG GC - 3' and 16S r RNA gene sequences was characterized at (Amnion Bio Sciences Pvt. Limited) and the aligned sequence were compared with GenBank nucleotide database with

BLAST [22] Sequences were deposited at the GenBank database and under EMBL (European Nucleotide Archive). The 16s r RNA gene sequences determined were aligned along with the sequences of the type strains obtained from the Gene Bank using the CLUSTAL W program version 2.1 [23]. A phylogenetic tree was constructed by neighbor- joining method [24] with the programme Tree view (Version 1.6.6). Individual branches in phylogenetic tree were determined by bootstrap analysis based on 1000 samplings [25].

Optimization of process parameters on α-amylase production in SmF and SSF by Using Classical One Variable at a Time Method (OVAT-Method)

The process was carried out systematically in such a way that the parameter optimized in one process was maintained at its optimum level in the subsequent process. The cultural conditions optimized for alpha amylase productivity included incubation period (24, 48, 72, 96h), Temperature (20, 25, 30, 35and 40°C), pH (6, 6.5, 7, 7.5 and 8.0) and inoculum size (1- 5%) for α-amylase enzyme production by SmF and SSF.

Production and extraction of α-amylase by SmF

The bacterial strainVITMDS2 was inoculated in nutrient broth followed by incubation at 37ºC for 24 h to get a standardized inoculum (0.5 OD at 600 nm with 3.5×10^5 CFU/ml). For the production of amylase enzyme, production media was prepared which contains (g/L) 10g starch, 5g peptone, 5 g yeast extract, 1 g KH_2PO_4, 0.2g $MgSO_4.7H_2O$, 0.2 g $CaCO_3$, 5 g NaCl. Maximum α-amylase was produced by optimized parameters such as incubation period, temperature, pH and inoculum size [26, 27]. After incubation, fermented broth was centrifuged at 10000 rpm for 10 min at 4 °C and supernatant was collected. Cell free supernatant recovered by centrifugation was used as crude enzyme.

Production and extraction of α-amylase by SSF

Production were conducted in 250 ml Erlenmeyer flasks containing 5 g of the substrate(wheat bran) with 10ml of sterile liquid nutrient medium containing (%): [KH_2PO_4 - 0.1, NaCl- 0.25, $MgSO_4.7H_2O$- 0.01, $CaCl_2$- 0.01]. Maximum α-amylase was produced by optimized parameters such as incubation period, temperature, pH and inoculum size. For the extraction, 20 ml of distilled water was added to the culture flasks and mixed well in a rotary shaker (120 rpm) at room temperature for 30 min [28]. After that it was filtered and centrifuged at 10,000 rpm for 20 min at 4°C. The pellet was discarded and supernatant was used as the crude enzyme source.

Purification of α-amylase

To concentrate the supernatant, prechilled acetone was added slowly to the solution of crude culture supernatant up to 6 times the sample volume and vortex the solution for 5min. The proteins were allowed to precipitate overnight at 4ºC. The precipitates were centrifuged at 10000 rpm for 15 min at 4ºC then decant and properly dispose of the supernatant being careful to not dislodge the protein pellet. The pellet was briefly washed with 100μl of cold 90% acetone and again centrifuged for 5 min at 10000 rpm for 15 min at 4ºC. The pellet was air dried at room temperature for 15-30 min and resuspended in a

minimum volume of (0.1M) phosphate buffer (pH 6.5).The resulting solution was loaded on Sephadex G-100 matrix, equilibrated with (10mM Tris buffer-pH 7.2) and eluted with the same buffer at a flow rate of 0.5ml/min. Fractions of (3 ml) were collected and monitored for protein (280 nm) and amylase activity. The molecular weight of purified enzyme was determined by SDS-PAGE using BSA as standard. The Lowry method was applied to determine the protein concentration in crude and purified samples [29]. The fractions showing the maximum specific activity was further subjected to High Performance Liquid Chromatography (HPLC) and Fourier transform infrared spectroscopy (FT-IR) analysis.

Assay of α-amylase

Estimation of amylase activity was carried out according to the di nitro salicylic acid (DNS) method of [30]. According to the procedure 1.0ml of culture extract 'enzyme' and 1ml of 1% soluble starch in (0.1M) phosphate buffer (pH 6.5) was added in test tube. The test tubes were covered and incubate at 50°C for 30 minutes in water bath. Then 2.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 10 minutes. After cooling at room temperature, the absorbance was read at 540 nm by colorimeter against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µ mole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min).

High Performance Liquid Chromatography (HPLC)

For all analysis that involved HPLC, the conditions were set as follows: the column employed was of analytical column Supelcosyl LC-NH2 (5 µm) with length of 250 mm and 4.6 mm internal diameter. The mobile phase consists of acetonitrile and water in ratio 75:25 (v/v). The flow rate was set at 1 ml/min. The internal column temperature was maintained at 30ºC and the evaporation temperature was 60ºC [31]. The eluent from the column was monitored at 280 nm by refractive index detector, and the data were recorded by the integrated computer system attached to the HPLC (Waters).

Fourier Transformation-Infra Red Spectroscopy (FT-IR)

The FT-IR spectrum of the amylase was obtained at a resolution of 4 cm^{-1}. The sample was incorporated into KBr (spectroscopic grade) and pressed into a 2 mm pellet. IR spectra were recorded in the transmittance mode from 4000 to 400 cm^{-1}.

RESULTS AND DISCUSSION

Isolation and Identification of amylase producing bacteria

Bacteria isolated from starch rich materials may have better potential to produce enzyme under adverse conditions [32]. Microorganisms that produce amylases could be isolated from places such as soil around mills, cassava farms and flour markets as well as soil contaminated with decaying materials including kitchen waste, bakery waste [33]. Similarly, in the present research work α-amylase producing bacterial strains was isolated from soil contaminated with kitchen waste. Five *Bacilius* sp were isolated from soil and screened for

amylase production, among them only one strain VITMDS2 showing clear hydrolytic zone on starch agar plate at 37 ºC. Further bacterial isolate was characterized on the basis of colony characteristics, microscopic appearance and biochemical tests (Table1).

