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# Isolation, optimization and characterization of α-amylase producing bacteria from spent mushroom compost

**Neerja Rana, Neha Verma, Devina Vaidya and Arti Ghabru**

**Abstract**
Amylases constitute a class of industrial enzymes representing approximately 30 per cent of world enzyme production. The amylases have applications in juice processing, starch processing, desizing of textiles, paper sizing, detergent additives, utilization of waste biomass for valuable products, treatment of waste water and other fermentation processes including malting barley and bakery industries. Amylases can generally extract from plants, microorganisms, fruits etc. Microorganisms serve as a potential source of amylase production. The study was carried out to isolate the amylase producing bacteria from spent mushroom compost from Nauni, of Himachal Pradesh. Subsequently, their screening and characterization was done on the basis of morphological, biochemical and molecular parameters. Out of 3isolates 1 bacterial isolate viz., M13 was identified as amylase producing. The highest amylase activity was obtained in apple pomace as a substrate at pH 9.0, 72 hrs of incubation and 45ºC temperature. Among different carbon sources starch followed by maltose was found to be the best. Among the nitrogen sources, Yeast extract (organic source) followed by $NaNO_3$ for both the isolates was found best. The phylogenetic analysis of 16S rDNA sequence showed isolate M13 belongs to *Bacillus* sp. vide accession number [KY962809] obtained from NCBI gene bank.

**Keywords:** carbon source, nitrogen source, solid state fermentation, 16s rDNA, mushroom compost, bacillus, morphological, biochemical

## Introduction
Extremophilic microorganisms are adapted to survive in ecological niches such as at high temperatures, extremes of pH, high salt concentrations and high pressure. These microorganisms produce unique biocatalysts which function under extreme conditions comparable to those prevailing in various industrial processes. The commercial application of enzyme has constraints due to their thermolabile nature, narrow pH range and high cost of production. Most industrial processes are designed to operate at elevated temperature. Therefore, thermophilic microorganisms have gained a great deal of attention. Hence, the enzyme from these microrganisms is of special interest because they are not denatured at high temperature.

α-amylase (E.C 3.2.1.1) catalyses the hydrolysis of α-D-(1,4) glycosidic linkages in starch componentsand related carbohydrates. They can specifically cleave the O-glycosidic bonds in starch (Becker *et al*., 1997; Beg *et al*., 2000) [9, 10]. Advances in the use of microbial amylases in industry have been possible with the isolation of thermophilic microorganisms from ecological niches of earth and subsequent extraction of useful enzymes from them (Groboillot, 1994; Bharat and Hoondal, 1998; Bauer *et al.*, 1999; Kohilu *et al.*, 2001) [21, 11, 8, 27].

The production and stability of α- amylase in the medium is affected by several factors. It is influenced by the components of medium and physical factors such as pH, temperature, agitation, dissolved oxygen and inoculums density (Babu and Satyanarayana, 1993; Dey *et al*., 2001; Gigras *et al*., 2002) [7, 17, 20]. Submerged fermentation has been traditionally used for the production of industrially important enzymes because of the ease of handling and greater control of environmental factors such as temperature and pH (Ramachandran *et al*., 2010).

The cost of enzyme is one of the major factors for determining the economics of the process which can be partially achieved by standardizing fermentation media. The content of synthetic media is very expensive and these contents might be replaced with more economically available wastes. The waste substrates like agricultural, industrial and forest residues can be used for the production of enzyme which not only lower the cost of the enzyme but also recycle the waste to this high value product besides lowering the environmental pollution.

Mushroom compost provides an excellent source of various kind of micro organism which contains such enzymes due to their varied physical and chemical conditions. The amylases have found applications in juice processing, starch processing, desizing of textiles, paper sizing, detergent additives, bread improvement, utilization of waste biomass for valuable products, treatment of waste water and other fermentation processes including malting barley and bakery industries (Haki and Rakshit, 2003; Rana *et al.*, 2012 ; Couto and Sanroman, 2006) [22, 16].

Keeping in view the current status of knowledge it is understood that amylase enzyme from thermophilic bacteria of mushroom compost which are supposed to be unexploited niches may have wide industrial applications. Therefore, the proposed study was undertaken for isolation, cheap production, characterization and evaluation of amylase enzyme from above mentioned source.

## Materials and Methods

The present investigation was carried out in the laboratory of Microbiology section, Department of Basic Sciences, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP).

### Isolation, Enumeration and Screening of Amylase Producing Bacterial Isolates

#### Collection of mushroom compost

Compost samples were collected from mushroom fields of university campus, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh (Longitude 30.51N, Latitude 77.10E). Compost samples for the isolation of amylase producing bacteria were collected from 5 different places. Samples were taken after pasteurization i.e. Stage III of compost formation and homogenized to form bulk sample after physico-chemical analysis.

#### Physico-chemical analysis of mushroom compost

Mushroom compost samples from various sites of mushroom fields of university campus, Nauni were studied for various physico-chemical characters. Various parameters i.e. pH, Nitrogen, Carbon, moisture content were recorded.

#### Isolation and Screening of amylase producing bacteria

Isolation for amylase producing bacteria was done by using serial dilution method (Aneja 2003) [4] by standard protocol. The samples (0.1 ml each) from each dilution were mounted by spread plate method on sterilized petri plates containing solidified nutrient agar medium for isolation of bacterial colonies. Plates were incubated at 35± 2°C.

After the isolation bacterial colonies producing clear zones were selected and purified using streak plate technique on the starch medium. The isolates were primarily examined according to their colony morphology.

### Production of Amylase

#### Preparation of seed culture and production of enzyme

The seed culture was prepared by inoculating a loopful of pure culture and incubated for 24 h at 35°C. 2 ml of seed culture was inoculated in 50 ml of production medium containing beef extract (0.25%), peptone (0.15%) and starch (1%). The medium was grown for 48 h at 35°C at 150 rpm in shaking incubator. Production media was centrifuged at 5,000 rpm for 10 minutes. The supernatant was collected and used further.

