

Production and Purification of Amylase from *Bacillus subtilis* Isolated from Soil

G. Kalyani¹ and E.M. Rajesh²

¹Research Scholar, PG & Research Department of Microbiology, PSG College of Arts and Science, Coimbatore, INDIA

²Assistant Professor, PG & Research Department of Microbiology, PSG College of Arts and Science, Coimbatore, INDIA

¹Corresponding Author: nithyaideas09@gmail.com

ABSTRACT

In spite of progress in biotechnology and enzymology, the enzymes have been industrialized in recent years for the mounting up the product development in various arena. The ultimate goal of this study comprises the production and purification the amylase enzyme from the bacterial strain. A powerful amylase producer, *Bacillus subtilis* ISOLATE-4 was isolated, screened and identified from the soil sample. In order to produce extracellular amylase, various physico-chemical parameters were optimized. During optimization, the maximal production of amylase by the isolate at 48 hrs of incubation in 100 rpm was found to be 6.93U/ml, 5.94U/ml, 6.0U/ml at 45°C, pH 6 with 1% substrate concentration respectively. Ammonium sulphate fractionation was done for rapid precipitation of the amylase at a concentration of 60% and exposed to dialysis showed the 25% purification fold of an enzyme. The dialyzed product was further subjected to DEAE-Cellulose column chromatography resulted in an increase up to 75% purification fold than crude enzyme. The amylase enzyme might be suitable for the liquefaction of starch, detergent, textile and several additional industrial applications.

Keywords--- *Bacillus Subtilis*, Amylase, Ammonium Sulphate Fractionation, Dialysis, DEAE-Cellulose Column Chromatography

industrial than analytical fields since it possesses many advantages than chemical methods. Utilization of highly specific enzymes for various sectors and applications are gaining a momentum because of their ability to replace harsh organic/inorganic chemicals currently used in all industries (Dhiman *et al.*, 2008). The source of enzymes is animal, plant and microorganisms; however, the industrial applications of commercial enzymes, microorganisms are the foremost vital source of assorted enzymes because they are inexpensive to produce and their enzyme contents are more expected, convenient to handle and reliable (Shanmugasundaram *et al.*, 2015). Enzymes are mainly performed in the conversion of macromolecules to body energy and new materials, also for growth, repair and cell maintenance.

Amylases are amongst the most studied worldwide attractions in an attempt to exploit their physiological and biotechnological applications (San-Lang Wang *et al.*, 2011 & Saini *et al.*, 2017). The major sources of alpha amylase have been recognized as different groups of microorganisms, especially bacteria and fungi leading into industrial solicitations. It has been widely studied due to a relative increase in large scale application. Among bacterial species, *Bacillus* sp is widely used and famous for the alpha amylase production and several *Bacillus* strains such as *B. stearothermophilus*, *B. subtilis*, *B. cereus*, *B. licheniformis*, and *B. amyloliquefaciens* are isolated and screened for amylase production (Simair *et al.*, 2017). Since *Bacillus* sp found ubiquitous nature, requiring optimal nutritional requirements for its

I. INTRODUCTION

Biotechnology and enzymology are well-thought-out a useful alternate to ancient procedures, especially in

amplification and also it produces maximal amount of alpha amylase (Singh *et al.*, 2012).

Amylase has been reported that 20-30% of individually produced enzymes are exploited in the textile and detergent industries worldwide due to having good thermal stability, low requirement of water and energy and the labors during processing (Rani *et al.*, 2015 & Rani *et al.*, 2015). In textile, sizing agents could be removed by an alpha amylase mainly for the removal of starch from the grey cloth before its further wet processing. It is so far widely used in pharmaceutical industries for the preparation of stimulants, the hydrolysis of starch for yielding several sugars namely glucose, maltose etc., implied in numerous applications. Amylases could degrade the polymer, starch results in the production of dextrans where the yeasts will easily act on that substrate during dough fermentation in bread industry. Alpha amylase plays a vital role in chocolate industry also for the preparation of choccos. Moreover, it has utmost importance in detergent for sizing the clothes. Meanwhile, in the paper industry, α – amylase hydrolyzed starch could be used for a sizing agent and coating agent instead of expensive chemically modified starches.

