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Isolation and optimization of amylase producing bacterial strains (mw6) isolated from hot water springs of Manikaran in Kullu district of Himachal Pradesh

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Abstract
The hot water springs provide an excellent source of microorganisms which contain such enzymes useful for industrial application. Amylase producing thermo philic bacterial strain was isolated from hot water springs of Manikaran falling in Kullu district of Himachal Pradesh. Among the 8 isolates, MW-6 showed maximum amylase activity (73.75 IU) with specific activity (3.05 IU/mg). Optimum activity of isolate MW-6 showed enzyme production (262.15 IU) at a pH of 9.0 and temperature of 45°C after 72 h of incubation. It could be claimed that significant thermostability of the isolate MW-6 makes it a good candidate for amylase production for use in the industrial applications.

Keywords: amylase, hot water spring, starch and yeast extract

Introduction
Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose (Sundarram *et al.*, 2014) [1]. Among the enzymes, amylase are most widely used industrial enzyme that exhibit great significance having approximately 65 per cent of the world enzyme market (Ali *et al.*, 2017) [13] Amylases have attracted global enzyme market due to their vast applications in starch processing, detergent, alcohol, textile, food, paper and pharmaceutical industries (Mageswari *et al.*, 2012 and Couto and Sanroman, 2006) [3, 4]**.** The amylases can be obtained from various natural resources such as plants, animals and microorganisms (Mageswari *et al.*, 2012; Saranraj and Stella, 2013) [3, 5]. Microorganisms are most important sources of enzyme production which can be used for various purposes by humans. Microbial enzymes have several advantages which comprise lower production cost, large-scale production in industrial fermenters, wide range of physical and chemical characteristics, scope of genetic manipulation and rapid culture development (Duza and Mastan, 2013) [6]. The enzymes produced by microorganisms are also more active and stable than plant and animal counterparts (Anbu *et al*., 2013) [7].
The thermostable enzymes display unique characteristics such as temperature, chemical and pH stability. Thermophillic microorganisms are the source of industrial important thermostable enzymes (Pathak *et al*., 2015) [8]. Thermophillic microorganisms prefer living at higher temperatures and these organisms can even survive in boiling water. Isolation of thermophiles has received considerable importance because of their industrial applications. Several workers have reported amylase production by *Clostridium* (Kilic *et al.*, 2005) [9], *Pseudomonas* (Khannous *et al*., 2014) [10], *Rhodotorula* (Carrasco *et al*., 2016) [11], *Bacillus* (Fentahun and Kumari, 2017) [12] and *Aspergillus flavus* (Ali *et al*., 2017) [13].
The thermostable enzymes mainly isolated from thermophillic organisms, have found a lot of commercial application because of stability and are active at elevated temperature (Haki and Rakshit 2003) [14]. The hot water springs of Himachal Pradesh provide an excellent source of various kind of microorganism which contains such enzymes due to their varied physical and chemical conditions.
Therefore, the present study was mainly focused on the production of amylase from microbial source isolated from hot water spring and optimizing various parameters to enhance the amylase production.

Materials and methods
Isolation, enumeration and screening of amylolytic microorganisms from hot water springs
5 water samples were collected from hot water spring of Manikaran of Kullu district of Himachal Pradesh (Longitude77° 20' 52.95"E, Latitude32° 01'40.34"N) in sterilized screw capped tubes and physico- chemical analysis and isolation of amylase producing bacterial isolates were carried out in the lab of microbiology section, Department of basic Sciences, Dr Y. S. Parmar University of Horticulture and forestry, Nauni, Solan (H.P).

Isolation and enumeration of amylolytic bacteria
Isolation of amylolytic bacteria from the hot water samples were carried out using serial dilution method of Subba Rao, (1999) [15] as standard protocol. The plates were incubated at 35± 2°C.

Screening of amylase producing bacterial isolates
Qualitative assay
Starch hydrolysis test was performed by Shaw *et al.*, (1995) [16]. The starch agar plates were spot inoculated with the isolated strains and incubated at 45°C for 72 h. The growth

thus obtained was flooded with 2 ml of iodine solution. Bacterial colonies producing clear zones were selected and purified using streak plate technique on the starch medium and refrigerated at 4°C for further studies.