#### Amylase assay and Protein Determination

Enzyme assay was carried out by the method described by Sadasivam and Manickam, 1997. The standard curve was made from the stock solution of maltose (10-100µg $ml^{-1}$). The enzyme activity was expressed in terms of International Unit (IU) and specific activity (SA) represents µ mole of glucose released $min^{-1}mg^{-1}$ of protein. Total protein was estimated using BSA (bovine serum albumin) as standard, as described by Lowry *et al*. (1951) [31]. All experiments were carried out in triplicate and the data presented are average values.

### Optimization of Cultural Conditions for Cost Effective or Maximum Amylase Production

**Amylase production in Solid state fermentation:** Apple pomace, wood dust and wheat bran were used as substrate for amylase production. These materials were obtained from Microbiology Laboratory of the Department of Basic Science. Basal salt medium was used for production of amylase enzyme. Twenty milliliter of the medium was dispense into 250 ml flask containing 10 g of substrate and autoclaved at 15 psi pressure for 20 min. The medium was supplemented with 0.5 per cent yeast extract. The inoculum was prepared by making a suspension of 24 h old growth of organism on starch nutrient medium slants containing 0.3 percent starch. The flasks were inoculated with 2ml of bacterial suspension (O.D 1.0 at 660 nm) and incubated in orbital incubator. At the desired interval the flask were taken out and the content were extracted with 45 ml sterilized buffer (0.1 M, pH 10.0, bicarbonate). The flask content was centrifuged at 5300 rpm for 30 min at $4^0$C. The culture supernatant was used as crude amylase preparation. Prior to centrifugation the sample were withdrawn for determining viable number of cells.

#### Substrates for amylase production

The amylase production in presence of different substrate under SSF was studied. The substrate giving maximum amylase production was used for further experiment.

### Optimization operational conditions for *α*-Amylase Producing Bacterial Isolate

#### 1. Optimization of incubation period

The bacterial isolates using best substrate were grown on medium for different incubation period viz. 24, 36, 48 and 72 h and incubated at 37±2°C for maximum amylase production

#### 2. Optimization of pH

The pH of the medium was adjusted ranging from 3 to 9 with the help of pH meter. The autoclaved liquid media was inoculated with the bacterial isolates and incubated at 37±2°C.

#### 3. Optimization of incubation temperature

In order to determine the optimum temperature for maximum enzyme production, the selected pH of the media was adjusted and inoculated with bacterial isolates. The flasks were then incubated at different temperature ranging from 25°C to 80°C with the interval of 5°C.

#### 4. Optimization of carbon sources

Different carbon sources viz. sucrose, maltose and glucose each rate of 1.0 per cent were added in the submerged media and their effect on the growth and amylase production was studied after incubating at 45±2°C.

#### 5. Optimization of nitrogen source

Different nitrogen sources viz. yeast extract, casein, urea

$NaNO_3$ and $NH_4Cl$ were added in submerged media at the rate of 1.0 per cent in the and their effect was studied on growth and amylase production after incubating at 45±2°C.

**Characterization of Selected Amylase Producing Bacterial Isolate**

**1. Morphological and taxonomic characterization**

Morphological based identification of selected amylase producing bacterial isolates was done as per Bergey's Mannual of Systematic Bacteriology (Claus and Berkley, 1986) [14].

**2. Biochemical characterization**

In order to determine the biochemical characterization of the selected bacterial isolates following biochemical tests were performed (Holt *et al.* 1994) [24], which includes: Oxidase test, Catalase test, Indole test, Methyl red test, Voges proskauer test, Citrate test, Glucose test, Sucrose test, Lactose test, Urease test and $H_2S$ test

**3. Molecular characterization of selected isolate by 16S rDNA sequence analysis**

Molecular identification of the selected bacterial isolate was done by 16S rDNA sequence analysis (Pace, 1997) [34].

**Genomic DNA extraction**

Genomic isolation was carried out by conventional method of Sambrook *et al*, (1989) [40]. Bacterial isolates was grown overnight at 37±2°C in nutrient broth at 200 rpm. The cells were harvested and processed for DNA isolation.

**Gel electrophoresis**

The isolated DNA was finally suspended in 100µl of elution buffer and quantified on 1% agarose gel.

**PCR amplifications of 16S rDNA**

Universal 16S rDNA primers were used for the amplification of amylase producing selected bacterial isolates.

PCR reaction was carried out in 20µl reaction containing ~50ng of template DNA, 20 pmoles of each primer, 0.2mM dNTPs and 1 U Taq polymerase (Banglore Genei) in 1x PCR buffer. Reaction were cycled 35 times as 94°C for 30s, 58°C for 30s, 72°C dor 1 min 30 sec. Followed by final extension at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel in 1x TAE buffer, run at 100V for 1 hr. Gels were stained with ethidium bromide and photographed. Amplified PCR products were eluted from the gel extraction kit (Hi Yield Gel/ PCR DNA Extraction Kit from Real Genomics).

**Gel elution was done by Hi Yield Gel/PCR DNA Extraction Kit**

Agarose gel slice containing relevant DNA fragments was excised and extra agarose was removed to minimize the size of the gel slice. 300 mg of the gel slice was transferred into microcentrifuge tube, 500 µl of DF buffer was added to the sample and mixed by vortexing. Incubation was done at 55°C for 10-15 min until the gel slice gets completely dissolved sample mixture get cool down to room temperature.

**DNA binding**

A DF column was placed in 2 ml collection tube. 800 µl of sample mixture (from above step) was applied into the DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube.

**Wash**

600 µl of wash buffer (ethanol added) was added into DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube. Centrifuged again for 3 min at 13,000 rpm to dry the column matrix.

