© 2019 IJSRSET | Volume 6 | Issue 1 | Print ISSN: 2395-1990 | Online ISSN : 2394-4099
Themed Section : Science and Technology
DOI : https://doi.org/10.32628/IJSRSET196122

Production, Purification and Optimisation of Amylase by Submerged Fermentation Using Bacillus Subtilis

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ABSTRACT

Amylase is abundantly present in nature. The main source of this enzyme is the microbial origin. It is known that about two-third of the industrial enzymes (amylase, protease, cellulose, penicillinase, chitinase, etc,) are produced by the Bacillus spp. The present work comprised in the identification of amylase producing Bacillus spp and exposure of the producers to various parameters for the maximum yield of the enzyme. To isolate and identify the amylase and protease producing strain, soil samples were collected from different vegetation from the altitude at 4367.35 feet above sea level. The isolates were screened and various biochemical tests and morphological observations were done to identify the isolates. The enzymes were produced by the submerged state fermentation (SmF) from the isolates and purified by dialysis. Effects of temperature, pH, and different carbon and nitrogen sources of the medium using SmF were optimized. Among 95 isolates, 36 were identified. Among the identified isolates, Bacillus subtilis produced the maximum yield and thus, it was optimized for the amylase production. The maximum amylase production was found at 42$_0$C temperature, in fructose as a carbon sugar, peptone as a nitrogen source and at pH 7. Almost all the enzyme producers inhabited the roots of leguminous plants. In the present study, starch is used with the nutrient agar medium to help in cell immobilization for maximum production of amylase by strains of Bacillus. More sophisticated process of purification such as chromatography and electrophoresis will yield more enzyme as compared to the dialysis.

Keywords : Amylase, Bacillus spp, Production, Purification, Optimisation, Vegetations

I. INTRODUCTION

Soil has been defined as the region on the earth's crust where geology and biology meet [1]. The vast difference in the composition of soil together with the difference in the physical characteristics and agricultural practices by which they are cultivated result in the microbial population both in total numbers and kind [2] .The bacterial population of the soil exceeds the population of all other groups of microorganisms in both number and variety [3]. The metabolic abilities of bacteria play a critical role in geochemical nutrient cycling and producing a wide range of products of industrial significance. *Bacillus* is the most abundant genus in the rhizosphere [4].

The genus *Bacillus* is frequently known as aerobic spore bearers. They are ubiquitous and are present in soil, dust, air, and water. Most of the 70 or so species of *Bacillus* are found in soil and water and are usually encountered in the medical laboratory as airborne contaminants [5].Most members of the genus *Bacillus* are able to produce low molecular weight antibiotics,

which possess different biological activities, including antimicrobial, antiviral, and antitumor activities [6].

Amylase is an enzyme that catalyses the hydrolysis of starch by breaking the bonds between sugar molecules. Many microorganisms have a potential to produce amylases including *Bacillus subitilis, Bacillus megaterium, Bacillus cereus, Lactobacillus* spp, *Escherichia coli*, *Pseudomonas* spp, *Streptomyces* spp, etc [7]. According to the article published by Priest (1977), *Bacillus* species that are involved in the production of amylase include *B. amyloliquefaciens, B. caldolyticus, B. coagulans, B. licheniformis, B. macerans, B.stearothermophilus, B. subtilis, B. cereus, B. megaterium, B. polymyxa* and Alkalophilic *Bacillus* spp [8].

Different strategies for purification of enzymes have been investigated, exploiting specific characteristics of the target biomolecule [9]. Enzyme purification can be done by using the following techniques: Ammonium Sulphate, Precipitation, Dialysis, Ultra filtration nd Lyophilization [10]. The optimization of fermentation conditions, particularly physical and chemical parameters, are important in the development of fermentation processes due to their impact on the economy and practicability of the process [11].The nature and amount of carbon sources in culture media are important factor for the production of extracellular enzymes [12]. Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and this is usually varied from one organism to another [13]. The pH range observed during the growth of microbes also affects product stability in the medium [14].

The industrial demand in view of the diversity in applications for amylase enzymes continues to stimulate the search for new enzyme source. Hence the present study is undertaken to isolate *Bacillus* from the rhizospheric region of various vegetations, and assess their ability to produce protease and amylase enzymes. The purpose of this study is to investigate the effectiveness of production of enzyme (protease and amylase) by incubating test *Bacillus* spp in media differing in one of the aspects: carbon source, nitrogen source, pH, temperature. The set of conditions which support higher degree of enzyme production can be used for more economical and higher yield of enzyme.