Table 1- Characterization of VITMDS2 strain

Mode of characterization	Characteristics of bacterial isolates	MDS02 bacterial strain
Cultural	Colony morphology on nutrient agar	Small, round, regular, mucoid, creamy yellow and fast growing colonies
Microscopic	Gram staining and Spore staining	Gram positive and Spore forming
Biochemical	Indole test	Negative
	Methyl Red test	Positive
	Voges Proskauer test	Negative
	Citrate utilization test	Positive
	Catalase test	Positive
	Oxidase test	Positive
	Urease test	Negative
	Nitrate reduction test	Positive
	Gelatin liquefaction test	Positive
	Starch hydrolysis test	Positive
	Sugar fermentation test Lactose, Fructose	Positive
	Xylose, Mannitol,	Negative
	Ramnose , Galactose	Negative

Molecular characterization of VITMDS2

Molecular techniques were used to prove and further confirm the identification of the isolate VITMDS2 to the species level. 16S rRNA phylogeny studies were primarily carried out. Sequencing results of 16S rRNA was exported to the database and checked for homology alignment. Based on the alignment results VITMDS2 was found as *B. pumilus*. The 16S rRNA sequence (1318 nucleotides) of VITMDS2 strain has been submitted to NCBI database and the accession number was KC991039. The 16S rRNA analysis of isolate showed 98% similarity with *B. pumilus*. Fig.1 shows the phylogenetic tree based on 16S rRNA sequences of strains.

Optimization of fermentation parameters for α-amylase production by SmF

Effect of incubation period on α-amylase production

Our results indicated that the optimum incubation period for amylase production was 72 h (Fig.2). This result is in complete accordance with finding of many investigators [34, 35, 36]. Incubation beyond 72 h was undesirable as this resulted in decreased enzyme activity. The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium and probably due to depletion of nutrients available to microorganism. The maximum specific activity of α-amylase was recorded at 72 h incubation period (0.071U/mg) as shown in Fig. 2.
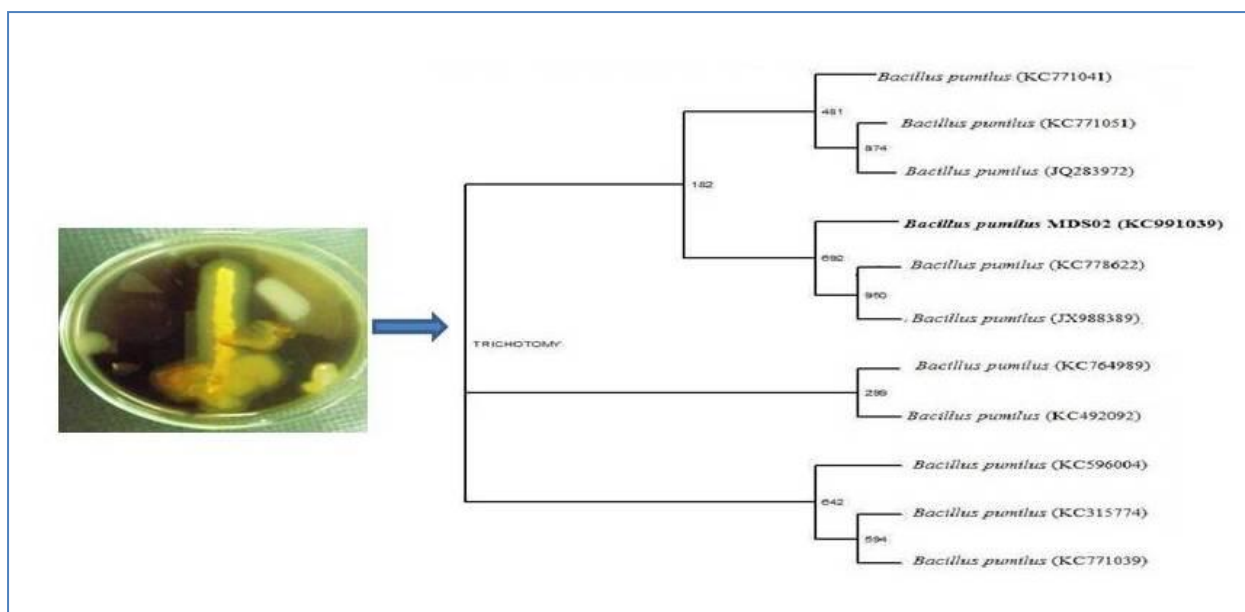
Fig. 1 The neighbor-joining method cladogram showing a phylogenetic relationship between *Bacillus pumilus* VITMDS2 and other related *Bacillus sp* based on the 16s rRNA gene sequence analysis. The numbers at branching point refer to bootstrap values based on 1000 re-samplings. The branch lengths in the cladogram are not to scale
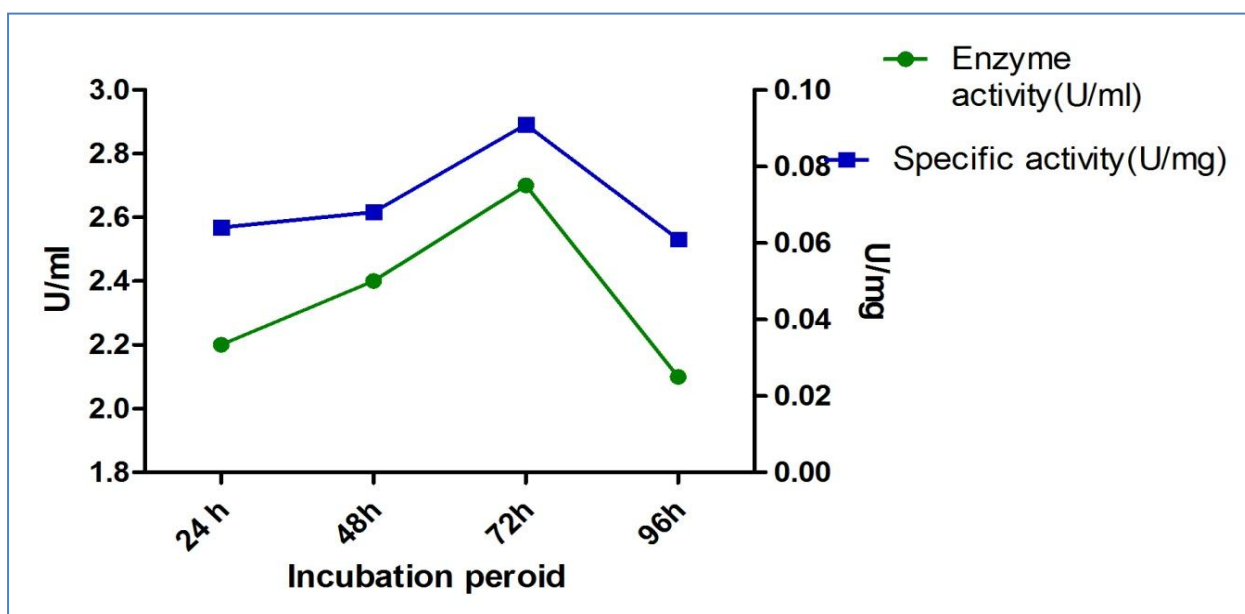