**DNA elution**

Dried column was transferred into new microcentrifuge tube. 15-30 µl of elution buffer or distilled water was added into the center of column matrix. Stand for 2 mins until elution buffer or distilled water was absorbed by the matrix. Centrifugation was done for 2 mins at 13,000 rpm to elute purified DNA eluted fragment was then sequenced using PCR primers.

**Sequence and phylogenetic analysis**

Sequencing was done as per manufacturer instructions (Xcleris lab. Ahmadabad). The sequence was aligned with corresponding sequences of 16S rDNA from the database using BLAST from the website http://www.ncbi.nlm.nih.gov/blast (Altschul *et al.* 1997) [3]. Multiple alignments were generated by the CLUSTUL W program and phylogenetic tree was constructed by neighbor-joining algorithm using MEGS 6 software.

**Results and discussion**

The Studies were conducted to produce and evaluate amylase enzymes from bacteria inhabiting mushroom compost. Possible attempts were made to standardize cultural conditions for maximum enzyme production.

**Variation in pH and Conductance of Spent Mushroom Compost**

The pH and conductance of the samples collected from compost yard of Mushroom section, Department of Mycology and Plant Pathology were determined and presented in Table 1 and 2. The variability in pH was studied in all the samples collected and the data revealed that the pH ranged from 5.0-7.4. The results also revealed that the pH in all samples were not above 7.5 neutral pH (Table 1). Barlett chi-square test was applied to test the homogeneity among the samples. Chi-square calculated value was less than the chi-square table value which indicated that the variances were homogenous. Wuest and Fahy (1991) [49] have shown the initial pH of spent mushroom compost around 7.28 which increases during weathering.

The data recorded on the conductance of 5 samples from compost unit are presented in Table 2. The data revealed that the conductance ranged from 5.0 to 8.3 m mhocm-1. Barlett chi-square test was applied to test the homogeneity among the samples. Chi-square calculated value was less than the chi-square table value which indicated that the variances were homogenous. Gerrits (1987) [19] has also reported variation in conductance of spent mushroom compost in the range of 1.9 to 8.3 m mho $cm^{-1}$.

**Table 1:** Variability in pH of collected mushroom compost

| Site | Mushroom compost samples | | | | |
|---|---|---|---|---|---|
| Nauni | I | II | III | IV | V |
| L1 | 4.5 | 4.5 | 4.5 | 5.4 | 4.5 |
| L2 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |
| L3 | 5.4 | 5.4 | 5.4 | 4.5 | 5.4 |
| Chi-square value$_{(0.05)}$ = 0.03 | | | Chi-square value$_{(0.05)}$ = 5.99 | | |

**Table 2:** Variability in Conductance of collected mushroom compost

| Site | Mushroom compost samples | | | | |
|---|---|---|---|---|---|
| Nauni | I | II | III | IV | V |
| L1 | 5.2 | 5.2 | 5.2 | 5.2 | 5.2 |
| L2 | 3.6 | 3.6 | 3.6 | 3.6 | 3.6 |
| L3 | 6.2 | 6.2 | 6.2 | 6.2 | 5.2 |
| Chi-square value$_{(0.05)}$ =2.29 | | | Chi-square value$_{(0.05)}$ =5.99 | | |

**Isolation, Enumeration and Screening of Amylase Producing Bacteria**

The natural niche i.e. mushroom compost is considered as rich sources of industrially important enzyme producing micro-organisms. The isolation of bacteria was done from the pooled samples of spent mushroom compost from Nauni of Solan district of Himachal Pradesh by serial plate dilution method and spread plate method on Nutrient agar (NA) incubating at 37±2°C. A total of 8 samples were isolated from mushroom compost. The selected colonies were grown on the starch nutrient agar medium. The plates were flooded with iodine reagent for the appearance of clear zone post incubation, which indicated the presence of amylase producing strains. In total 3 isolates were found to be the amylase producers (M 13, M15 and M51) showed clear zones of starch hydrolysis with varying diameters from the pooled samples of compost.

The zone size of bacterial colonies ranged between 4.7 to 10.30 mm with enzyme index of 0.07 to 30.60. The isolate M13 from spent mushroom compost gave highest zone size of 10.00 mm with enzyme index of 37.00 (Table 3). The isolate M13 have luxuriant growth of isolate when flooded with iodine (Plate1).

Chauhan *et al*. (2011) [13] have found seven bacterial strains of amylase producer by utilizing starch and Vyas and Sharma (2015) [47] isolated 12 hyper amylase producing strain from the mushroom compost.

All the thermophilic amylase producing isolated were preliminary characterized based on their morphology as well as on the basis of their clear zone of hydrolysis.

**Table 3:** Characteristics of amylase producing bacterial isolate from mushroom compost

| Isolates | Zone size (mm) | Enzyme Index |
|---|---|---|
| M13 | 10.00 | 37.00 |
| M31 | 6.70 | 18.30 |
| M51 | 5.00 | 18.40 |
| S.E.$_m$ | 0.43 | 0.07 |
| CD | 1.26 | 0.20 |

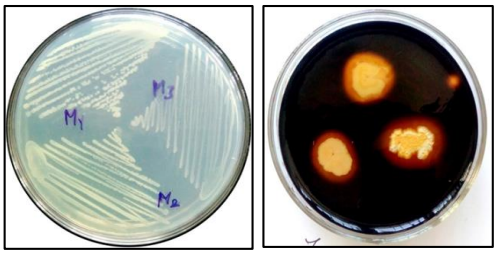


**Plate 1:** Growth of isolates on Nutrient agar (1) and starch nutrient agar (2) media of selected isolate M13

**Screening of Bacterial Isolate for Maximum Amylase Production**

Different substrates viz., apple pomace, wheat bran and wood dust were studied for the growth and amylase production by the selected bacterial isolates. The results revealed that the growth expressed in terms of viable count as well as amylase activity (61.35 IU) were recorded highest in apple pomace in comparison to wood dust, and wheat bran in M13 isolate from mushroom compost. This isolate recorded with protein content of 20.11 mg ml$^{-1}$ and specific activity of 2.87I U mg$^{-1}$ (Fig 1). Based on these observations isolate M13 was selected for further studies. The highest amylase activity in apple pomace may be due to high non reducing and total sugars which induces the enzyme activity.