Despite of these many advantages, the use of enzymes in industrial applications has been limited by several factors, mainly the high cost of enzymes, their instability and availability in small amount, soluble in aqueous media and it is difficult to recover them from reactor effluents at the end of catalytic process. With the above background information, the current research was planned with the primary goal to isolate, screen, production and purification studies for amylase production in an effective manner.

II. MATERIALS AND METHODS

Isolation and screening of amylase producing Bacillus sp from soil

Bacillus sp was isolated from soil environment using substrate enriched medium and maintained on nutrient agar slants for further analysis. The isolates were then screened for the amylolytic activity by using starch hydrolysis method. The starch agar media were sterilized, plated and inoculated with the test isolates aseptically and then incubated at room temperature. The plates were then flooded with 1% w/v aqueous iodine solution and kept undisturbed for 2 – 3 min. The stain was poured off and the plate was observed in the zone of clearance (Sonia Sethi & Saksham Gupta, 2015).

Identification of an isolated organism

The selected isolate was identified by morphological and biochemical characteristics according to the Bergey's Manual of Systematic Bacteriology.

Enzyme production medium

A volume of 500ml Erlenmeyer flasks containing 100ml of fermentation medium comprises 1% Tryptone, 0.5% Yeast extract, 1% sodium chloride and 1% soluble starch and pH of the medium was adjusted to 7.0. Subsequently, it was autoclaved at 121°C for 15 min. Next, it was inoculated with 5 ml of the overnight starter culture and incubated at 37°C for 24 hrs. Bacterial cells were separated by centrifugation at 5000 rpm for 15 mins. The supernatant was then used for enzyme assay.

Protein Estimation of amylase

Protein content of the enzyme extract was determined by using Lowry *et al.*, (1951) method where Bovine Serum Albumin used as a standard. The protein concentration was estimated using values extrapolated from the standard graph of protein.

$$\text{Protein concentration (mg/ml)} = \frac{\text{Absorbance value}}{\text{Gradient}}$$

III. QUANTITATIVE ASSAY OF ENZYME

The crude enzyme sample was incubated with 1 % soluble starch in 0.1M Citrate buffer at 45°C in pH 5 and left it for 10 minutes. The liberated reducing sugars were estimated by using 3 ml of 3,5- dinitrosalicylic acid (DNS) and heated for 15 minutes in boiling water bath. One ml of Rochelle salt (40% potassium sodium tartarate) was added prior cooling to room temperature and the final volume was made with the addition of 7 ml distilled water (Miller, 1959). Two ml of 0.1 M Citrate buffer was used as a reference blank. The color developed was read by measuring its optical density using a spectrophotometer at 575 nm. The results were compared with standard curve using 0.10 to 1.0 mg of glucose/ml. One unit of enzymatic activity (IU) was defined as the amount of enzyme releasing 1 μ mol of sugar in 1 minute under standard assay conditions (Puri *et al.*, 2013).

Optimization of culture conditions for enzyme production

In order to produce the higher level of amylase production, the optimization of the cultural conditions for the isolated bacterial culture such as incubation time, pH, temperature and substrate concentration was done according to Sheoran and Dhankhar (2016).

Effect of incubation time on amylase production

To study the time interval for maximum amylase production, the culture was grown in 250 ml flasks with 50 ml of fermentation medium supplemented with starch as a substrate under submerged conditions. These flasks were incubated on a rotary shaker at 120 rpm at 37°C up to 5 days. Then, the cell free supernatant was assayed at interval of every 24 hrs for amylase production.

Effect of temperature on amylase production

The optimum temperature for maximum production of amylase was determined by preparing the fermentation medium and incubated at varying temperature from 25°C, 30°C, 35°C, 40°C, 45°C and 50°C with 100 rpm shaking condition at optimized incubation time. Then, the cell free supernatant was assayed for amylase production.

Effect of initial pH on amylase production

The optimum pH required for enhanced production of amylase was determined by preparing the fermentation medium, where the pH was adjusted previously from 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using 1 N NaOH or 1 N HCl and incubated at optimized temperature with 100 rpm shaking condition. Then the cell free supernatant was assayed for amylase production.