Fig. 2 Enzyme activity and Specific Enzyme activity of α- amylase at different incubation period for SMF

Effect of pH on amylase production

In our study maximum specific activity of α-amylase by *B. pumilus* VITMDS2, was found maximum at pH 7.0 (0.121U/mg). Further increase in the pH resulted decrease in the activity of amylase (Fig.3). When pH is altered below or above the optimum, the activity is decreased. Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth [37].
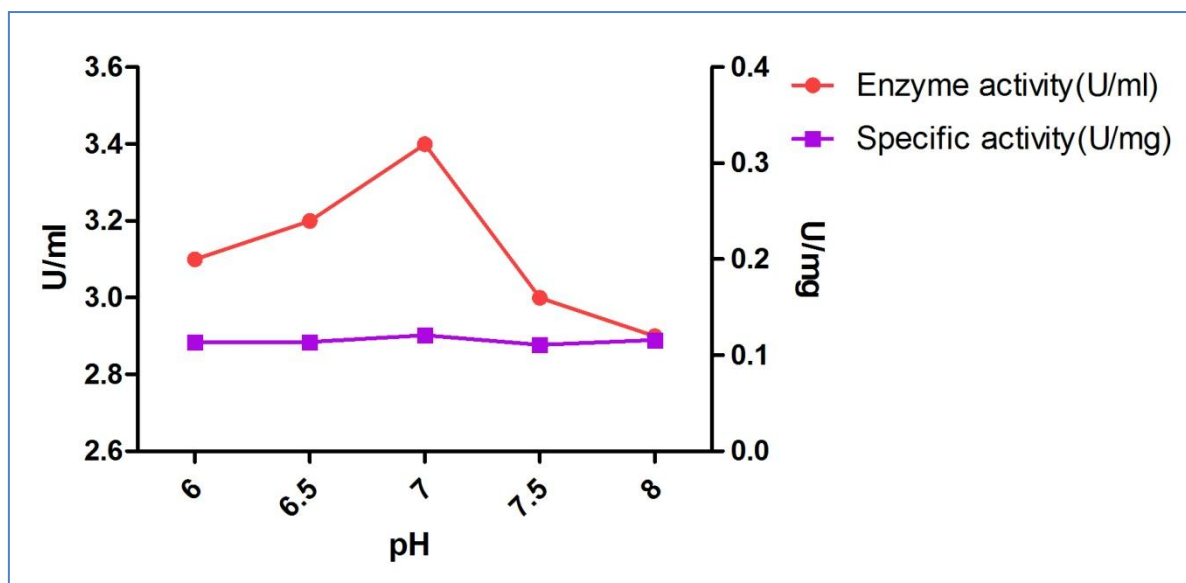
Fig. 3 Enzyme activity and Specific Enzyme activity of α- amylase at different pH for SMF

Effect of temperature on amylase production

Results show the effect of different incubation temperature on the production of amylase by *B. pumilus* VITMDS2. The maximum specific enzymatic activity 0.25U/mg was obtained at 30 °C as shown in Figure 4. Increase in incubation temperature, decreased the production of enzyme. The production of the enzyme was greatly inhibited at 40 °C. It might be due to that at high temperature, the growth of the bacteria was greatly inhibited and hence, enzyme formation was also prohibited.

Fig. 4 Enzyme activity and Specific activity of α- amylase at different temperature for SMF

Effect of inoculum size on α-amylase production

Fig.5 shows the effect of different size of inoculum on the production of α-amylase by *B pumilus* VITMDS2 in shake flask. The inoculum at the level of 1-5% was studied for the