Quantitative assay

Amylase activity was determined by measuring the amount of hydrolyzed starch using the method of Xiao *et al.*, (2006) [17]. 0.5 ml of enzyme solution was incubated with 0.2 per cent starch at 37°C for 15 min. 3 ml of DNSA reagent was added to it and the mixture was heated on boiling water bath for 15 min. After cooling down to room temperature, absorbance of reaction mixture was read at 540 nm.

Production of amylase by amylolytic bacterial isolate

Preparation of seed culture and production of enzyme

The prepared seed culture was inoculated in production media 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. In the supernatant the amylase activity, protein content and specific activity were estimated as per the method of Bernfeld (1955) [18] and Lowry *et al.* (1951) [19], respectively. One International Unit (IU) of amylase activity is defined as the disappearance of an average of 1µmol of iodine binding starch material per minute in the assay reaction.

Morphological and biochemical characterization of mw6

Morphological and biochemical identification of MW6 were performed as the method of Sherman and Cappuccino (2005) [20].

Optimization of cultural conditions for maximum amylase production

Effect of incubation period

The bacterial isolates were grown on medium for different incubation period viz. 24, 36, 48, 72 and 96 h and incubated at 37±2°C for maximum amylase production.

Effect of pH

The pH of the medium was adjusted at viz. 3, 4, 5, 6, 7, 8 and 9 with the help of pH meter. The autoclaved liquid media was inoculated with the bacterial isolates and incubated at 37±2°C for selected incubation period i.e. 72 h.

Effect 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 respectively. The flasks were incubated at different temperature viz. 25°C, 30°C, 35°C, 40°C, 45°C and 50°C with the interval of 5°C.

Effect of carbon sources

Different carbon sources viz. sucrose, maltose, starch, fructose, lactose and glucose each rate of 1.0 per cent were added in the media and their effect on amylase production was Studied after incubating at selected temperature.

Effect of nitrogen source

Different nitrogen sources viz. yeast extract, peptone, urea, beef extract and ammonium chloride were added in media at the rate of 1.0 per cent and their effect was studied by enzyme activity.

Results and discussion

In order to achieve maximum amylase production of selected amylolytic isolate, a proper combination of various cultural conditions and nutrients was established. One single independent culture variable was altered while others were maintained at a constant level and level of extracellular amylase production was monitored. The results of present study are presented and discussed under different headings as under:

Variation in pH and temperature of water collected from hot spring

The pH of collected water ranged between 6.3 and 7.9 and temperature ranged from a minimum of 88.0°C to a maximum of 98.0°C. The variation in pH and temperature of water collected from different hot spring may be attributed to the type of microorganism present in them. Our results are in confirmation with Fooladi and Sajjadian (2010) [21] who reported temperature ranged between 46°C to 82°C and the pH ranged from 6.5 to 7.0 of three Iranian hot springs namely, Larijan, Mahallat and Meshkinshashr.

Isolation and screening of amylase producing bacteria

A total of 8 isolates were found to be the amylase producers. These are MW1, MW2, MW5, MW6, MW7, MW10, MW13 and MW26 which showed clear zones of starch hydrolysis with varying diameters.

Fig 1: Growth of isolates on nutrient agar media and starch nutrient agar media of selected isolates.

The zone size of bacterial colonies ranged between 3.00 to 8.43 mm with enzyme index of 10.39 to 24.62. The isolates MW6 from the hot spring exhibited highest zone size of 7.23 mm with enzyme index of 24.62.