Effect of substrate concentration on amylase production

The effect of substrate concentration on enzyme production was determined by incubating the inoculated culture flasks in fermentation medium with varied substrate (starch) concentration of 0.5%, 1%, 1.5%, 2% and 2.5% under optimized incubation time, temperature and pH at 100 rpm in an orbital shaker- incubator. The optimum substrate concentration of amylase production was determined by assaying the cell free supernatant.

Effect of static and agitated condition on amylase production

To study the effect of static and agitated condition on amylase production by selecting five sets of fermentation medium was prepared and sterilized by autoclaving at 121°C at 15 mins. In all the sets, all the conditions (incubation time, pH, temperature and substrate concentration) applied were kept constant. One set of inoculated medium was kept in an incubator without shaking; another 4 sets were kept in different rpm (50, 100, 150 and 200) in an orbital- shaker incubator. Then the cell free supernatant was assayed for amylase production.

Enzyme bulk production and extraction

An amount of 500 ml fermentation media was prepared in 1000 ml ehrlenmeyer flask and autoclaved at 121°C for 15 minutes. After sterilization, it was inoculated with 10 ml culture broth of test isolate. All the contents of flasks were mixed well and incubated under optimized

conditions. Following incubation, the contents of the flasks were extracted using 1:20 ratio of substrate to phosphate buffer (0.05M, pH 6.5). The flasks were kept in an incubator shaker for one hour at 120 rpm and 30°C. Further, the enzyme extract was centrifuged at 10,000 rpm for 15 minutes at 4°C to remove cells and debris. The clear supernatant was used as crude enzyme content and it was assayed for its quantification (Sheoran and Dhankhar, 2016).

IV. PURIFICATION OF ENZYME

Partial purification of amylase by ammonium sulphate fractionation

The crude enzyme source was subjected with 20, 30, 40, 50, 60, 70, 80% of ammonium sulphate and stirred for one hour forcefully and allowed to stand at 4°C for 24 hrs until it precipitates. Followingly, it was centrifuged at 10,000 rpm for 15 minutes in 4°C and the pellet was collected. The pellet was added with 0.05 M phosphate buffer (pH 6.5) in the ratio of 1: 0.5. Yet again, it was centrifuged at 10000 rpm for 15 minutes at 4°C. The supernatant was collected and repeatedly the pellets subjected in buffer for 2 - 3 times and continued the same experiments. The yield of an enzyme was noted for all concentrations and found out the optimized concentration of ammonium sulphate for amylase enzyme precipitation.

Dialysis against buffer

Partially purified enzyme precipitate was resuspended in 0.05 M sodium acetate buffer at pH 5.5 and dialyzed against a large volume of 0.005 M sodium acetate buffer (pH 5.5) using a dialysis membrane. Dialyzed product was stored in the refrigerator at 4°C (Vantamuri & Kaliwal, 2016).

Ion exchange chromatography

The dialyzed protein was subjected to ion-exchange column chromatography on a DEAE-cellulose column. The column was pre-equilibrated with 0.05 M sodium acetate buffer at pH 5.5. The protein was eluted (flow rate 60 ml h⁻¹) with a linear gradient of NaCl (0.1–1 M) in the same buffer. Number of fractions were collected and assayed for protein and enzyme activity (Pushpa *et al.*, 2013).

V. RESULTS AND DISCUSSION

Isolation, screening and identification of amylase producing Bacillus sp from soil

In the present investigation, a total of 13 bacterial strains (Isolate 1 – Isolate 13) were isolated from the

garden soil sample at PSG College of Arts and Science, Coimbatore. Among which, six strains were satisfactory for amylase production identified through starch hydrolysis with a promising result, a zone of clearance. The maximum diameter of zone of clearance was found at 12.6 mm produced by isolate 4 and it was identified as *Bacillus subtilis* while subjected to identification in response to biochemical reaction represented in the Table 2 and the results were compared with standard chart (Bergey's Manual of systematic Bacteriology, 2005). Amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. Among bacteria, *Bacillus* sp is widely used for thermostable α -amylase production to meet industrial needs. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α -amylase and these have been widely used for commercial production of the enzyme for various applications (Pandey *et al.*, 2002).