Mamatha *et al.* (2012) [32] observed that sugarcane bagasse was found to be to be the superior substrate than wheat bran, molasses bran, rice bran and maize meal. They reported the amylase activity of 15.64 µg min$^{-1}$ using sugarcane bagasse as a substrate which was attributed to high non reducing sugar content in this substrate. Pathak and Rekadwad (2013) [35] reported tamarind seeds and Chauhan *et al* (2011) [13] found deodar (*Cedrus deodara*) wood dust as the best substrate.

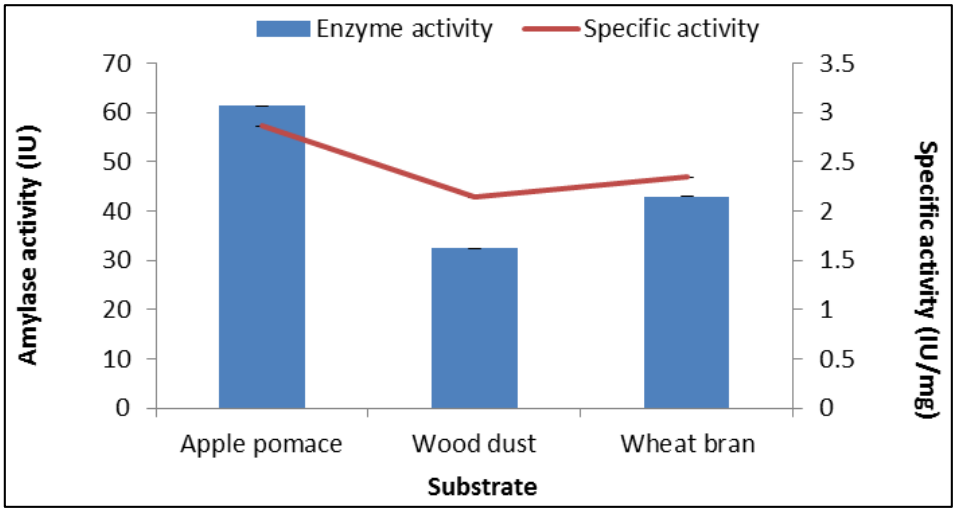


**Fig 1:** Effect of substrate on enzyme activity and specific activity of amylase produced from M13

**Identification of selected amylase producing bacterial isolate**

The selected amylase producing bacterial isolate M13 from mushroom was marked on the basis of their morphological and biochemical characteristics and the results are shown in the table 4. The Gram staining reaction for pigment producing bacterial isolates was performed to check the gram's staining. The results revealed that all the bacterial isolates exhibited blue-purple color by gram staining. The selected pigment producing bacterial isolates were identified as gram positive and coccus shaped. These bacterial isolates were found to be capsule forming. The results on various biochemical tests indicated that the two amylase producing bacterial isolates selected in the present study does not exhibit catalase activity and $H_2S$ production. According to the Bergey's manual the M13 isolates were identified as *Bacillus* sp. on basis of various biochemical and morphological studies.

The characterization of selected amylase producing bacterial isolates has been supported by the findings of Kumar *et al.*, (2011) [28]; Akpomie *et al*., (2012) [2] and Verma *et al.*, (2014) [45].

**Table 4:** Morphological and Biochemical characteristics of the selected amylase producing bacterial isolates

| Characteristics | $M_{13}$ |
|---|---|
| Gram's reaction | + |
| Color | Purple |
| Catalase test | - |
| Spore formation | - |
| Simmon citrate test | - |
| Oxidase test | |
| Catalase test | - |
| Indole test | - |
| Methyl red test | + |
| Voges proskauer test | - |
| Glucose test | + |
| Sucrose test | + |
| Lactose test | + |
| Urease test | + |
| $H_2S$ test | + |

**Optimization of Growth Conditions For Maximum Amylase Production**

**Effect of incubation period on the growth and amylase production of selected bacterial isolate**

The amylase activity was measured at regular intervals from 24 h to a period of 96 h. (Fig 2). The data showed that the bacterial isolate M13 exhibited highest enzyme activity of 55.87 IU with a specific activity of 2.61 IU/mg at 72 h of incubation. The selected bacterial isolates during initial 24h low enzymatic activity was noticed which increased up to 72h and the activity followed a declining trend thereafter. The growth and activity were found to be highest at 72 h.

The low amylase activity during the initial 24 h could be due to the lag and log phase of the growth where the time was taken by the microorganism to get stabilized in a new medium and then started multiplying. Amylase being an inducible enzyme is generally produced during stationary phase. Thus, the peak in enzyme yield was recorded at 72$^{th}$ h by both of the isolates. The incubation period after72 h resulted in a decreased enzyme activity which may be due to the depletion of nutrients thereby causing a stressed microbial physiology (Flores *et al.*, 1997) [18]. Production of amylase activity during the exponential growth phase is also in agreement with the findings of Moral (1995) [33], Chauhan *et al* (2011) [13] and Vyas and Sharma (2015) [47].