production of enzyme. The maximum specific activity of enzyme was obtained at 2% level of inoculum (0.155U/mg). As the level of inoculum was increased, the production of the enzyme was decreased. Thus the inoculums level of 2.0% (v/v) was found optimum for fermentation Studies of alpha amylase [38].
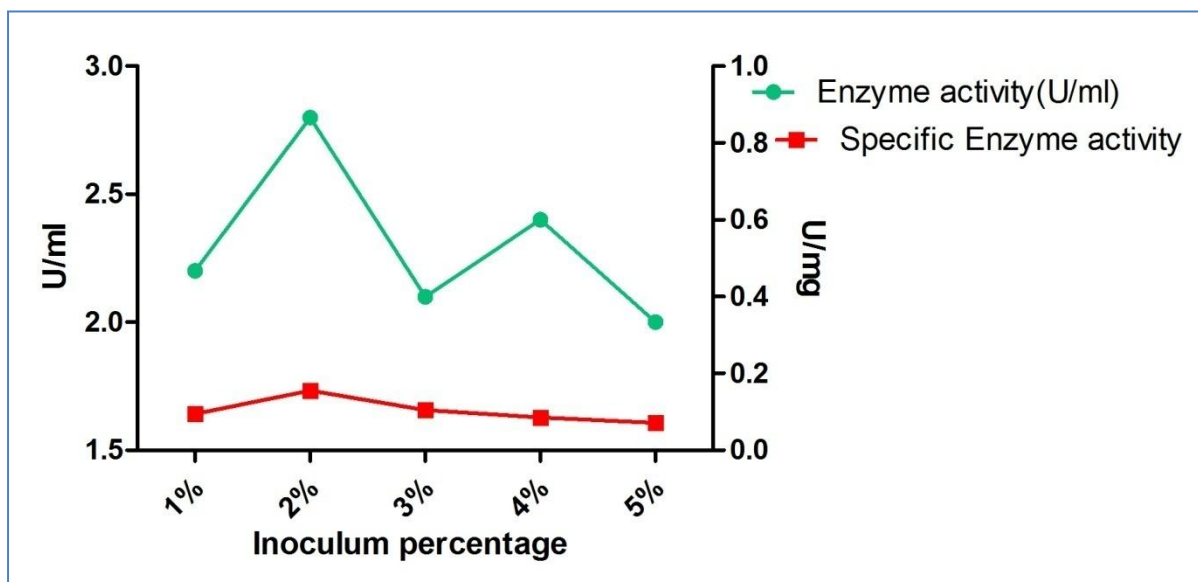

Fig. 5 Enzyme activity and Specific activity of α- amylase at   different size of inoculum percentage for SMF

Optimization of fermentation parameters for α-amylase production by SSF

Effect of incubation period on α-amylase production

The *B pumilus* VITMDS2 showed maximum specific activity 0.155U/mg at 24 h. The production of enzyme decreased after 24 h for wheat bran as shown in Fig.6. Enzyme production is related to growth of the microorganism. Growth of the organisms would have reached a stage (due to insufficient nutrients) that indirectly stimulates production of secondary metabolites [39].
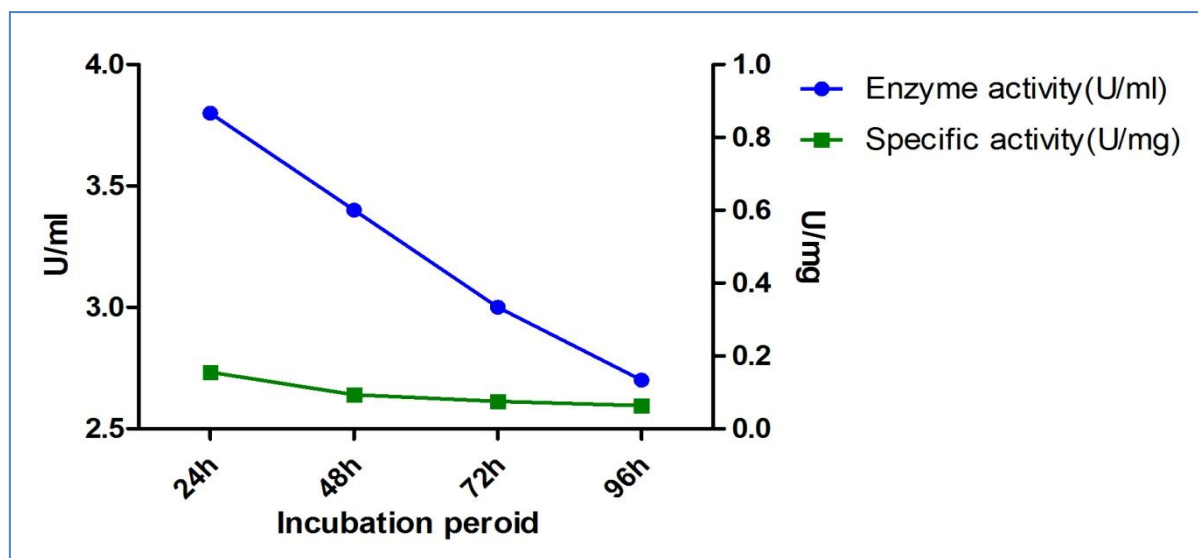

Fig. 6 Enzyme activity and Specific activity of α- amylase at different incubation period for SSF

Effect of pH of the medium on α-amylase production

Among the physicochemical parameters, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. The specific activity was maximum, when initial medium pH was 6.5 (0.097 U/mg) for wheat bran as shown in Fig.7. Further increase in pH resulted in decrease of α-amylase production by the *B. pumilus* VITMDS2. Generally, agro industrial wastes possess unique buffering action and have advantages for enzyme production. Therefore, in the subsequent process, the initial pH of the fermentation medium was adjusted to 6.5.
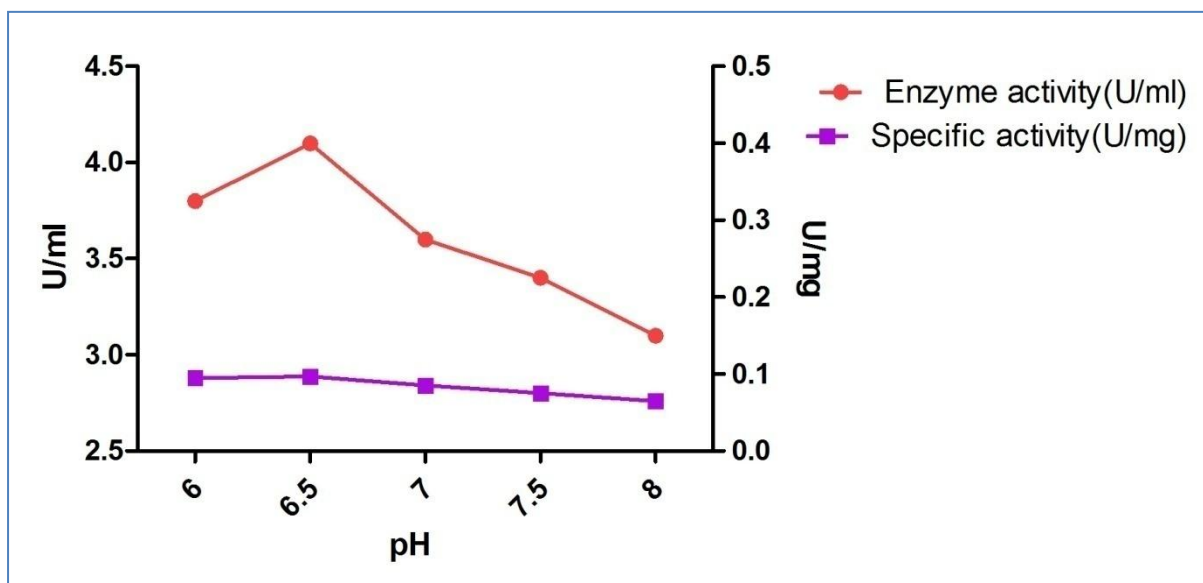