II. MATERIALS AND METHODS

Study Area

The study area for sample collection was from Kathmandu Valley, Nepal which was located at an altitude of 4367.35 feet above sea level. Soil was collected using random sampling method from different places of Kathmandu.

Study Method

Random sampling method was applied for the collection of the samples. Soil samples were collected from various wet and dry land.

Sample Size

A total of 25 samples were collected from different vegetations of Balaju Nursery House, Kathmandu, Nepal which is at an altitude of 4367.35 feet above sea level.

Study Duration

The project was carried out in microbiology laboratory of St. Xavier's College, Maitighar, Kathmandu, Nepal from 15th October, 2016 to 10th June, 2017.

Sample Collection

Soil samples were collected in an aluminum foil from a depth of 5 cm.

Isolation of Bacteria from Soil

One gram of soil sample was taken and serially diluted up to 10-6 in sterile distilled water and spread plate technique was performed for the isolation of bacteria. For this 0.1 ml of sample was dispensed in petri plate containing nutrient agar and HiCrome *Bacillus* agar and was spread using sterile glass rod.

Samples from dilution 10-6 were subjected to spread plate into respective plates. The plates were then incubated at temperature 28˚C for 24 hours [15].

Primary screening of Amylase Producer

Different isolated colonies from the nutrient agar plates were chosen and sub cultured in 1% Starch agar plate and some bacterial isolates were sub cultured in nutrient broth as stock culture and was incubated at temperature 28˚C for 24 hours. After incubation, the starch agar plate was flooded with iodine solution. Iodine was then dispensed with caution and zone of hydrolysis was observed [23].

Characterization of Amylase Producer

Starch hydrolyzing bacterial isolates were then characterized by using standard microbiological technique which included colony morphology, Gram's stain and biochemical reactions and pure colonies were examined for *Bacillus* with standard description of Bergey's Manual of Determinative Bacteriology.

Production of Extracellular Enzyme (Submerged Fermentation)

The starch hydrolyzing isolates were inoculated in conical flask containing 50 ml of and 1% starch broth. The flasks were then kept in shake flask rotary incubator. Time, temperature and speed (in rpm) were set at 24 hour, 28˚C and 120 rpm respectively.

Extraction of Extracellular Amylase

The broth cultures were transferred in tubes and labeled. It was centrifuged at 2000 rpm for 20 minutes and the obtained supernatant was transferred to another test tube and pellets were discarded. The tube containing supernatant was mixed with double volume of acetone and was left overnight and again centrifuged at 3000 rpm for 20 minutes.

Supernatant was transferred to to other test tube(s) and pellet was dissolved in minimum volume of phosphate buffer. These enzymes were labeled as crude enzyme [16].

Purification of Crude Enzyme

The crude enzyme obtained was purified by using dialysis bag. The crude enzyme was kept in the dialysis bag and was introduced in a beaker with distilled water and purification was done using magnetic stirrer for 40 minutes.

Determination of Amylase Enzyme Activity

Amylase activity in the crude enzyme was determined by estimation of the reducing sugar liberated by the action of amylase on soluble starch. Reducing sugars were estimated by 3,5 – Dinitrosalicylic acid (DNS) method [17].

Enzyme Optimization

For enzyme optimization, multiple sets of conical flask- each containing 50 ml broth media differing uniquely in carbon, nitrogen, pH and temperature and time of incubation were inoculated with test organism. The enzyme so produced were assayed using corresponding methods as described above.

Quality Control

Laboratory equipments like incubator, hot air oven, autoclave, refrigerator, etc. were regularly monitored for their performance and immediately corrected if any deviation occurred. The temperature of the incubator and refrigerator were mentioned every day. For consistency in results, any reagents and media were prepared fresh and stored under appropriate conditions. Any media or reagents to be used were prepared in sterile (if not aseptic) condition.

Data Analysis

Data were statistically analyzed using various graphs and tables.

Validity and Reliability

For the valid test results standard protocol was followed and pre-testing of the tests was performed. Similarly, reliability of the study depended on expert's opinion, guidance of supervisor's and literature review.