Table 1 – Zone of clearance in the starch agar medium by isolates

S. No	Isolated strains	Zone of clearance
1	ISOLATE1	-
2	ISOLATE2	-
3	ISOLATE3	4 mm
4	ISOLATE4	12.6 mm
5	ISOLATE5	-
6	ISOLATE6	1.4 mm
7	ISOLATE7	3.5 mm
8	ISOLATE8	-
9	ISOLATE9	9.7 mm
10	ISOLATE10	5.3 mm
11	ISOLATE11	-
12	ISOLATE12	-
13	ISOLATE13	-

(-) indicates that the absence of zone formation

Table 2 – Identification of the amylase producing strain using standard biochemical tests

S. No	Identification test	Result
1	Cell size and shape	Long rod
2	Gram's reaction	Gram (+)
3	Starch hydrolysis	(+)
4	Indole production test	(+)
5	Methyl red test	(-)
6	Voges-Proskauer test	(-)

7	Citrate utilization test	(-)
8	Glucose fermentation	(+)
9	Lactose fermentation	(-)
10	Sucrose	(-)
11	Mannitol	(-)
12	Catalase	(+)

(+) indicates a positive result and (-) indicates a negative result

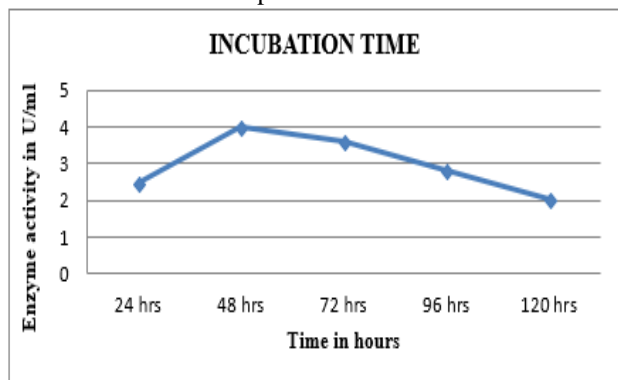
VI. OPTIMIZATION OF CULTURAL CONDITIONS FOR ENZYME PRODUCTION

Effect of incubation time

Physico-chemical parameters play a crucial role in the growth of the microorganism and determine the production of secondary metabolites. Each and every organism will differ from each other based on its physiology. The incubation time for achieving the maximal enzyme level is administered by the features of the isolates chiefly based on the growth rate and enzyme production. The maximal production of amylase by the isolate *Bacillus subtilis* ISOLATE4 was found to be 4U/ml at 48 hrs of incubation was depicted in Figure 1. Prolonged incubation time declines the production of amylase enzyme have been clearly shown. Minimum level of production was attained at 24 hrs of incubation because the organisms still in lag phase might not entered in to a log phase. Higher incubation time decreases the amylase production might be the organisms had entered into stationary phase or less nutritional supply especially in 96 hrs and 120 hrs of incubation.

Lonsane and Ramesh, 1990 had reported that average incubation time for the maximal enzyme production is about 48 – 50 hrs in common. Duochuan reported that byproducts produced during fermentation would also inhibit not only bacteria, but also the enzyme production. Nevertheless, a higher inoculum concentration might fall down the incubation period for enzyme production. Similar results were obtained in this state that the production of amylase increased with increasing incubation time, and it was found to be maximal up to 48 hours following inoculation. A further increase in the incubation period resulted in a decrease in the production of amylase. This result might be due to the production of by products and depletion of nutrients after 48 hrs.