Fig. 7 Enzyme activity and Specific activity of α- amylase at different pH for SSF

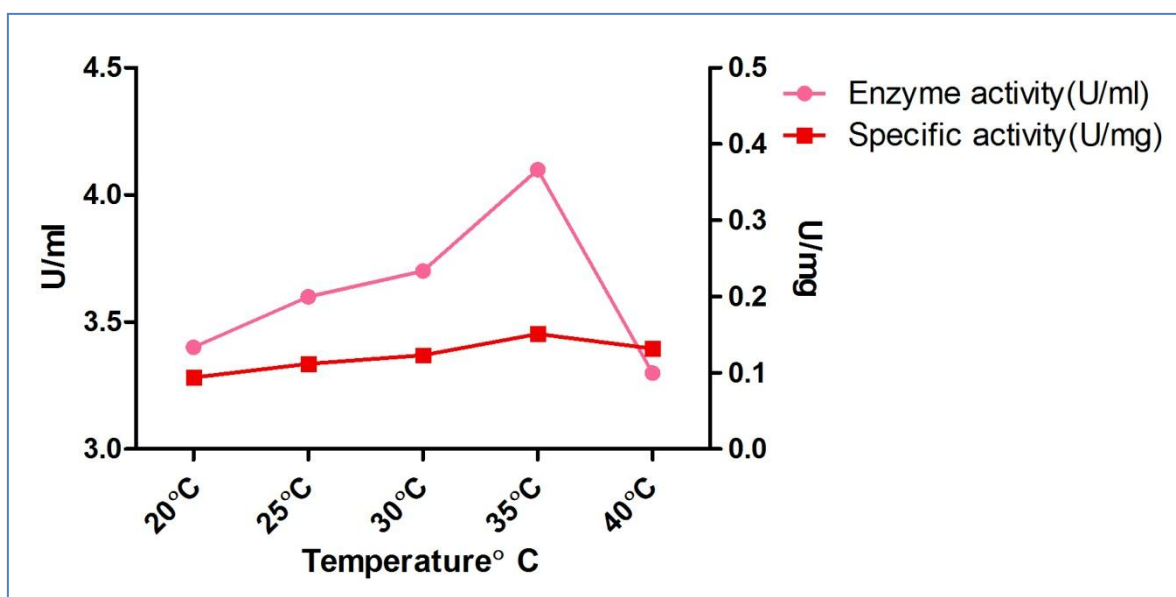**Effect of incubation temperature on α-amylase production**

Fig. 8 Enzyme activity and Specific activity of α- amylase at different temperature for SSF

Temperature plays a significant role in development of the biological process as it influences protein denaturation, enzyme inhibition and cell growth [40]. Investigation of the effect of temperature on enzyme production during fermentation showed that the optimum temperature for maximum specific activity of α-amylase was 35 °C as shown in Fig.8. It has also been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial activity and thus the initial set temperature is vital [41].

Effect of inoculum size on α-amylase production

The inoculum size was also an important factor for the production of α-amylase. The highest specific enzyme activity (0.105U/mg) was obtained at an inoculum size of 9% (v/w) as shown in Fig.9. A higher inoculum size may increase moisture content and lead to a decrease in growth and enzyme production; this may be due to the limiting nutrients at higher inoculum size and a lower inoculum size may require a longer time for fermentation to form the desired product [42, 43].
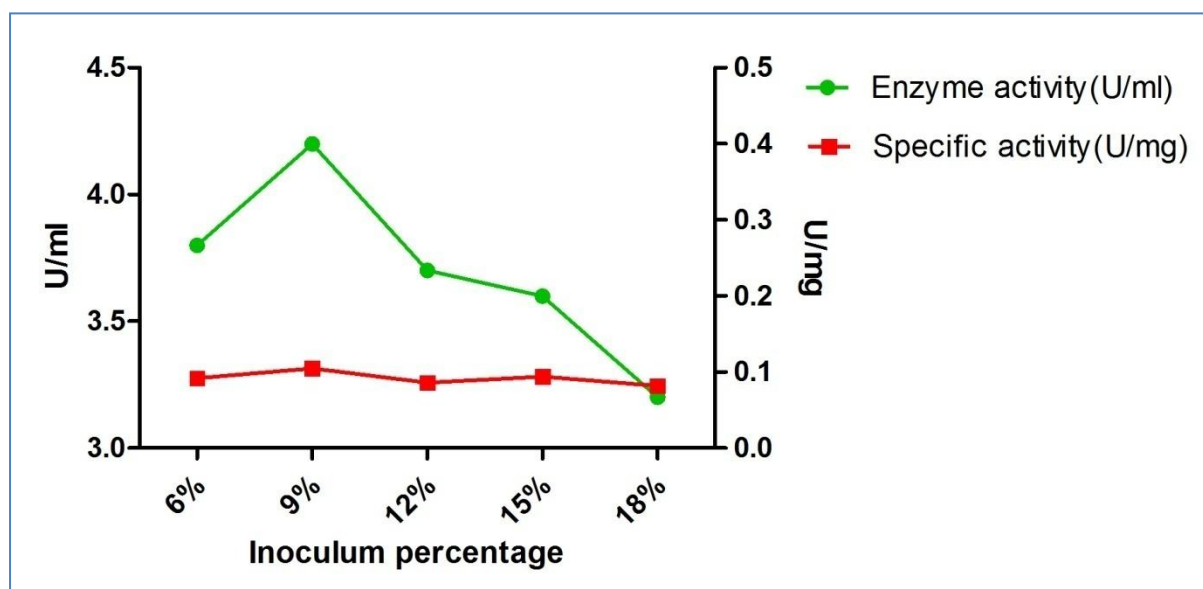