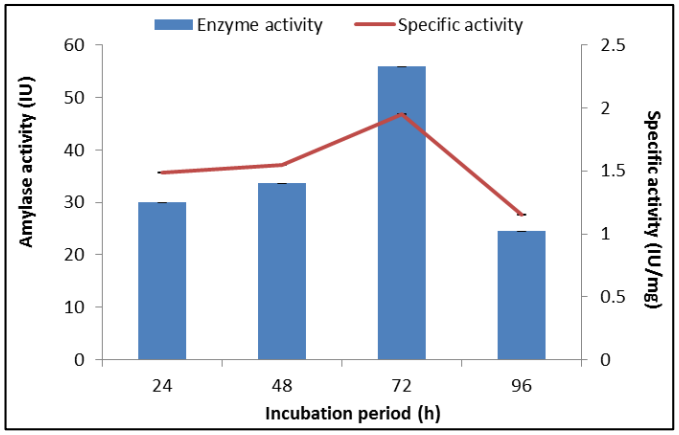


**Fig 2:** Effect of Incubation period on enzyme activity and specific activity of amylase produced from M13

**Effect of pH on growth and amylase production by selected bacterial isolates**

The effect of varying the initial pH (5.0-10.0) for amylase production on apple pomace was studied. Figure 3 showed that the highest amylase activity of 75.94 IU for M13 isolate at pH 9.0 with specific activity of 2.85 IU $mg^{-1}$. At lower pH of 5.0, a respective amylase activity of 30.24 IU and 1.80 IU $mg^{-1}$ specific activity was recorded. Similarly, at pH 10.0 low enzyme activity of 46.39 IU was recorded. The production of enzyme activity initially increased with increased in pH of medium up to 9.0 and thereafter the activity decreased. The pH of the growth medium plays an important role in terms of inducing enzyme production, overall metabolic activity and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannam, 2006; Willey *et al.*, 2008) [36, 25, 48].

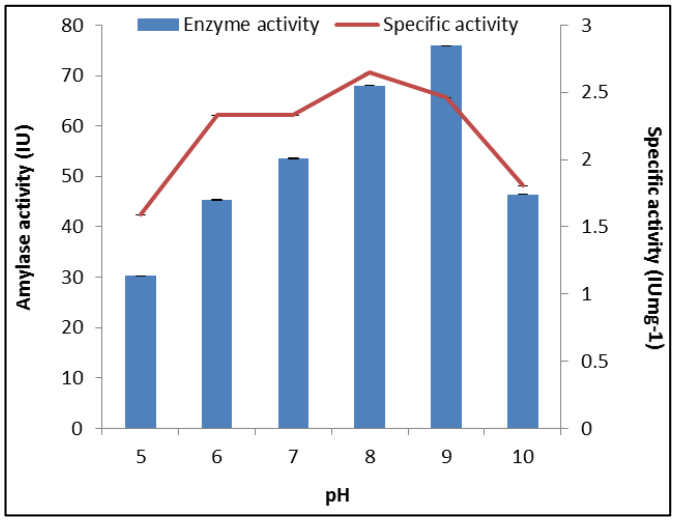


**Fig 3:** Effect of pH on enzyme activity and specific activity of amylase produced from M13

**Effect of temperature on the growth and amylase production by selected isolates**

A study of amylase production was conducted at 25 to 50°C with an interval of 5°C using apple pomace with 9.0 pH. The figure 4 showed that amylase production was affected by the

temperature. The bacterial isolate M13 exhibited highest activity of 60.64 IU followed by specific activity of 2.13 IU/mg at an optimum temperature of 45ºC which was significantly higher than temperature under study. Further, it was noticed from the present investigation that amylase production improved with the increase in temperature from 25 to 50ºC and attained a peak value at 45ºC which was also maximum for growth of isolates.

However, with the further increase in temperature the amylase activity decreased significantly. Maximum enzyme activity at optimum temperature may be due to an increased metabolic activity of the cells resulting in enhanced enzyme production. Lowered incubation temperature of 25 and 30ºC resulted in a decreased amylase activity of 34.41 and 43.94 IU respectively for M13 isolate. At temperatures lower than optimum, membranes solidify, and transport carriers and other proteins thus lower the enzyme activity (Willey *et al*., 2008) [48].

Incubation temperature is the most important physical factor which effects structure and function of macromolecules. Khalil (2011) [26], Senthilkumar *et al.*, (2012) [41] and Vyas and Sharma (2015) [47] reported maximum amylase activity at 50ºC by a thermophilic bacterial strain.

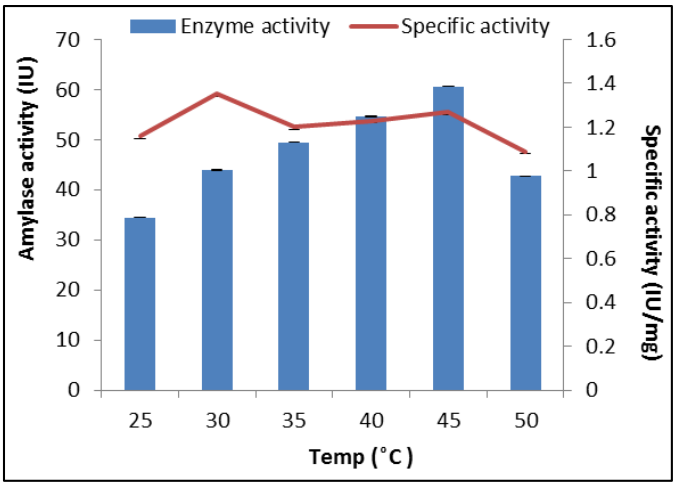


**Fig 4:** Effect of temperature on enzyme activity and specific activity of amylase produced from M13

## Effect of carbon sources on the growth and amylase production of selected isolates

A study of amylase production was conducted to check that use of apple pomace was appropriate as low cost substrate as carbon source. The different carbon sources were tested under submerged fermentation. Amylase is an inducible enzyme and is produced in the presence of starch or its hydrolytic products. Figure 5 depicts the effect of various carbon sources on amylase production from M13.