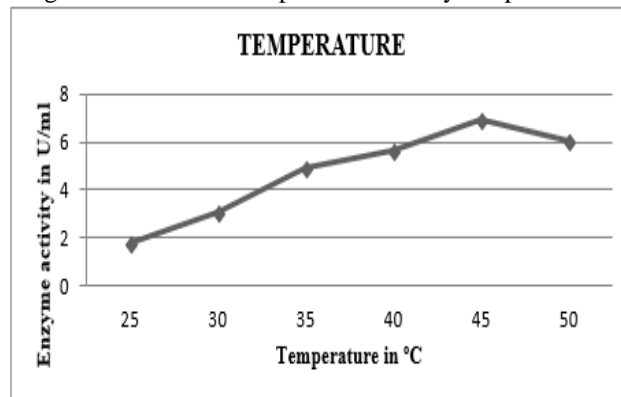
Figure 1 – Effect of incubation time for amylase production



Effect of temperature

Next to incubation period, the temperature was regarded as most important among various parameters. The influence of temperature on amylase production is related to the growth of the organism. The effects of various incubation temperatures on the growth of *Bacillus subtilis* isolate 4 and its amylase production were investigated. Most amylase production studies have been done with mesophilic bacteria within the temperature range of 25- 37°C. Higher yield of α -amylase production by the *B. subtilis* was found to be 6.93U/ml at 45°C depicted in Figure 2. Further increase in the temperature resulted in the decreasing yield of the enzyme might be due to the loss of stability for enzyme production. Among bacterial species, a wide range of temperatures, from 35 to 80°C has been reported popularly for their maximal growth and amylase production. A similar study conducted by Castro *et al.*, 1992, amylase production from *B. amyloliquefaciens* was found at maximum when it was incubated at 36 - 48°C. According to Sivaramkrishnan *et al.*, 2006 *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus stearothermophilus* are the most frequently used *Bacillus* species that reported amylase production at the temperature of 37– 60°C (Chand *et al.*, 2014). However, temperatures as high as 80°C have been used for amylase production from the hyperthermophile *Thermococcus profundus* (Chung *et al.*, 1995).

Figure 2 – Effect of temperature for amylase production

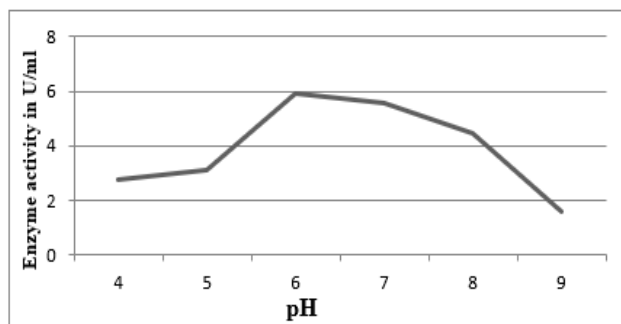


Effect of pH

One among the physical parameters, the pH plays a dynamic role in the growth by inducing morphological changes in the organism and in enzyme secretion. Since microorganism growth and enzymes are very sensitive to pH, determination of the optimal pH is essential to the production of amylase.

In the present study, the effect of pH on the production of enzymes was thus studied by carrying out fermentation over a range of pH (4.0 - 9.0). As observed in Figure 3 and, the isolated strain can grow very well and obtained its maximal enzyme production at a pH 6.0; pH values outside this range significantly decreased the growth a production rate. At pH 4.0, the strain did not grow well and a longer lag period was detected. At pH 5.0, minimum log phase was observed. Gupta *et al.*, 2003 reported that most of the *Bacillus* strain used commercially for the production of bacterial α -amylases by submerged fermentation have an optimum pH between 6.0 and 7.0 for growth and enzyme production. Chand *et al.*, 2014 assumed that the optimum production of amylase was achieved at an initial pH range of 7.5–8.0 for the bacteria, *Rhodothermus marinus*. The thermophilic anaerobic bacteria, *Clostridium thermo-sulfurogenes* exhibited maximum production at pH 7.0. But in this study, the optimal production of amylase was recorded at pH 6 by the indicated that the organisms required slightly acidic pH for maximal enzyme production by *Bacillus subtilis* was shown. The change in pH observed during the growth of the organism which disturbed the stability of the product present in the medium.