Fig. 9 Enzyme activity and Specific activity of α- amylase at different percentages of inoculum for SSF

Production of α-amylase using *B. pumilus* VITMDS2 with Optimized parameters by submerged fermentation (SmF)

Production media was adjusted to optimized pH 7.0 and inoculated with optimized 2% of 24 h inoculum of VITMDS2 and incubated at optimized incubation period of 72 h at optimized temperature 30 ºC. The enzyme activity and specific enzyme activity (crude) observed as 3.9U/ml and 0.390U/mg respectively.

Production of amylase using *B Pumilus* VITMDS2 with Optimized parameters by Solid state fermentation (SSF)

Production media was adjusted to optimized pH 6.5 and inoculated with optimized 9% of 24 h inoculum of VITMDS2 and incubated at optimized incubation period of 24 h at

optimized temperature 35 ºC. The enzyme activity and specific enzyme activity (crude) observed as 4.4U/ml and 0.162 U/mg respectively.
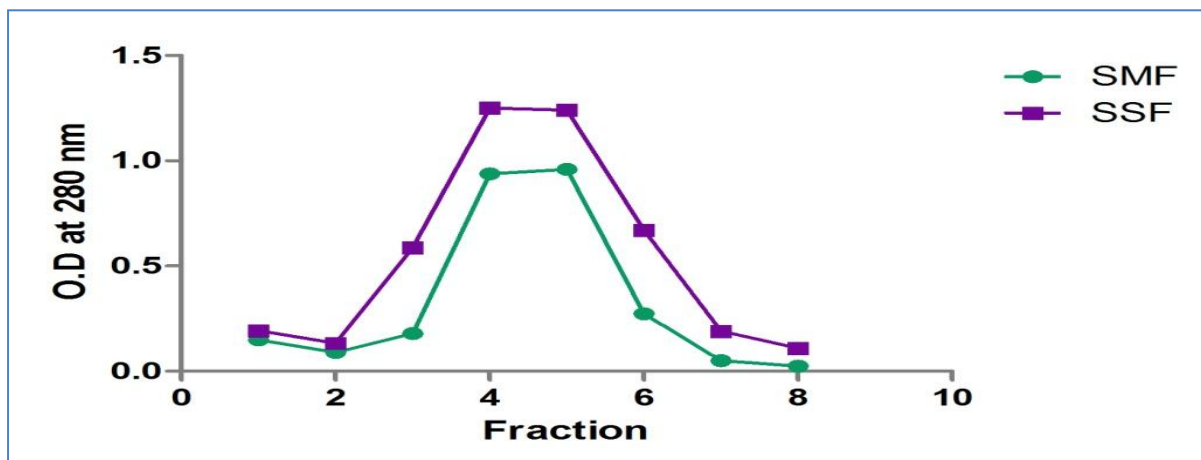

Fig. 10 Protein elution profile for SmF and SSF

Partial purification of the enzyme

Purified enzyme exhibits increased specific activity from (0.39 U/mg) to (0.75 U/mg) for SmF. Moreover in SSF also there is increase in specific activity from (0.162 U/mg) to (0.956 U/mg). The elution profile of the enzyme on Sephadex G-100 chromatography was shown in the Fig. 10.The purification results are summarized in Table 2 for SmF and Table 3 for SSF. The purification sequence allowed for the recovery of 15.3% (SmF) and 25% (SSF) of the original crude enzyme activity. The molecular mass of the amylase present in the corresponding fraction was estimated to be around 38 kDa(SmF) and 42kDa(SSF), which was confirmed by SDS-PAGE analysis (Fig. 11).
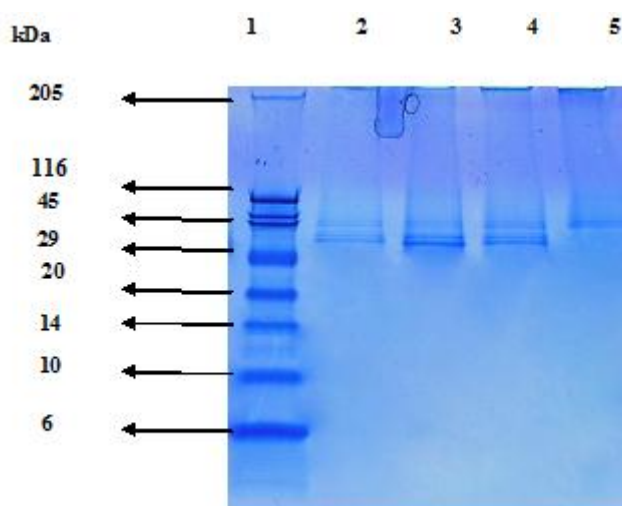