Morphological and biochemical characterization of MW6

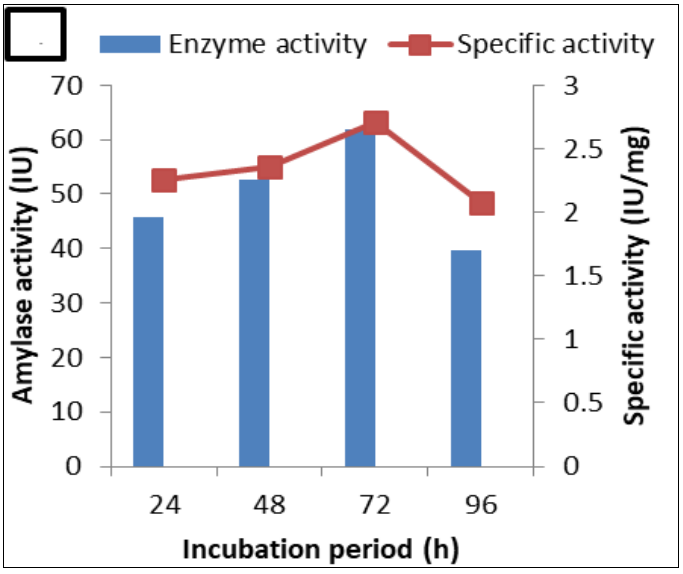
On the basis of morphological and biochemical characteristics, the MW6 isolate was identified as per the criteria of Bergey's Manual of Systematic Bacteriology (Table 2). Similar morphological and biochemical characteristics have been reported by Kirti *et al.*, (2016) [22].

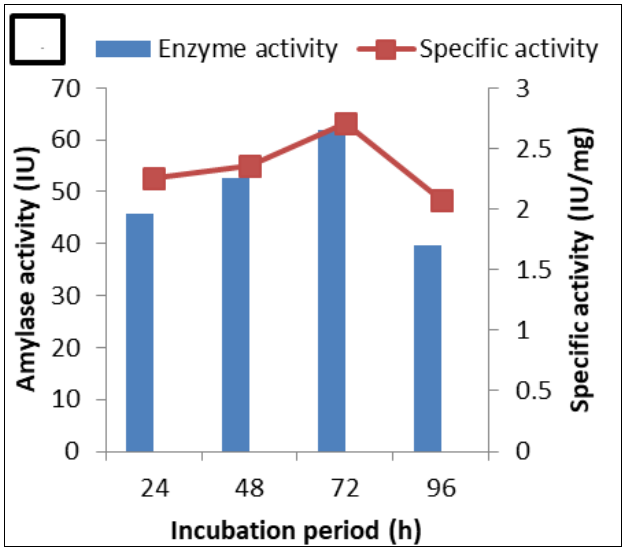
Table 2: Morphological and biochemical characterization of MW6

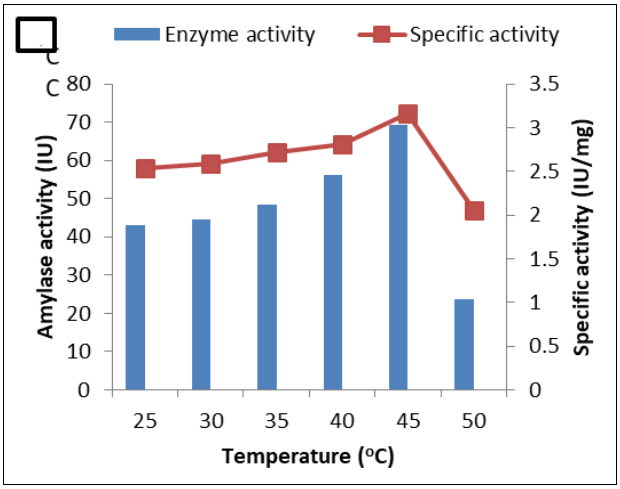
Characteristics	MW6
Morphological	
Form	Circular
Elevation	Raised
Margin	Entire
Color	Creamish
Gram's reaction	+
Shape	Rod
Endospore formation	-
Motility	+
Capsule formation	-
Biochemical	
Oxidase test	+
Catalase test	-
Indole test	-
Methyl red test	-
Voges Proskauer test	+
H_2S test	-
Simmon citrate test	-
Glucose test	+
Sucrose test	-
Lactose test	-
Urease test	-