III.RESULTS AND DISCUSSION

All together 25 soil samples were taken from the rhizospheric region of different vegetations from the Balaju Nursery which is at an altitude of 4367.35 feet above sea level. Of the 95 isolates obtained, 36 species were identified. producer. The most frequently occurring amylolytic bacteria with greatest enzyme activity was *Bacillus subtilis* followed by *B. polymyxa, B. megaterium, B. coagulans, B. licheniformis* and *B. cereus.* In a similar study performed by [18] *Bacillus subtilis* was found to be most frequently occurring amylase producer.

Of the 36 species isolated *B. polymyxa* was observed to be the most prominent one with overall of 20% population followed by *B. circulans* with 14%, *B. megaterium* 11%, *B. coagulans* and *B. licheniformis* with 9%. From the identified species, *B. subtilis* was optimized for the amylase production under different parameters.

Identification of the isolates

Based on the color of colonies observed on HiCrome *Bacillus* agar [19] and the biochemical tests based on the Bergey's Manual of Determinative Microbiology [20], the isolated colonies were identified. Since, the identification of *B. thuringiensis* was not mention in the manual, for its detection, biochemical tests were carried out based on the procedure described [21]. Similarly, for the identifaction and detection of *Bacillus thuringensis* among the isolates, the crystal protein staining was also performed as outlined by Sharif and Alaeddinoğlu [22]. The results are presented in table 1.

Table 1 : Identification of *Bacillus* spp from root nodules of different vegetables

S.N.	Batch	Vegetations	Code	Isolates Designation	Gram Staining	Catalase Test	Oxidase Test	MR	VP	Citrate	% NaCl	Size	Identified Species
1.	1st	Bakula	BBG	B1a	+	+	+	+	-	-	+	6.5 <1µm	*B. alcalophilus*
2.			BBB	B1b	+	+	+	+	-	-	+	<1 µm	*B. brevis*
3.		Tomato	BT_0B	B1c	+	+	+	+	-	-	+	<1 µm	*B. megaterium*
4.			BT_0R	B1d	+	+	+	+		-	+	<1 µm	*B. macerans*
5.			BT_0G	B1e	+	+	+	+	-	+	+	<1 µm	*B. megaterium*
6.		Turnip	BTG	B1f	+	+	+	+	-	+	-	<1µm	*B. circulans*
7.		Raddish	BRLG	B1g	+	+	+	+	-	+	-	>1µm	*B. coagulans*
8.			BRP	B1h	+	+	+	+	-	+	+	<1µm	*B. macerans*
9.			BRB	B1i	+	+	+	-	+	-	+	>1µm	*B. subtilis*
10.			BRY	B1j	+	+	+	-	+	-	+	<1µm	*B. licheniformis*
11.	2nd	Ginger	BGgY	B2a	+	+	+	-	+	-	-	>1µm	*B. circulans*
12.		Green onion	BG_0P	B2b	+	+	+	-	+	-	+	<1µm	*B. polymyxa*
13.		Gram	BGrY	B2c	+	+	+	+	-	-	-	<1µm	*B. circulans*
14.			BGrP	B2d	+	+	-	-	+	-		<1µm	*B. polymyxa*
15.	3rd	Gheu Simi	GSW	B3a	+	+	+	+	-	-	+	<1µm	*B. megaterium*
16.		Badam	BD	B3b	+	+	+	-	-	-	-	>1µm	*B. polymyxa*
17.			BDR	B3c	+	+	+	-	+	-	-	>1µm	*B. subtilis*