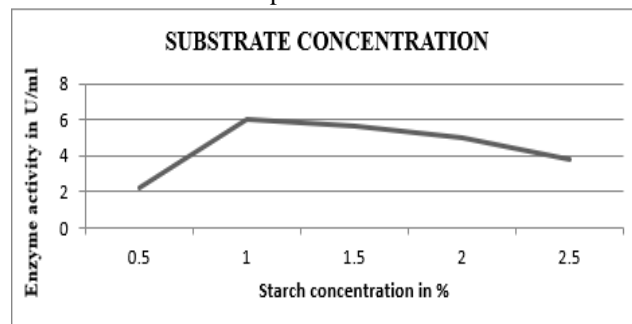
Figure 3 – Effect of pH for amylase production



Effect of substrate concentration

A change in the medium composition and molecular weight might be responsible for changes in the production of enzymes. When the amount of starch was increased, the production of enzymes was reduced. This result may be due to an increase in the amount of the carbon source above the optimal level, leading to a reduction in enzyme formation. The effects of different concentrations of the starch from 0.5-2.5% were determined. Maximal activity (6.02 U/ml) was exhibited by *Bacillus subtilis* isolate 4 with 1% substrate, the result of enzyme production at different substrate concentration was represented in Figure 4. When the amount of starch was increased from 0.5% (enzyme-substrate mixture), there was an increase in amylase production. Afterwards, when the substrate concentration exceeded 1%, a decline in enzyme production was noticed. This reduced enzyme production may have been associated with the saturation of the catalytic sites of the enzyme by the substrate. According to Gangadharan *et al.*, 2006 α -amylase production is induced by the presence of starch in the production medium. Amylase production by this strain was constitutive since biosynthesis of the enzyme took place not only in the presence of starch but also with other carbon sources. Moreover, the amylase yield was similar in all types of carbon sources such as soluble starch, potato starch, glucose, maltose, sucrose, etc. and therefore, this was not considered to reflect inducibility (Tonkova, 2006).

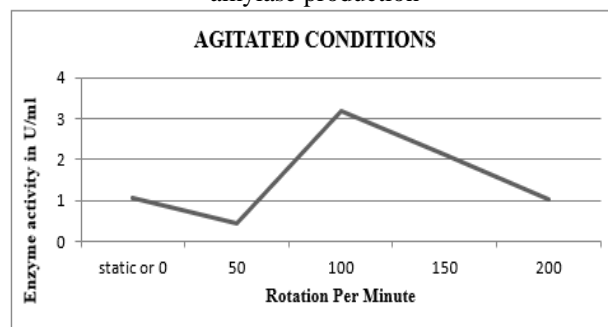
Figure 4 – Effect of substrate concentration of amylase production



Effect of static and agitated condition

The effect of agitation for the production of amylase was depicted in Figure 5. It was noticed that the optimum level of shaking needed for the maximum production of the enzyme was at 100 rpm. With an increase in the rpm level from 50 rpm with a gradation of 50 there has been an increase in the production of amylase and maximum enzyme activity was found to be 3.095 U/ml at 100 rpm. With further increase in rpm level, there was a decrease in enzyme activity; this could be due to the fact that the increase in rpm level has resulted in the coagulation of the organism to form as lumps and decrease in rate of mass transfer. The optimum shaking speed was observed at 100 rpm. Based on the optimal results of physico-chemical parameters, the higher amount of amylase was produced by the isolate 4 *Bacillus subtilis*.

Figure 5 – Effect of static and agitated conditions of amylase production



Purification of Amylase Enzyme

Purification of α -amylases from microbial sources in most cases has involved classical purification methods. These methods involve separation of the culture from the fermentation broth, precipitation using ammonium

sulphate and then subjected to chromatography usually affinity, ion exchange and/or gel filtration.

Cell free extract or crude filtrate was subjected to ammonium sulphate precipitation. Ammonium sulphate concentration was optimized for amylase purification using various concentrations from 20%-80%. Maximal activity was observed in 60% fractions having 6.92 U/ml, which were subjected to further purification by ion exchange chromatography containing DEAE-cellulose

column was shown in Table 3. Therefore, it could be concluded that the addition of 60% saturation of ammonium sulphate is recommended for the first step in the purification process of the enzyme. Subsequently, the precipitate was undergone the desalting process, dialysis. The enzyme and protein concentration were found to be 7.1U/ml and 1.06 mg/ml respectively which linked to 25% purification fold represented in the Table 4.