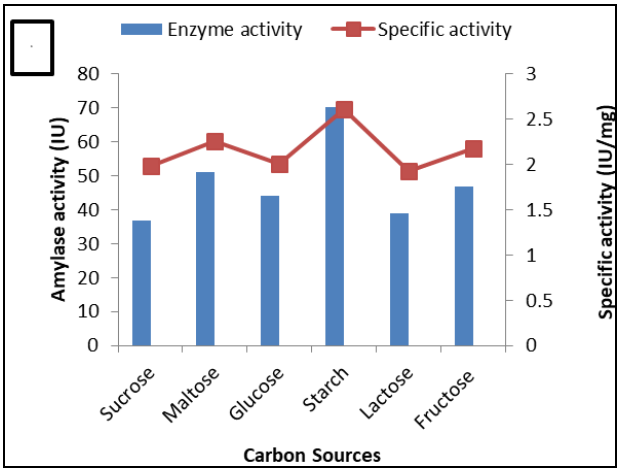
Effect of incubation period, ph and temperature on amylase activity

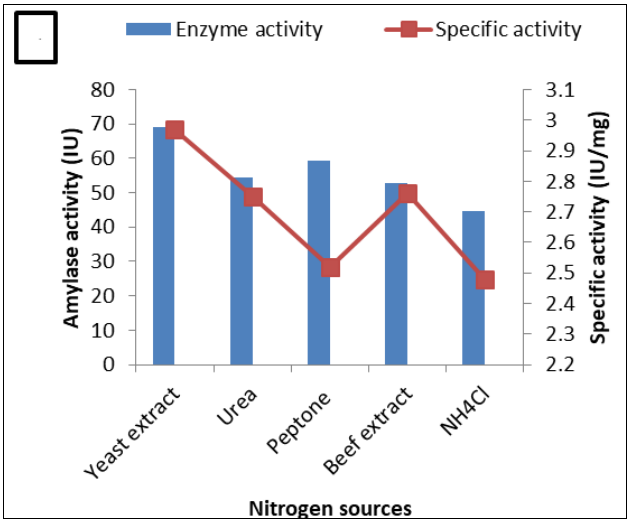
MW6 exhibited highest amylase activity of 55.37 IU with specific activity of 2.43 IU/mg at 72 h of incubation [Fig. 2 (B)]. At 24 h, a low enzymatic activity was noticed which increased up to 72 h and the activity followed a declining trend thereafter.











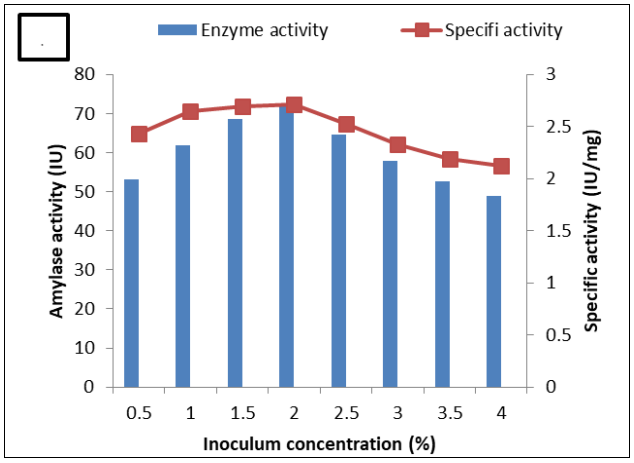


Fig 2: Effect of (A) Incubation period (h), (B) pH, (C) Temperature (°C), (D) Carbon sources, (E) Nitrogen sources and (F) Inoculum concentration on amylase production of MW6

Maximum production of amylase at 72 h of incubation has also been reported by various workers Vidyalakshmi *et al.*, (2009) [23], Chauhan *et al.*, (2011) [24] and Rana *et al.*, (2017) [23]. Possible reason for amylase activation after 24 h might be due to release of high levels of intracellular proteases and/or secondary metabolites in the culture medium at the end of exponential phase. After 72 h, there was a decrease in the amylolytic activity, which may be due to the depletion of nutrients, thereby causing a stressed microbial physiology resulting in an inactivation of enzyme (Flores *et al.*, 1997) [25]. Another reason could be the catabolite repression i.e. the increase in production of reducing sugars, which after a certain period of growth could exhibit inhibitory effect on enzyme production, since α-amylase is an inducible enzyme (Premila, 2013) [26]. The production of enzyme activity initially increased with increase in pH of medium up to 9.0 and thereafter the activity decreased [Fig.2 (A)] MW6 showed highest amylase activity of 60.84 at pH 9.0 with specific activity of 2.86 IU/mg.