18.			BDG	B3d	+	+	+	-	+	+	+	<1µm	*B. polymyxa*
19.		Corn	CoW	B3e	+	+	+	+	-	+	-	>1µm	*B. thuringiensis*
20.			CoG	B3f	+	+	+	+	+	+	+	>1µm	*B. cereus*
21.		Lamo Bodi	LBW	B3g	+	+	+	-	+	+	-	<1µm	*B. coagulans*
22.			LBR	B3h	+	+	+	-	+	+	+	>1µm	*B. licheniformis*
23.			LBG	B3i	+	+	+	-	+	-	+	>1µm	*B. polymyxa*
24.	4th	Barela	BBaC	B4a	+	+	+	+	-	-	+	<1µm	*B. alcalophilus*
25.			BBaLG	B4b	+	+	+	+	-	-	+	>1µm	*B. circulams*
26.			BBaP	B4c	+	+	+	-	+	+	-	>1µm	*B. coagulans*
27.		Chilli	BChW	B4d	+	+	+	-	-	+	+	>1µm	*B. subtilis*
28.			BChLG	B4e	+	+	+	+	-	-	+	>1µm	*Bacillus* spp
29.			BChCW	B4f	+	+	+	+	-	+	+	>1µm	*B. alcalophilus*
30.			BChP	B4g	+	+	+	-	+	-	+	>1µm	*B. polymyxa*
31.		SoopSag	BSoP	B4h	+	+	+	-	-	+	-	>1µm	*B. circulans*
32.			BSoG	B4i	+	+	+	+	-	-	-	>1µm	*B. thuringiensis*
33.			BSoY	B4j	+	+	+	-	-	+	+	<1µm	*B. megaterium*
34.		Taaro (Karkalo)	BKmY	B4k	+	+	+	+	+	+	+	>1µm	*B. licheniformis*
35.			BKmG	B4l	+	+	+	+	+	-	+	>1µm	*B. polymyxa*
36.		Ishkush	BIR	B4m	+	+	+	-	-	-	+	>1µm	*B. brevis*

Distribution of isolated species

A total of 36 species were identified on the basis of Bergey's Manual of Derterminative Bacteriology and are presented in the figure below. Highest Identification of selected Bacillus strain was on the amylase production was seen in Badam (Figure 1).

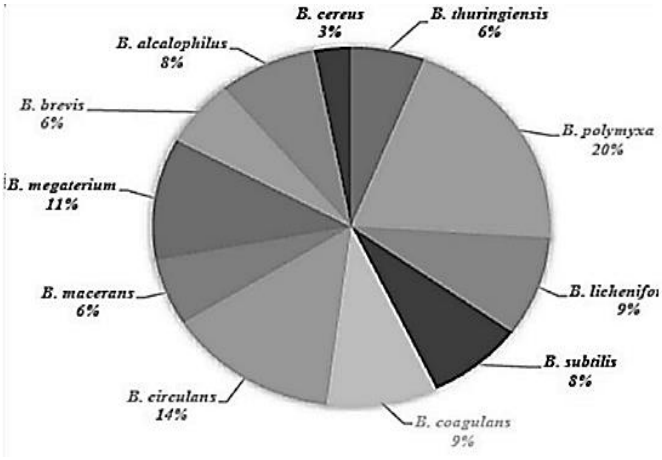

Figure 1 : Population of *Bacillus* strain from root nodules of different vegetations

The genus *Bacillus* produces a large variety of extracellular enzymes, of which amylases are of particularly considerable industrial importance [23]. Since *Bacillus subtilis* yield the maximum amylase production among other organism, it was associated with various parameters in shake- flask culture using starch (0.5%) as a substrate along with different forms of sugars (glucose, fructose, lactose and sucrose), nitrogen source (peptone, urea and ammonium sulphate) at different temperatures (27^0C, 32^0C,37^0C and 42^0C) and pH 7. Optimal culture conditions such as sources of nitrogen and carbon, their ratio, pH, temperature of incubation, etc. ensure maximum growth, metabolism and production of enzyme. Thus, optimization of growth condition is a prime step in fermentation technology. In this study, we observed 42°C as the optimum temperature for the enzyme production. Optimal temperature for the enzyme production was found to be 42^0 C. Our finding correlates with the study carried out by Md. M. Hasan et al [24].

Assay of amylase produced by *Bacillus subtilis* under different parameters

For the optimization of amylase, the organism was inoculated in media with variety of nitrogen source

(peptone, urea and ammonium sulphate) coupled with various carbon source (glucose, fructose, sucrose and lactose) and incubated under various temperatures. Peptone was considered suitable nitrogen source as compared to urea and ammonium sulphate according to the optical density readings at 540 nm (Figure 2). Similar readings were observed in case of fructose where maximum reading was observed when peptone was used along with fructose compare to other two nitrogen sources (Figure 3).

When glucose was used as a carbon source with respective nitrogen sources: urea, ammonium sulphate and peptone, varying results were observed. These variations in the results is given in the theory proposed by Szilard [25] which suggests that when there is an external manipulation in the enzyme system (in our case the temperature) when glucose is used as a carbon source, the maximum activity is seen at those temperatures that favors the enzyme production irrespective of the nitrogen sources used.