M13 isolate exhibited the highest amylase activity of 65.68 IU with starch followed by maltose with activity of 64.57 IU. However the differences was observed non-significant. The higher specific activity of M13 bacterial isolate was observed to be 3.07 IU $mg^{-1}$ in case of starch followed by maltose (2.88 IU $mg^{-1}$). The maximum amylase activity with starch was comparable with apple pomace which may be due to the presence of polysachharides, non-reducing sugars and maltose in the apple pomace. Other carbon source such as, sucrose and glucose had shown little lesser activity. Syed *et al* (2009) [43]; Hashmi *et al* (2010) [23]; Tsegaye *et al.* (2014) [44] and Vyas and Sharma (2015) [47] also reported starch as the best carbon source for amylase production.

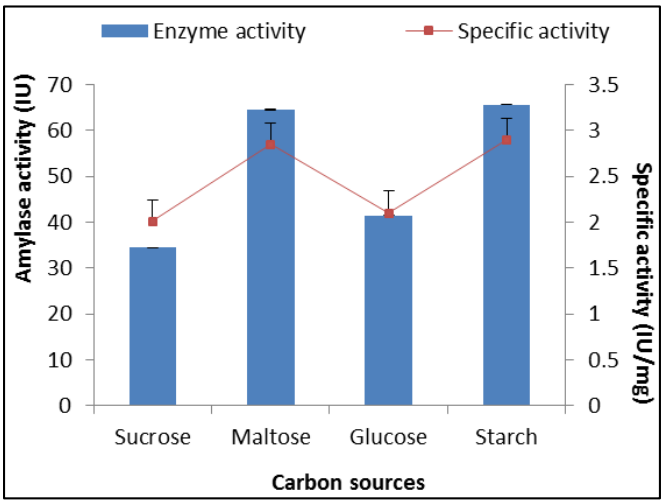


**Fig 5:** Effect of carbon sourceon enzyme activity and specific activity of amylase produced from M13

## Effect of nitrogen sources on the growth and amylase production by selected isolates

Nitrogen sources are the most important compounds for the growth and metabolism of microorganisms. The nature of these compounds and the concentration used may stimulate or down the production of enzymes (Sharma and Singh 2012) [42]. Figure 6 showed that M13 bacterial isolate exhibited the highest amylase activity with yeast extract 78.88 IU along with specific activity of 3.27 for M13. The other sources viz., urea and casein showed low activity of enzyme as well as growth.

In the present investigations, the yeast extract was found to be the best nitrogen source due to higher content of minerals, vitamins, and coenzymes as reported by several workers (Asgher *et al.*, 2007; Ashwini *et al.*, 2011; Vijayabhasker *et al.*, 2012) [5, 6, 46].

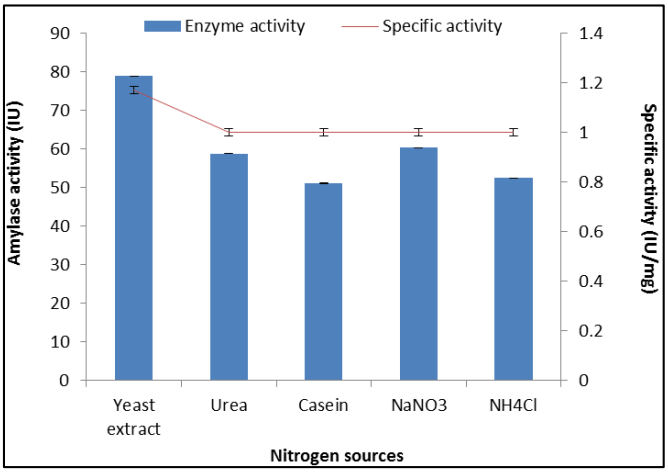


**Fig 6:** Effect of nitrogen source on enzyme activity and specific activity of amylase produced from M13

## Molecular characterization of the selected isolate M13

Molecular identification of the amylase producing bacterial isolate M13 was done by sequencing part of the 16S rDNA. The amplification of the 16S rDNA was confirmed by agrose gel electrophoresis. The PCR product of the amylase producing bacterial isolate was gel eluted and sequenced. The sequence data of 16S rDNA was subjected to BLAST analysis. The isolate M13 was identified as *Bacillus sp.* using BLASTN analysis and has been registered under the

accession number |KY962809| in National Centre for Biotechnology Information (NCBI). Nucleotide sequence of the isolate M13

GGGGCTAATACCGGATGCTTGTTTGAACCGCATGGT
TCAAACATAAAAGGTGGCTTCGGCTACCACTTACAG
ATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAA
TGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTG
AGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTT
AGGGAAGAACAAGTACCGTTCGAATAGGGCGGTAC
CTTGACGGTACCTAACCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCA
ACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGT
GCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGT
TAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGAC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACAATCCTA
GAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCT
TAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAATCATCATGCCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGACAGAACAAAGGGCAGCGAAACC
GCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGT
TCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTG
GAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC
ACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGT
AACCTTTT

Phylogenetic tree of M13 with respect to other *Bacillus* sp. as inferred by neighbor joining method which has been shown in Fig.7.