Fig. 11 - The SDS-PAGE analysis of the purified alpha amylase, Lane 1- Marker proteins; Lane 2- purified fraction 4(SmF); Lane 3- purified fraction 5(SmF), Lane 4- purified fraction 4(SSF), Lane 4- purified fraction 5(SSF)
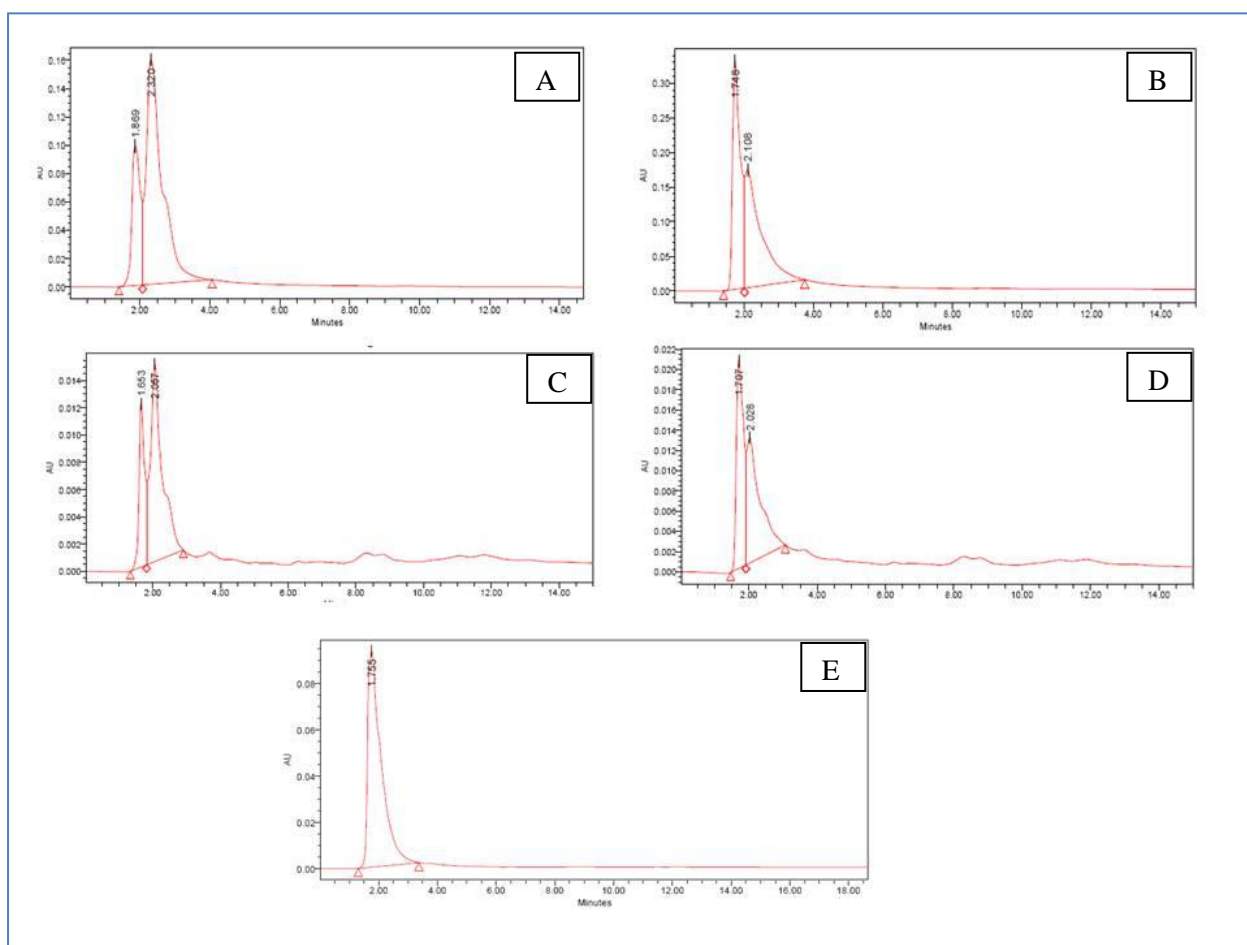
Table 2 - Summary of purification of α- amylase activity of *Bacillus pumilus* VITMDS2 for SmF

Purification step	Enzyme activity(U/ml)	Protein concentration(mg\ml)	Specific activity(U/mg)	Purification fold	% yield
Crude	3.9	10	0.39	1	100
Acetone Precipitation	1.9	4.2	0.452	1.15	48.7
Gel-permeation chromatography	0.6	0.8	0.75	1.92	15.3

Table 3- Summary of purification of α- amylase activity of *Bacillus pumilus* VITMDS2 for SSF

Purification step	Enzyme activity(U/ml)	Protein concentration(mg/ml)	Specific activity(U/mg)	Purification fold	% yield
Crude	4.4	27	0.162	1	100
Acetone Precipitation	2.3	11	0.209	1.29	52.27
Gel-Permeation chromatography	1.1	1.15	0.956	5.9	25.00

High Performance Liquid Chromatography of α- amylase

Fig. 12- HPLC Chromatogram of acetone precipitated α-amylase by SmF(A), Gel-permeation fraction α-amylase by SmF(B), Acetone precipitated α- amylase by SSF(C), Gel-permeation fraction α- amylase by SSF(D), Standard α- amylase (E) .

The purified fractions showing highest specific activities were characterized by HPLC [44]. In case of acetone precipitated α- amylase, retention time was found to 1.869 min for SmF (Fig.12A) and 1.653 min for SSF (Fig.12C) respectively. The retention time of the purified α-amylase after gel permeation chromatography was found to be 1.748 min for 5th fraction (Fig.12B) by SmF and 1.707 for 4th fraction (Fig.12D) by SSF. The retention time of the purified fractions were compared with the standard α-amylase (Hi-media). The retention time of the standard amylase was found to be 1.755 min as shown in Fig.12E. After comparing with the retention time of standard, the presence of amylase was confirmed in the purified fractions. The interference of other peaks in the chromatogram of acetone precipitated and Gel permeable purified fractions required further purification.