Comparably, the optimization of the different nitrogen source was observed with lactose and sucrose respectively. Lactose corporate media with the different nitrogen sources gave similar reading at all temperatures (Figure 4). Whereas, sucrose and urea gave the maximum result at 42^{0}C (Figure 5).

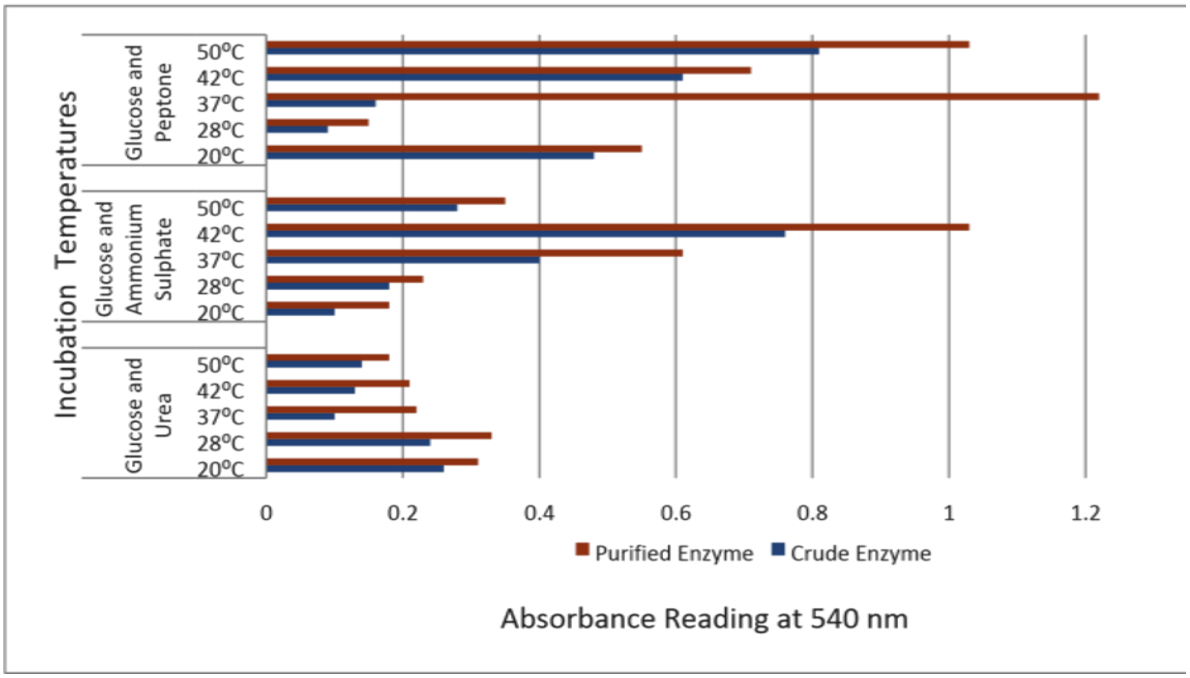

Figure 2: Amylase activity of *Bacillus subtilis* upon incubation with Glucose and different Nitrogen Sources (Peptone, Ammonium Sulphate and Urea) under various incubation temperatures

