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# Purification and Production Optimization of Alpha Amylases from Bacillus Species

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**Abstract:** Amylases are the digestive enzymes belong to the family of glycoside hydrolase capable of hydrolysing starch and polysaccharides. These are the recent breakthroughs in food, feed, pulp and pharma industries. Our aim of this study is to purify amylases from *Bacillus subtilis and Bacillus megaterium* and optimize the growth conditions to achieve the cost-effective production of amylases from bacillus species. Extensive research on amylases from different sources is essential in producing enzymes in large scale and in optimizing the growth conditions and minimize the production cost to meet the increased demand of these industrially important enzymes. Amylases were produced in the current study using submerged fermentation and purified the extracellular enzymes from bacillus species using ultracentrifugation, Ammonium sulphate precipitation and gel filtration chromatography. There was no effect on amylase production from bacillus species when the initial substrate concentration is increased from 1% to 5%. Increase in inoculum size from 1% to 5% and the addition of yeast extract to broth had significantly increased the amylase production. The study observed significantly higher levels of amylase production using rice starch when compared with wheat starch also higher levels of amylase production observed with *B. megaterium* when compared with *B. subtilis*. The study concludes 1% rice starch and 5% *B. megaterium* inoculum can be used for cost effective production of amylases. In the current study, cost effective amylase production was evaluated and able to optimize the yield of extracellular amylases from *Bacillus megaterium* to a concentration level of 120 IU/ml of fermentation broth.

**Keywords:** Amylases, Gel Filtration Chromatography, Bacillus Species and Saccharification

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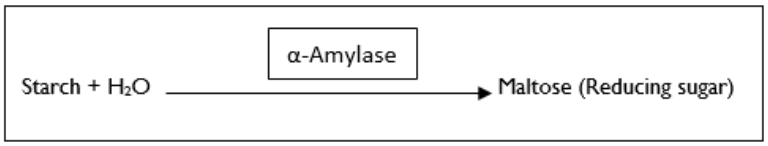
## 1. INTRODUCTION

Amylases are hydrolytic enzymes which catalyses the breakdown of glycosidic bonds in starch and other polysaccharides. There are three major classes of amylases i.e., α -amylases, β -amylases, γ -amylases.[1,2] All these three amylases act on different sites of starch molecule. α -amylases are extracellular enzymes produced by wide spectrum of organisms including humans, animals, plants and microbes. α -amylases are endo acting enzymes which catalyses the hydrolysis of α -1,4 bonds in polysaccharides leads to the production of maltose, glucose and dextrin's.[3,4] However, α -amylases cannot hydrolyse α -1,6 bonds in starch molecule. β -amylases are exo acting enzymes which catalyses the breakdown of α -1,4 bonds in starch and other polysaccharides leading to the production of maltose molecules.[5,6] γ -amylases are exo acting enzymes which can hydrolyse both α -1,4 and α -1,6 bonds in polysaccharides leading to the production of glucose molecules.[7,8] Amylases are one of the most widely used enzymes in the food and pharmaceutical industry[9,10]. Amylases are majorly used in detergent, textile, baking, starch, paper pulp and animal feed industries[11,12]. Large scale production of these enzymes with lower cost of production is required to meet the industrial demand. Amylase produced from microorganisms are widely used in industry due to their large productivity, low cost, and chemical stability.[13-15] Amylases from several bacterial species were purified, produced in bulk scale and used industrially for hydrolysis of starch.[16,17] Bacillus species is well known as workhorses for producing several industrially relevant enzymes including extracellular amylases. The purpose of this study is to purify amylases from *Bacillus subtilis and Bacillus megaterium* and optimization of the growth conditions to achieve the cost-effective production of amylases from bacillus species.




## 2. MATERIALS AND METHODS

### *2.1 Media and Growth Condition*

*Bacillus subtilis* and *Bacillus megaterium* culture was obtained from Vishwamitra bio agro pvt ltd. Guntur. 1 ml of culture of Bacillus species was inoculated to 100 mL LB broth and incubated at 37°C for 24 hr.

### *2.2 Amylase Production*

Fermentation media was prepared with 1-10% (w/v) initial substrate concentrations of rice and wheat powder with 1% yeast extract and autoclaved at 15 psi for twenty minutes. Inoculum size of 1-5% (v/v) grown on LB broth was then seeded into the fermentation media. Five-liter flasks were used to carry out submerged fermentation and incubated at 37°C for 78 hours. Broth was then centrifuged at 6000rpm for 10 min and supernatant was collected [18].