At lower pH of 3.0, amylase activity of 48.92 IU and specific activity 2.44 IU/mg was recorded at optimum pH 9 [Fig.2 (B)]. The pH of the medium influences the growth of Microorganisms and plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannam, 2006) [27, 28]. Our results are in agreement with Zaferanloo *et al.*, (2014) [29] who have reported maximum activity of amylase at pH 9.0 by *Preussia minima*.

Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and usually varies from one organism to another (Banerjee and Bhattacharyya, 1992; Kumar and Takagi, 1999) [29, 12]. The production of amylase was conducted at 25 to 50°C with an interval of 5°C. The bacterial isolate MW6 exhibited highest activity of 54.67 IU followed by specific activity of 2.57 IU/mg at temperature of 45ºC. [Fig.2 (C)]. However, with the further increase in temperature, the amylase activity decreased significantly. Similar optimum incubation temperature of 45°C for an enhanced amylase production has been reported by Matthias (2013) [31], Wang (2016) [32] and Rana *et al.*, (2017) [33].

Effect of carbon and nitrogen sources on amylase activity

MW6 exhibited highest amylase activity of 68.62 IU [Fig. 2 (D)] and specific activity of 2.65 IU/mg with supplementation of starch among all other carbon sources. The maximum amylase activity with starch was comparable with apple pomace which may be due to the presence of polysaccharides, non-reducing sugars and maltose in the apple pomace. Starch has also been found to increase enzyme production by *Bacillus sonorensis* GV2 and *Bacillus* sp. as reported by Vyas and Sharma (2015) [34] and Khusro *et al.*, (2017) [35], respectively.

Nitrogen is the most important compounds for the growth and metabolism of microorganisms. The nature of these compounds and the concentration used may stimulate or slow down the production of enzymes (Sharma and Singh, 2012) [36]. MW6 exhibited the highest amylase activity of 64.72 IU with specific activity of 2.97 IU/mg [Fig. 2 (E)] with Yeast extract. It is required as the best nitrogen source. this may be due to higher content of minerals, vitamins, and coenzymes as reported by several workers (Ashwini *et al.*, 2011; Demirkan *et al.*, 2011; Vijayabhasker *et al.*, 2012) [37, 38].

The MW6 exhibited highest amylase activity of 66.98 IU with specific activity of 3.02 IU/mg at 1% (v/v) of the production medium [Fig. 2 (F)]. As the inoculum size was further increased, no significant increase in the production of amylase was observed. Decline in enzyme production at 4% (v/v) of inoculum is likely due to the fact that at higher inocula, the bacteria grow rapidly, exhausting essential nutrients for growth at the initial stages (Dash *et al.*, 2016) [40]. Inoculum size at 2% might increase amylase activity *Brevibacillus borstelensis* R1 (Indriati and Megahati, 2016) [41].

Conclusion

Amylases are extensively used in industrial applications like starch modification and food processing. Amylase producing bacteria was isolated from hot water spring. The isolated bacteria was found to be a potent producer of amylase enzyme with high activity after 72 hours at 45° C. The present study concluded that hot water spring served as a rich source of

Numerous hydrolytic enzymes and can be a source of many potent microorganisms.