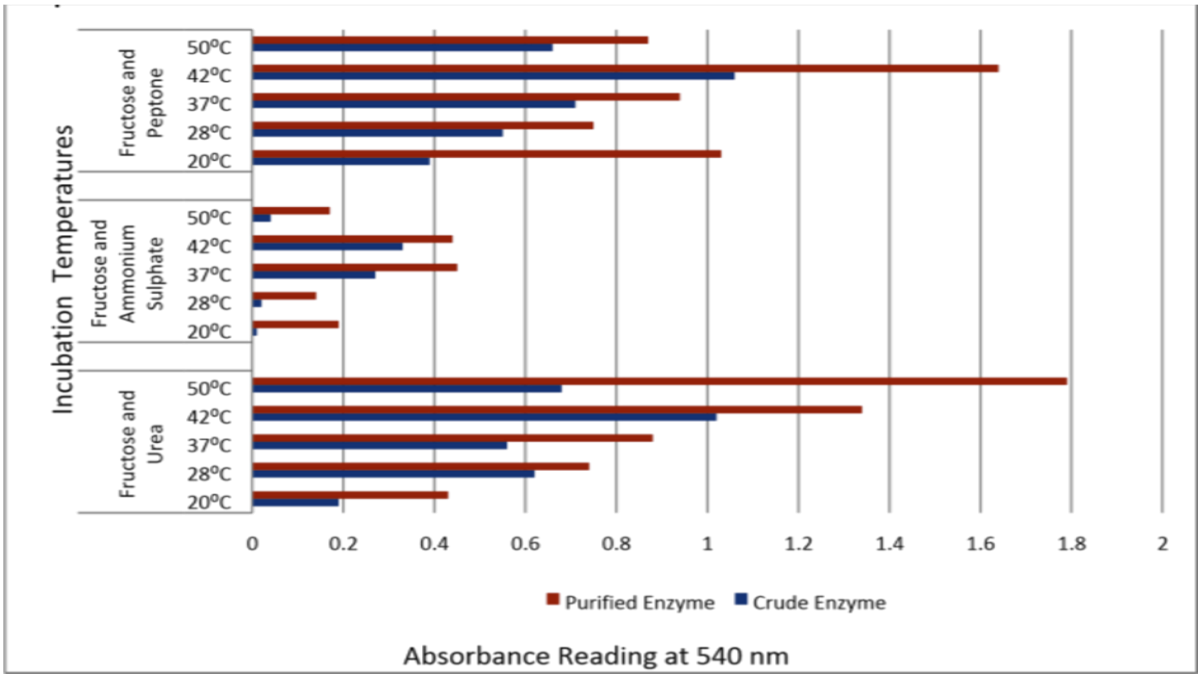

Figure 3 : Amylase activity of *Bacillus subtilis* upon incubation with Fructose and different Nitrogen Sources (Peptone, Ammonium Sulphate and Urea) under various incubation temperatur

Though fructose and glucose, both are simpler form of carbohydates, fructose yielded more effective and fruitful results. The maximum enzyme activity was observed at 42C with absorbance of 1.67 when it was used along peptone. This was unlike glucose where variable results were found. This is because, fructose is comparatively fermented faster than glucose because it skips one step in glycolysis and when it acts along with simpler form of nitrogen, it tends to give more efficient result. This agreed with the findings of Suribabu [26].

Among various pH ranges (3.6, 7.0 and 9.0), pH 7 was considered to be the best both in amylase and protease production in presence of glucose and sucrose as carbon source along with peptone as a nitrogen source at 37^{0}C which are shown in figure 6.

Most of the starch degrading bacterial strain revealed a pH range between 6.0 and 7.0 for normal growth and enzyme activity [12]. The optimum pH was found to be 7 which showed optimum enzyme activity despite the different sugars that were used.

The finding accord with the study done by Devi [13]. The nitrogen source, when it is utilized by bacteria, they break it down into simpler product and increase the pH ranges and thus increase the enzyme activity. This is evident from the facts that maximum enzyme activity was seen at pH 7 in all cases. Enzyme production was comparatively higher at pH 9 than at pH 3.6