Table 3 – Partial purification of amylase enzyme by ammonium sulphate precipitation

% of Ammonium sulphate	Volume (ml)	Enzyme activity (U/ml)	Protein content (mg/ml)	Purification fold
20	100	0.26	0.32	0.03
30	100	0.63	0.11	0.12
4	100	1.43	0.08	0.48
50	100	1.02	0.24	0.65
60	100	6.92	0.16	1.97
70	100	5.03	0.23	0.67
80	100	4.45	0.20	0.64

The dialyzed product was subjected to DEAE-Cellulose column chromatography and the fractions were collected. Amongst the fractions, 5th fraction showed maximum enzyme concentration found as 4.55U/ml and the protein content were determined as 0.15 mg/ml which linked to 75% purification fold. The commercial use of α -amylase generally does not require purification of the

enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases. Chakraborty *et al.*, purified a thermostable α -amylase enzyme by ammonium sulphate fractionation and ion exchange column chromatography on DEAE-cellulose to obtain a homogeneous product.

Table 4 – Partial purification of amylase enzyme by DEAE cellulose column

Purification methods	Fractions obtained	Enzyme activity (U/ml)	Protein content (mg/ml)	Purification fold
Dialysis	Single fraction	7.1	1.06	25%
Ion exchange chromatography	1	0.23	0.02	3.79%
	2	0.21	0.08	3.46%
	3	0.02	0.09	0.32%
	4	0.32	0.12	5.27%
	5	4.55	0.15	75%
	6	1.04	0.14	17.14%

VII. CONCLUSION

Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market. Studies carried out so far have shown that the *Bacillus subtilis* are good enough for the production of amylase found greatly in the environment. Amylases are one of the most

promisingly used enzymes chiefly required for the preparation of fermented foods. Apart from food and starch industries, in which demand for them is increasing continuously, they are also used in various other industries such as paper and pulp, textile, etc., Research is focused on developing thermo tolerant and pH tolerant α -amylase from microbes, modifying them genetically or applying site-directed mutagenesis to acquire desired properties in the enzyme. Commercially, most of the production of α -

amylase is carried out in submerged fermentation, but solid-state fermentation is being looked at as a potential tool for its production, especially applying agricultural-industrial residues as substrate. The use of agricultural waste residues and microorganisms in enzyme production will upsurge the industrial advantages in the area of research and development. Enzymes are not only beneficial from an ecological point of view, but they are also saving lot of money by recycling and energy consumption which ultimately reduce the cost of production or even exterminated. Upcoming prospects will be the efforts taken to minimize the reduction of cost by recycling the enzyme usage. It looks like in the future, it will be possible to do every process in each and every sector with the help enzymes. Future prospects will be the efforts taken to minimize the reduction of cost by recycling the enzyme usage.