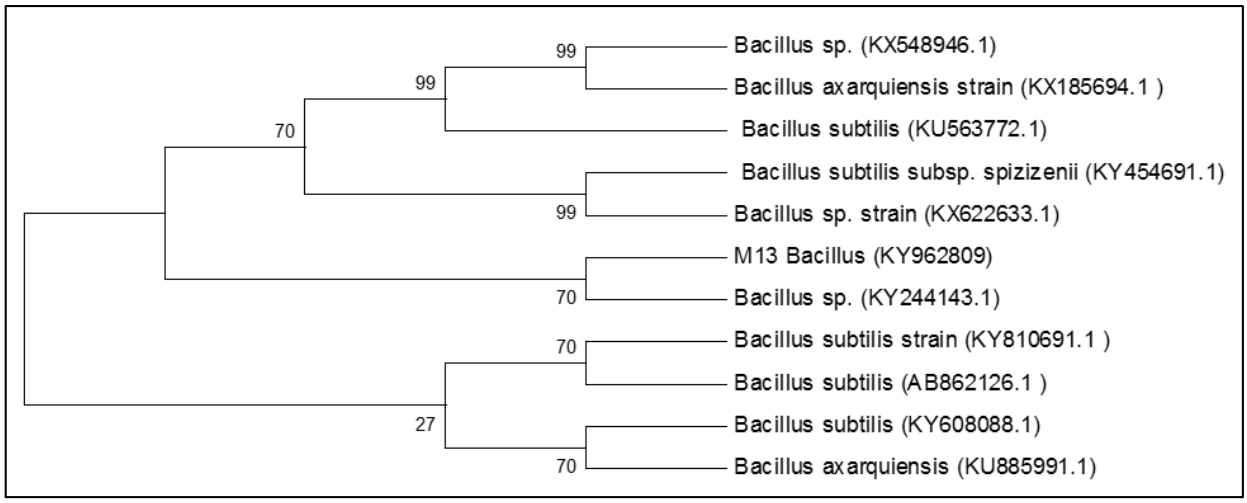


**Fig 7:** Neighbor joining tree based on 16S rDNA sequences showing the phylogenetic relationship of bacterial isolate M13 with the analyzed sequences

## Conclusion

It can be concluded from the present investigations that the M13 isolate from spent mushroom compost from mushroom yard of Dept. of MPP, Dr YS Parmar U.H.F, Nauni, Solan, Himachal Pradesh was found to be are potential sources for amylase production. The high amylase production was obtained with apple pomace as low cost substrate, after 72 h of incubation, at pH 9.0 and temperature of 45ºC. The crude amylase was characterized using RSM and gave optimum activity 2.75 starch concentration at 55°C at 9.70 pH. Hence, amylase yield, stability and the low cost substrate production supported the hypothesis that microbial enzymes have potential in food industries. These natural resources need to be exploited for commercial enzymes.

## References


1. Abdullah AGL, Sulaiman NM, Aroua MK, Megat MNMJ. Response surface optimization of conditions for clarification of carambola fruit juice using a commercial enzyme. Journal of Food Engineering. 2007; 81:65-71.
2. Akpomie OO, Akponah E, Okorawhe P. Amylase production potentials of bacterial isolates obtained from cassava root peels. Agricultural Science Research Journals. 2012; 2(2):95-99.
3. Altschul SF, Thomas LM, Alejandro AS, Jinghui Z, Zheng Z, Webb M, *et al*. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Research. 1997; 25:3389-3402.
4. Aneja KR. Experiments in microbiology, plant pathology and biotechnology. New Age International (P) Limited Publishers, New Delhi, 2003.
5. Ashger M, Asad MJ, Rahman SU, Legge RL. A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. Journal of Food Engineering. 2007; 79:950-955.
6. Ashwini K, Gaurav K, Kartik L, Bhaskara RKV. Optimization, production and partial purification of extra cellular α-amylase from *Bacillus* sp. *marini*. Archives of Applied Sciences Research. 2011; 3(1):33-42.
7. Babu KR, Satyanarayana T. Parametric optimization of extracellular α-amylase production by thermophilic *Bacillus coagulans.* Folia Microbiologica. 1993; 38:77-80.
8. Bauer M, Driskil L, Callen W, Snead M, Mathur E, Kelly