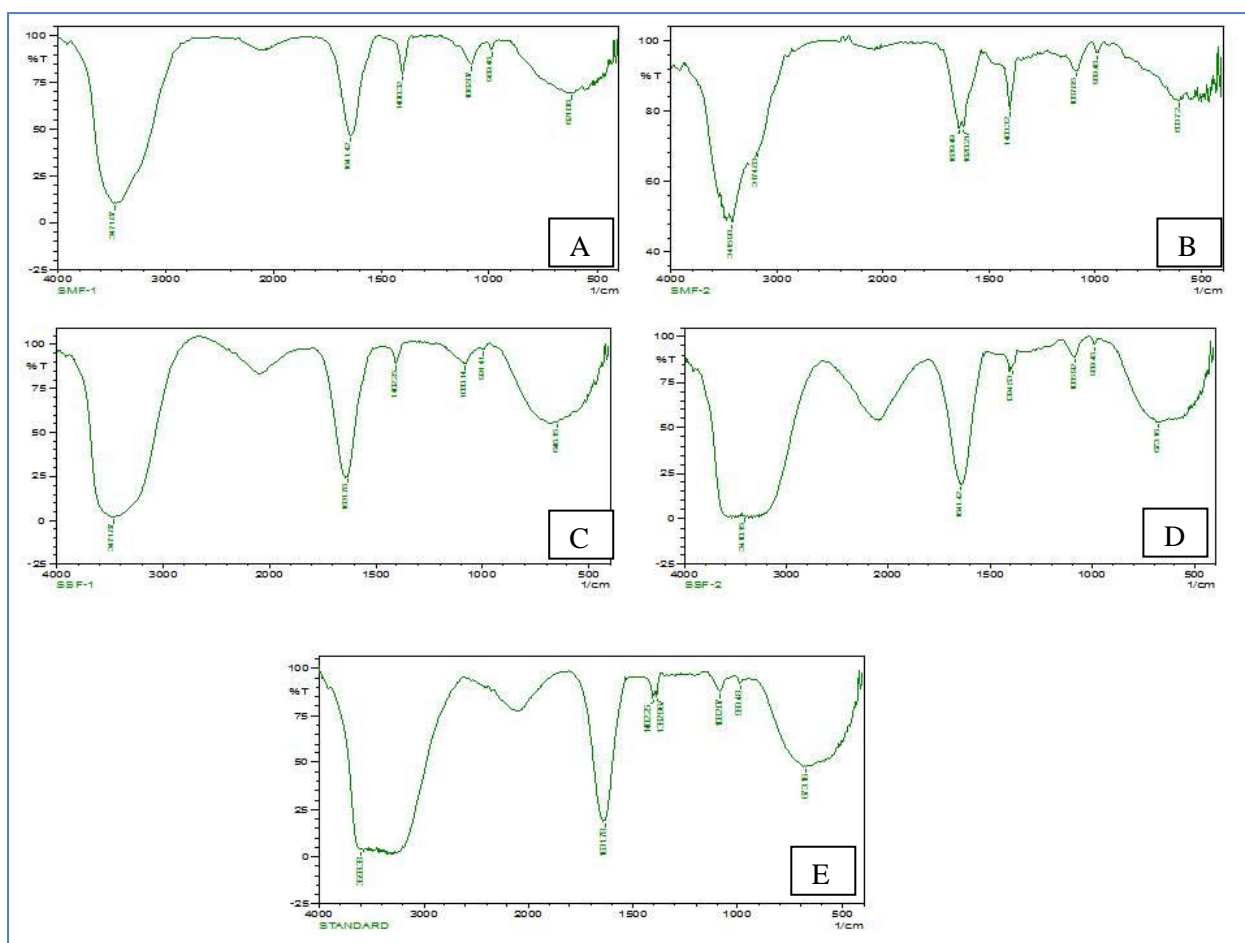**Fourier Transformation-Infra Red Spectroscopy (FT-IR) of α- amylase**

Fig. 13 FTIR spectrum of acetone precipitated α-amylase by SmF(A), Gel-permeation fraction α-amylase by SmF(B), Acetone precipitated α- amylase by SSF(C), Gel-permeation fraction α- amylase by SSF(D), Standard α- amylase (E)

FT-IR spectroscopy of the standard α- amylase (Himedia) was compared with the acetone precipitated and Gel permeated purified α- amylase. The characteristic signal around 3600- 3100 cm^{-1} that identifies the existence of OH functional group. The characteristic amide I band arises principally from the C=O stretching vibration of the peptide group at 1,641.97 cm-1, which is attributed to the lower shift in the normal α-helical frequency range 1630–1660 cm^{-1} for water soluble protein. The absorption bands

between 1000 and 1200 cm^{-1} are characteristic of the C-O bond stretching. The peak around 1400–1200 cm^{-1} corresponds to the N–H bending and peak between 1000-650cm^{-1} corresponds to =C-H bend (Benaki et al, 1998). All the above mentioned functional groups are present in standard α- amylase as well as acetone precipitated and gel permeated α-amylase from both the fermentation process. The similarity between the standard and rest of other samples (Fig 13 A, B, C, D, E) confirms the presence of α- amylase in the partially purified fraction. The presences of additional peaks imply the interference due to other functional groups.

CONCLUSION

The identification of the bacteria was confirmed by 16S rRNA gene sequencing studies. The phylogenetic tree revealed that the strain fit into an evolutionary cluster comprising members of *B. pumilus* VITMDS2. Here with that bacterial species can be exploited fully for the production of α-amylase being used industrially. Due to the importance of these findings, further studies will be carried on in order to commercialize the production process after necessary optimization for enhanced enzyme production. Commercial α-amylase production is usually produced by submerged fermentation; however, SSF appear promising due to the natural potential and advantages they offer. Based on the present study, it appears that wheat bran, which is inexpensive and readily available agricultural substance, could replace the commercial and more expensive substances in the development of a suitable economic fermentation medium for obtaining high yields of α-amylase. However, the present study was entirely a laboratory-scale study, and it has to be further improved for a large-scale SSF. The activity of the amylases purified here is comparable to the activities of the amylases purified earlier by various researchers. Further work includes further purification of the enzymes in order to attain higher specific activity.

ACKNOWLEDGEMENTS

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