REFERENCES

- [1] Castro GR, Ferrero MA, Mendez BS, & Sineriz F. (1993). Screening and selection of bacteria with high amylolytic activity. *Acta Biotechnologica Banner*, 13(2), 197-201.
- [2] Chakraborty, S., Khopade, A., Kokare, C., Mahadik, K., & Chopade, B. (2009). Isolation and characterization BACTERIA of novel α -amylase from marine Streptomyces sp. D1. *Journal of Molecular Catalysis B: Enzymatic*, 58(1), 17-23.
- [3] Chand, N., Sajedi, R. H., Nateri, A. S., Khajeh, K., & Rassa, M. (2014). Fermentative desizing of cotton fabric using an α -amylase-producing *Bacillus* strain: Optimization of simultaneous enzyme production and desizing. *Process Biochemistry*, 49(11), 1884-1888.
- [4] Chung, Y.C., Kobayashi, T., Kanai, H., Akiba, T., & Kudo, T. (1995). Purification and properties of extracellular amylase from the hyperthermophilic archaeon thermococcus profundus DT 5432. *Applied Environmental Microbiology*, 61(4), 1502-1506.
- [5] Dhiman, S. S., Sharma, J., & Battan, B. (2008). Industrial applications and future prospects of microbial xylanases: A review. *Bio Resources*, 3(4), 1377-1402.
- [6] Gangadharan D, Sivaramakrishnan S, Namboothiri KM, & Pandey A. (2006). Solid culturing of *Bacillus amyloliquefaciens* for α -amylase production. *Food Technology Biotechnology*, 44(2), 269-274.
- [7] Gupta R, Gigras P, Mohapatra H, Goswami VK, & Chauhan B (2003). Microbial α -amylases: A biotechnological prospective. *Process Biochemistry*, 38(11), 1599-1616.
- [8] Lawal, A. K., Banjoko, A. M., Olatope, S. O., Alebiosu, F. A., Orji, F. A., Suberu, Y. L., & Dike, E. N. (2014). Production and partial purification of amylase by *Aspergillus niger* isolated from cassava peel. *Journal of Basic & Applied Sciences*, 10, 287-291.
- [9] Li, D. C., Yang, Y. J., & Shen, C. Y. (1997). Protease production by the thermophilic fungus *thermomycetes lanuginosus*. *Mycological Research*, 101(1), 18-22.
- [10] Lonsane, B. K., & Ramesh, M. V. (1990). Production of bacterial thermostable α -amylase by solid-state fermentation: A potential tool for achieving economy in enzyme production and starch hydrolysis. *Advances in Applied Microbiology*, 35, 1-56.
- [11] Lowry, O.H., Rosebrough, N.J., Fan, A.L., & Randall, R.J. (1951). Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry*, 193(1), 265-275.
- [12] Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, 31(3), 426-428.
- [13] Pandey, A. (2001). Solid-state fermentation in biotechnology: Fundamentals and applications. *Biochemical Engineering Journal*, 13(2), 81-84.
- [14] Rani, K., & Chauhan, C. (2014). Biodegradation of cicer arietinum amylase loaded coconut oil driven emulsified bovine serum albumin nanoparticles and their application in washing detergents as eco-Friendly bio-Active additive. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3(12), 924-936.
- [15] Reeta Rani, Anil Kumar, Carlos R, and Soccol Ashok Pandey. (2009). Recent advances in solid state fermentation. *Biochemical Engineering Journal*, 44(1), 13-18.
- [16] Saini, R., Saini, H. S., & Dahiya, A. (2017). Amylases: Characteristics and industrial applications. *Journal of Pharmacognosy and Phytochemistry*, 6(4), 1865-1871.
- [17] Arun Kumar Sharma, Vinay Sharma, Jyoti Saxena, Bindu Yadav, Afroz Alam, & Anand Prakash. (2015). Optimization of protease production from bacteria isolated from soil. *Applied Research Journal*, 1(7), 388-394.
- [18] Shanmugasundaram, S., Annadurai, E., Mayavu, P., Surya, M., & Anbarasu, R. (2015). Screening and identification of amylase producing bacteria from marakkanam saltpan environment, tamilnadu, india. *Asian Journal of Biomedical and Pharmaceutical Sciences*, 5(48), 35-37.
- [19] Sheoran, S., & Dhankhar, R. (2016). Partial purification and characterization of amylase from *aspergillus flavus* using pod biomass of *pithecellobium*

dulce as substrate. *International Journal of Recent Scientific Research Research*, 7(4), 10413-10417.

[20] Simair, A. A., Qureshi, A. S., Khushk, I., Ali, C. H., Lashari, S., Bhutto, M. A., & Lu, C. (2017). Production and partial characterization of α -amylase enzyme from bacillus sp. bcc 01-50 and potential applications. *BioMed research international*, 2017, 1-9. Available at:

<https://www.hindawi.com/journals/bmri/2017/9173040/>

[21] M. J. E. C. van der Maarel, B. van der Veen, J. C. M. Uitdehaag, H. Leemhuis, & L. Dijkhuizen. (2002). Properties and applications of starch-converting enzymes of the α -amylase family. *Journal of Biotechnology*, 94(2), 137–155.

[22] Vantamuri, A. B., & Kaliwal, B. B. (2016). Purification and characterization of laccase from *Marasmius* species BBKAV79 and effective decolorization of selected textile dyes. *3 Biotech*, 6(189), 1-10.

[23] Wang, S. L., Liang, T. W., & Yen, Y. H. (2011). Bioconversion of chitin-containing wastes for the production of enzymes and bioactive materials. *Carbohydrate Polymers*, 84(2), 732-742.