R. An endoglucanase EgiA, from the thermophilic archaeon *Pyrococcus furiosus* hydrolyzes a-1, 4 bonds in mixed linkage (1-3), (1-4)-b-D-glucans and cellulose. Journal of Bacteriology. 1999; 181:284-290.
9. Becker P, Abu-Reesh I, Markossian S. Determination of the kinetic parameters during continuous cultivation of the lipase producing thermopile *Bacillus* sp. IHI-91 on olive oil. Applied Microbiology and Biotechnology. 1997; 48:184-190.
10. Beg QK, Bhushan B, Kapoor M, Hoondal GS. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. Journal of Industrial Microbiology and Biotechnology. 2000; 24:396- 402.
11. Bharat, Hoondal G. Isolation, purification and properties of thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11. Biotechnology Letters. 1998; 20:157-159.
12. Box G, Wilsen KB. On the experimental attainment of optimum conditions. Journal Royal Statistics. 1951; 13(1):1-45.
13. Chauhan A, Mehta P, Mahajan R, Walia A, Shirkot CK. Deodar (*Cedrus deodara*) wood dust: An alternative substrate for amylase production in solid state fermentation by alkalophilic *Bacillus* spp. A1 isolated from mushroom compost. Asian Science. 2011; 6(1-2):41-47.
14. Claus D, Berkeley RCW. Genus *Bacillus* Cohn. In: Bergey's manual of systematic bacteriology. Sneath PHA, Nair NS, Sharpe ME and Holt JG (eds.). MD Baltimore, Williams and Wilkins, USA. 1986-1872; 174(2):1105-1139.
15. Coman G, Bahrim G. Optimization of xylanase production by *Streptomyces* sp. P12-137 using response surface methodology and central composite design. Annals of Microbiology. 2011; 61:773-779.
16. Couto SR, Sanroman MA. Application of solid state fermentation to food industry: a review. Journal of Food engineering. 2006; 76:291-302.
17. Dey G, Mitra A, Banerjee MBR. Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. Biochemical Engineering Journal. 2001; 7:227-231.
18. Flores ME, Perez R, Huitron C. β-xylosidase and xylanase characterization and production by *Streptomyces* sp. CH-M-1035. Letters in Applied Microbiology. 1997; 24:410-416.
19. Gerrits JPG. Biedt composteren in tunnels perspectief? De Champignoncultuur. 1987; 31:357-365.
20. Gigras P, Sahai V, Gupta R. Statistical media optimization and production of alpha-amylase from *Aspergillus oryzae* in a bioreactor. Current Microbiology. 2002; 45:203-208.
21. Groboillot A. Immobilization of cells for application in the food industry. Critical Reviews in Biotechnology. 1994; 14:75-107.
22. Haki GD, Rakshit SK. Developments in industrially important thermostable enzyme: a review. Bioresource Technology. 2003; 89:17-34.
23. Hashmi M, Razavi SH, Shojaosadati SA, Mousavi SM, Khajeh K, Safari M. Development of solid state fermentation process for production of an alpha amylase with potentially interesting properties. Journal of Biosciences and Bioengineering. 2010; 110(3):333-337.
24. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's manual of determinative Bacteriology, 9th ed. Williams and Wilkins, Baltimore, (MD), 1994.
25. Kaithiresan K, Manivannan S. Alpha- amylase production by *P. Fellutanum* isolated from mangrove rizospheric soil. African Journal of Biotechnology. 2006; 5(10):829-832.
26. Khalil A. Isolation and characterization of three thermophilic bacterial strains (lipase, cellulose and amylase producers) from hot water springs of Saudi Arabia. African Journal of Biotechnology. 2011; 10(44):8834-8839.
27. Kohilu U, Nigam P, Singh D, Chaudhary K. Thermostable alkalophilic and cellulase free xylanases production by *Thermoactinomycetes thalophilus* subgroup C. Enzyme and Microbial Technology. 2001; 28:606-610.
28. Kumar S, Nussinov R. How do thermophilic proteins deal with heat? A review. Cellular and Molecular Life Sciences. 2011; 58:1216-1233.
29. Kunamaneni A, Kumar SK, Singh S. Response surface methodological approach to optimize the nutritional parameters for enhanced production of amylase in solid state fermentation by Thermomyces lanuginosus. African Journal of Biotechnology. 2005; 4:708-716.
30. Liu BL, Tzeng YM. Optimization of growth medium for production of spores from *Bacillus thuringienesis* using RSM. Bioprocess Engineering. 1998; 18:413-418.
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. Journal of Biology and Chemistry. 1951; 193:265-275.
32. Mamatha J, Suresh V, Vadamurthy AB, Shilpi B, Shruthi SD. Production of α-amylase from *Aspergillus flavus* under solid state fermentation with optimum conditions. International Research Journal of Pharmacy. 2012; 3(8):135-140.
33. Moral R, Navarro PJ, Gomez I, Mataix J. Effects of chromium on the nutrient element content and morphology of tomato. Journal of Plant Nutrition. 1995; 18:815-822.
34. Pace NR. A molecular view of microbial diversity and the biosphere. Science. 1997; 276:734-740.
35. Pathak PA, Rekadwad NB. Isolation of thermophilic *Bacillus* sp. strain EF_TYK1-5 and production of industrially important thermostable α-amylase using suspended solids for fermentation. Journal of Scientific & Industrial Research. 2013; 72:685-689.
36. Pederson H, Neilson J. The influence of nitrogen sources on the alpha amylase productivity of *Aspergillus niger* in continues cultures. Applied Microbiology and Biotechnology. 2000; 3:278-281.
37. Rana N, Walia A, Gaur A. α- amylases from microbial sources and its potential applications in various industries. a review: National Academy Science Letters, 2013, 4009-012.
38. Ramachandran S, Patel AK, Nampoothiri KM, Chandran S, Szakacs G, Soccol CR *et al*. Alpha amylase from a fungal culture grown on oil cakes and its properties Braz Arch Biol Technol. 1999; 47:309-317.
39. Sadasivam S, Manickam A. Biochemical methods. 2nd ed. New Age International (P) Limited, Publishers. New Delhi, India, 1996.
40. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory, 1989.

41. Senthilkumar PK, Uma C, Saranraj P. Amylases production by *Bacillus* sp. using cassava as substrate. International Journal of Pharamaceutical and Biological Archives. 2012; 3(2):300-306.
42. Sharma V, Singh PK. Strain improvement of *Bacillus coagulans* and *Geobacillus stearothermopilus* for enhanced thermostable cellulase production and the effect of different metal ions on cellulase activity. International journal of engineering science and Technology. 2012; 4(11):4704-4709.
43. Syed DG, Agasar D, Pandey A. Production and partial purification of alpha-amylase from a novel isolate *Streptomyces gulbargensis*. Journal of Industrial Microbiology and Biotechnology. 2009; 36(3):189-194.
44. Tsegaye NK, Gessesse A. Amylase production under solid state fermentation by a bacterial isolates W74. African Journal of Biotechnology. 2014; 13**(**21):2145-2153.
45. Verma A, Gupta M, Shirkot P. Isolation and characterization of thermophilic bacteria in natural hot water springs of Himachal Pradesh (India). The Bioscan. 2014; 9(3):947-952.
46. Vijaybhaskar P, Jayalakshmi D, Shankar T. Amylase production by moderately Halophilic *Bacillus cereus* in solid state fermentation. Africian Journal of Microbiology Research. 2012; 6(23):4918-4926.
47. Vyas G, Sharma N. Production and Optimization of α-amylase from a novel thermoalkalophilic *Bacillus sonorensis* GV2 isolated from mushroom compost. Proceedings of the National Academy of Sciences. 2015; 81:1207-1221.
48. Willey JM, Sherwood LM, Woolverton CJ. Prescott, Harley and Kleins Microbiology, 7th ed. McGraw Hill Co. Inc. Boston, 2008.
49. Wuest PJ, Fahy HK. Spent mushroom compost traits and uses. Mushroom News. 1991; 39(12):9-15.