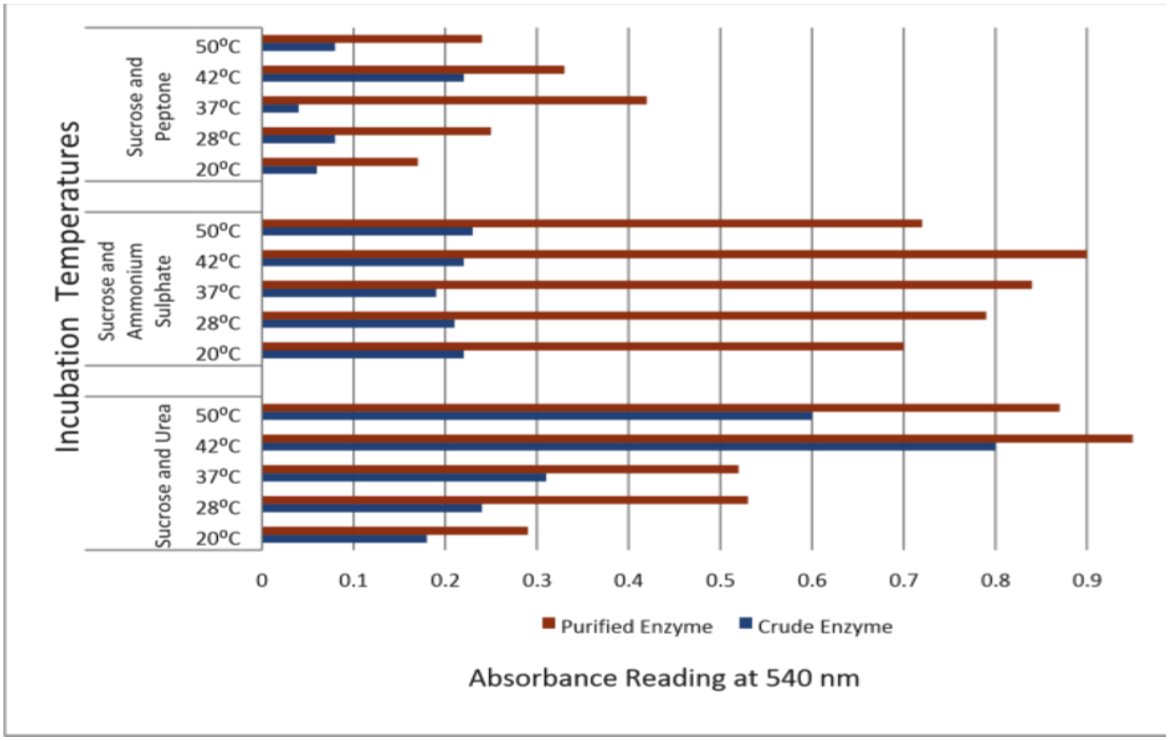

Figure 5: Amylase activity of *Bacillus subtilis* upon incubation with Sucrose and different Nitrogen Sources (Peptone, Ammonium Sulphate and Urea

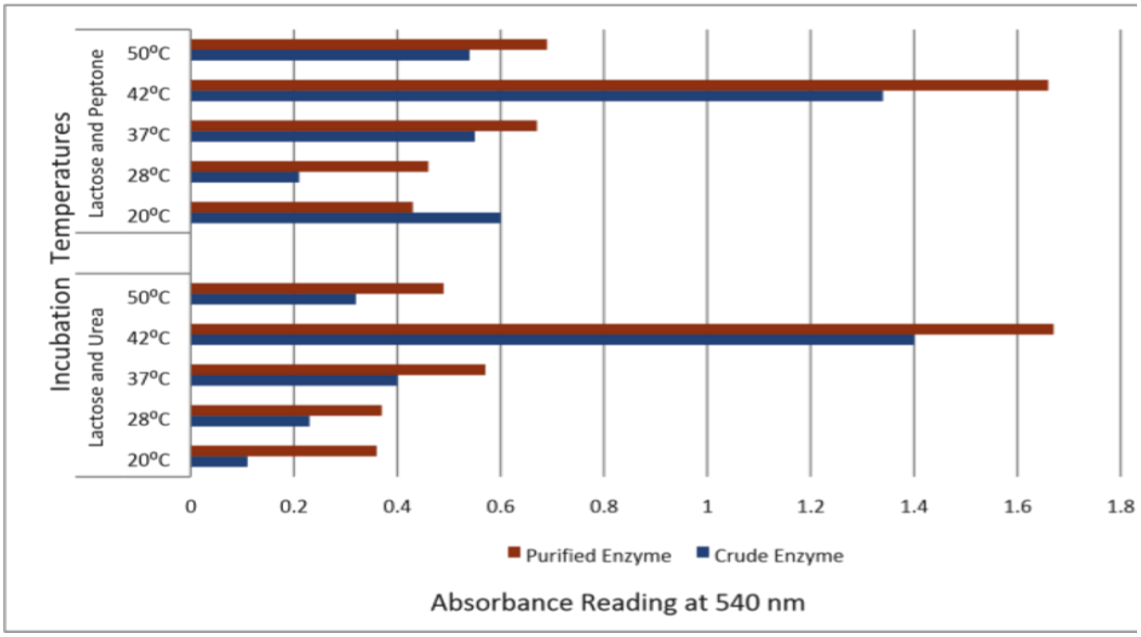

Figure 4: Amylase activity of *Bacillus subtilis* upon incubation with Lactose and different Nitrogen Sources (Peptone and Urea) under various incubation temperatures