References

1. Sundarram A, Murthy TPK. α-Amylase Production and Applications: A Review, Journal of Applied and Environmental Microbiology, 2014, 166-175.
2. Ali EH, El-Nagdy MA, Al-Garni SM, Ahmed MS, Rawaa AM. Enhancement of alpha amylase product ion by *Aspergillus flavus* AUMC 11685 on mandarin (Citrus reticulata) peel using submerged fermentation. European Journal of Biological Research. 2017; 7:154-164.
3. Mageswari A, Subramanian P, Chandrasekaran S, Sivashanmugam K, Babu S, Gothandam KM. Optimization and Immobilization of Amylase Obtained from Halotolerant Bacteria Isolated from Solar Salterns, Journal of Genetic Engineering and Biotechnology, 2012, 201-208.
4. Couto SR, Sanroman MA. Application of solid state fermentation to food industry: a review. Journal of Food Engineering, 2006, 291-302.
5. Saranraj P, Stella D. Fungal amylase: A Review, International Journal of Microbiological Research, 2015, 203-211.
6. Duza B, Mastan A. Microbial Enzymes and their Applications: a review, Indoamerican Journal of Pharmaceutical Research, 2013, 6208-6219.
7. Anbu P, Gopinath SCB, Cihan AC, Chaulagain BP. Microbial Enzymes and their Applications in Industries and Medicine, BioMedical Research International, 2013, 1-2.
8. 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 and Industrial Research, 2013, 685-689.
9. Kilic D, Apar FH, Ozbek B. Amylase inactivation during rice starch hydrolysis, Process Biochemistry, 2005, 1367-1379.
10. Khannous L, Mouna J, Mouna D, Miladi R, Chaaben N, Khemakhem B, *et al*. Isolation of a Noval Amylase and Lipase-Producing *Pseudomonas luteola*strain: Study of Amylase Production Conditions, Lipids in Health and Disease, 2014, 1-9.
11. Carrasco M, Villarreal P, Barahona S, Alcaino J, Cifuentes V, Baeza M. Screening and Characterization of Amylase and Cellulase Activities in Psychrotolerant Yeasts. BMC Microbiology, 2016, 1-26.
12. Fentahun M, Kumari PV. Isolation and Screening of Amylase Producing Thermophilic Spore Forming *Bacilli* from Starch Rich Soil and Characterization of their Amylase Activity, African Journal of Microbiology Research, 2017, 851-859.
13. Ali EH, El-Nagdy MA, Al-Garni SM, Ahmed MS, Rawaa AM. Enhancement of Alpha Amylase Production by *Aspergillus flavus* AUMC 11685 on Mandarin (*Citrus reticulata*) Peel using Submerged Fermentation, European Journal of Biological Research, 2017, 154-164.
14. Haki GD, Rakshit SK. Developments in Industrially Important Thermostable Enzymes: a review, Bioresource Technology, 2003, 17-34.
15. Subba Rao NS. Soil Microorganisms and Plant Growth, 4th Ed., New Delhi: Academic, 1999.
16. Shaw JF, Lin FP, Chen SC, Chen HC. Purification and Properties of an Extracellular α-Amylase from *Thermus* sp, Botanical Bulletin of Academia Sinica, 1995, 195-200.
17. Xiao Z, Storms R, Tsang AA. A Quantitative Starch Iodine Method for Measuring Alpha Amylase and Glucoamylase Activities, Analytical Biochemistry, 2006, 146-148.
18. Bernfeld P. Amalyses α and β. In: *Methods in Enzymology* (Colowick S and Kaplan N ed). Academic Press Inc, New York, 1955, 149-158.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein Measurement with the Folin-phenol Reagent, Journal of Biology and Chemistry, 1951, 265-275.
20. Sherman N, Cappuccino JG. Microbiology: a laboratory manual, 6th ed., ISBN: Academic, 2005.
21. Fooladi J, Sajjadian A. Screening the Thermophilic and Hyperthermophilic Bacterial Population of Three Iranian Hot Springs to Detect the Thermostable α-amylase Producing Strain, Iranian Journal of Microbiology, 2010, 46-50.
22. Kirti S, Dipta B, Bhardwaj S, Pawar R, Kaushal R. Screening and Characterization of Plant Growth Promoting rhizobacteria Associated with Cherry (*Prunusavium* L.), The Bioscan, 2016, 2111-2115.
23. Vidyalakshmi R, Paranthman R, Indhumathi J. Amylase Production on Submerged Fermentation by *Bacillus* sp, World Journal of Chemistry, 2009, 89-91.
24. Chauhan A, Mehta P, Mahajan R, Walia A, Shirkot CK. Deodar (*Cedrusdeodara*) Wood Dust: an Alternative Substrate for Amylase Production in Solid State Fermentation by Alkalophilic *Bacillus* spp. A1 Isolated from Mushroom Compost, Asian Science, 2011, 41-47.
25. Flores ME, Perez R, Huitron C. β-Xylosidase Xylanase Characterization and Production by *Sreptomyces* species CH-M-1035, Letters of Applied Microbiology, 1997, 410-416.
26. Premila JS, Dhandayuthapani K. Optimization of α-Amylase Production of *Bacillus stearothermophilus* KDP from Sago Industry Waste, International Journal of Applied Biological Research, 2013, 17-21.
27. Pederson H, Nielson J. The Influence of Nitrogen Sources on the Alpha Amylase Productivity of *Aspergillus oryzae*in Continues Cultures, Applied Microbiology and Biotechnology, 2000, 278-281.
28. Kathiresan K, Manivannan S. α-Amylase Production by *Penicillium fellutanum* Isolated from Mangrove rhizosphere soil, African Journal of Biotechnology, 2006, 829-832.
29. Zaferanloo B, Bhattacharjee S, Ghorbani MM, Mahon PJ, Palombo EA. Amylase Production by *Preussia minima*, a Fungus of Endophytic Origin: Optimization of Fermentation Conditions and Analysis of Fungal Secretome by LC-MS, BMC Microbiology, 2014, 14-55.
30. Banerjee R, Bhattacharyya BC. Extracellular Alkaline Protease of Newly Isolated *Rhizopusoryzae*, Biotechnology Letters, 1992, 301-304.
31. Matthias CO. Optimization of α-Amylase and Glucoamylase Production from Three Fungal Strains

Isolated from Abakaliki, Ebonyi State, European Journal of Experimental Biology, 2013, 26-34.
32. Wang S, Lin C, Liu Y, Shen Z, Jeyaseelan J, Qin W. Characterization of a Starch-Hydrolyzing α-amylase Produced by *Aspergillus niger* WLB42 Mutated by ethyl methanesulfonate Treatment, International Journal of Biochemistry and Molecular Biology, 2016, pp1-10.
33. Rana N, Verma N, Vaidya D, Dipta B. Production of Amylase from *Bacillus thuringiensis* J2 Using Apple Pomace as Substrate in Solid State Fermentation, International Journal of Current Microbiology and Applied Sciences, 2017, 3465-3474.
34. Vyas G, Sharma N. Production and Optimization of α-Amylase from a Novel Thermoalkalophilic *Bacillus sonorensis* GV2 Isolated from Mushroom Compost, Proceedings of the Indian National Academy of Science, 2015, pp1207-1221.
35. Khusro A, Barathikannan K, Aarti C, Agastian P. Optimization of Thermo-Alkali Stable Amylase Production and Biomass Yield from *Bacillus* sp. Under Submerged Cultivation, Fermentation, 2017, 1-19.
36. 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, pp4704-4709.
37. Ashwini K, Gaurav K, Kartik L, Bhaskara RKV. Optimization, Production and Partial Purification of Extracellular α-Amylase from *Bacillus* sp. *marini*, Archives of Applied Science Research, 2011, 33-42.
38. Demirkan E, Dincbas S, Sevinc N, Ertan F. Immobilization of *Bacillus amyloliquefaciens* α-amylase and comparison of some of its enzymatic properties with the free form. Romanian Biotechnological Letters, 2011, 6690-6701.
39. Vijaybhaskar P, Jayalakshmi D, Shankar T. Amylase Production by Moderately Halophilic *Bacillus cereus* in Solid State Fermentation, Africian Journal of Microbiology Research, 2012, pp4918-4926.
40. Dash BK, Rahman MM, Sarker PK. Molecular identification of a Newly Isolated *Bacillus subtilis* BI19 and Optimization of Production Conditions for Enhanced Production of Extracellular Amylase, BioMedical Research International, 2015, 805-859.
41. Indriati G, Megahati RRP. Optimization Medium of Amylase Production by bacillus licheniformisstrain MGI Originated from Pariangan Hot Spring, West Sumatera, Indonesia, International Journal of Advance Reseaech, 2017, 660-664.