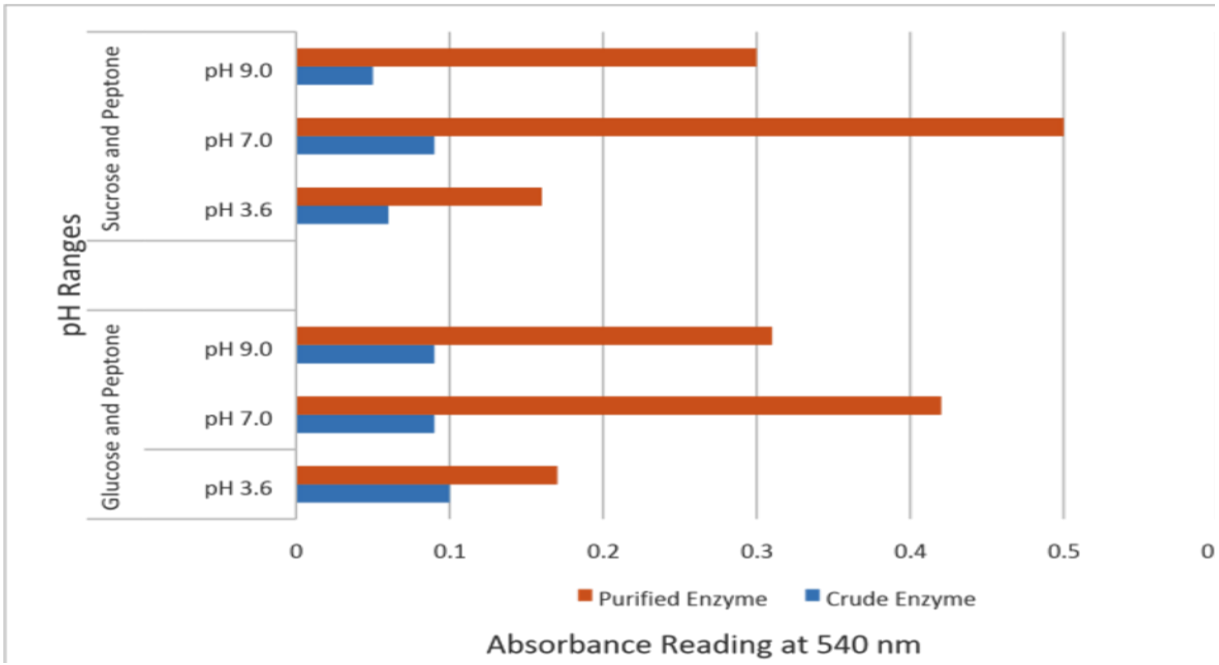

Figure 6: Amylase activity of *Bacillus subtilis* upon incubation with Carbon source (Glucose and Sucrose) and Peptone under various pH.

IV.CONCLUSION

The use of amylase in industries has been prevalent for many decades and a number of microbial sources exist for the efficient production of this enzyme, but only a few selected strains of fungi and bacteria meet the criteria for commercial production. The search for new microorganisms that can be used for amylase production is a continuous process. More recently, many authors have presented good results in developing enzyme purification techniques, which enable applications in pharmaceutical and clinical sectors which require high purity amylases. *Bacillus* species can be used for large-scale production of alkaline enzymes such as amylase and to meet present-day needs in the industrial sector. The highest amylase activity was given by *B. subtilis* coded by B3c. The highest yield organisms belonged to the nodules of leguminous plant which points to the prospect of optimizing the organisms inhabiting nodules for greater result.

V. ACKNOWLEDGMENT

We would like to express our profound gratitude and deep regards to our guide and supervisor **Mr. Anup Basnet** for his guidance, monitoring, constructive suggestions and constant support to make this project a success. We appreciate our colleagues for discussions and communications for the formulation of the ideas and layout of this project. We also wish to express my gratitude to other staff members and lab technicians for their help and support.We are very much grateful to the principal **Father. Jiju Varghese, S.J.** and the head of department of Microbiology **Mr. Sudhakar Pant** for providing us opportunity to embark on this project.

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Cite this article as :
Aastha Acharya, Amit Khanal, Mrishal Ratna Bajracharya, Aashish Timalsina, Arjun Bishwokarma, Anup Basnet, "Production, Purification and Optimisation of Amylase by Submerged Fermentation Using Bacillus subtilis", International Journal of Scientific Research in Science, Engineering and Technology (IJSRSET), ISSN : 2456-3307, Volume 6 Issue 1, pp. 265-275, January-February 2019.
Available at doi :
https://doi.org/10.32628/IJSRSET196122
Journal URL : http://ijsrset.com/IJSRSET196122