### *2.3 Steps Involved in Amylase Purification*

Ammonium sulphate precipitation: Supernatant was then subjected to ammonium sulphate precipitation with a saturation of 70% salt concentration. The solution after precipitation was centrifuged at 6000rpm for 10 min. Pellet was then resuspended in 5 ml of 10mM potassium phosphate buffer and dialysis was performed to eliminate salts. Dialysis: Dialysed crude enzyme extract was concentrated using sucrose powder. Gel filtration chromatography: Crude protein sample was then fractionated using a gel filtration column. Sephadex G-100 matrix soaked in distilled water overnight and column (50 cm X 2 cm) was packed with a bed volume of 50 ml. The column was washed with distilled water, equilibrated with 10mM Potassium phosphate buffer and the sample was loaded on to the column. Flow rate was fixed to 1ml/minute and the fraction size was restricted to 1ml. Absorbance of these fractions were measured at 280 nm and the protein concentrations were estimated. Chromatogram was plotted against the absorbance values with the fraction number. Purified amylase enzyme was run on 12% denaturing Page along with protein molecular weight ladder [19]. (Figure 1).

### *2.4 Amylase Activity Assay*

Amylase reduces polysaccharide starch to its subsequent sugar maltose. Standard graph of maltose was prepared using 0–1 mg/ml maltose concentrations. Amylase activity was tested incubating 100µ L purified amylase enzyme with 1ml of 10 mg/ml starch solution. 1.5mL of 3,5-Dinitrosalicylic acid (DNS) reagent was then added and incubated in hot water bath at 50°C for 5 min. Optical density was measured at 540 nm. Amylase activity was measured in terms of amount of maltose released during the enzymatic reaction. A standard graph of dextrose of concentration ranging from 0-1000 µ g/ml was plotted and used for estimating the maltose released during the reaction (Figure 2). One unit of amylase activity was estimated as the amount of enzyme required to release 1µmol of reducing sugar i.e., maltose per minute under the assay conditions described [10].

### *2.5 Effect of Temperature On Amylase Activity*

Purified amylase enzyme activity was examined after exposing enzyme to different temperatures ranging from $25^0$ C to $80^0$ C for 10 minutes in hot water bath.

### *2.6 Effect of* pH *On Amylase Activity*

To determine the optimal pH level purified amylase enzyme was incubated at different pH solution ranging from pH 4-9 for 10 minutes and then performed amylase activity assay. The following buffers were used to estimate the effect of pH on enzyme activity. Glycine-HCL buffer (pH-3&4), Acetate buffer (pH-5), Succinate buffer (pH-6), Phosphate buffer (pH-7), Tris HCL buffer (pH-8), Glycine NaoH buffer (pH-9)[20]

### *2.7 Effect of Solvent and Chemicals On Amylase Activity*

To investige the effects of solvents on amylase enzyme purified amylase were incubated with Methanol, Isopropanol,

Acetonitrile for 10 minutes and then tested amylase activity. Similarly, the effect of different chemicals on amylase enzyme was investigated by incubating the purified enzyme for 10 minutes with EDTA, PMSF, DTT, SDS and Tween20 and then examined the amylase activity under assay conditions [21].

### *2.8 Determination of Kinetic Parameters*

Amylase enzyme kinetics were performed using different substrate concentration of soluble starch ranging from 0.5 mg/ml to 5 mg/ml. One unit of enzyme activity was equalled to release of 1 micromole of reducing sugars release during enzyme activity. Kinetic parameters Km and Vmax was measured using double reciprocal plot i.e., Lineweaver burk plot [22].

### *2.9 Statistical Analyses*

Results are reported as means ± SEM. Results of all analyses were significantly different at $P < 0.05$.

## 3. RESULTS AND DISCUSSIONS

### *3.1 Production of Beta Amylase Using Submerged Fermentation*

*Bacillus subtilis and Bacillus megaterium* inoculum sizes ranging from 1-5% was used to optimize the production of amylases. Similarly different initial substrate concentration was maintained with rice starch (1-10% concentration), wheat starch (1-10% concentration) to optimize the amylase production. Submerged fermentation was carried out with above mentioned variations in initial substrate concentration with and without yeast extract as nitrogen source in five-litre flask for 72 hours. Amylase released into the media were separated by centrifuging the broth at 10,000 rpm. Amylases available in the supernatant was then concentrated using ammonium sulphate precipitation.

### *3.2 Amylase Purification Using Gel Filtration Chromatography*

Crude extract of amylase was fractionated using gel filtration column chromatography. Chromatogram obtained after purification was represented in Figure 1. Three protein peaks (represented as P I, P I and P III) were obtained after purification. Proteins present in the peak fraction were selected and tested for amylase activity. Proteins present in peak II reduced soluble starch to maltose. Amylase activity of the purified fractions were calculate using maltose standard curve (Figure 2).

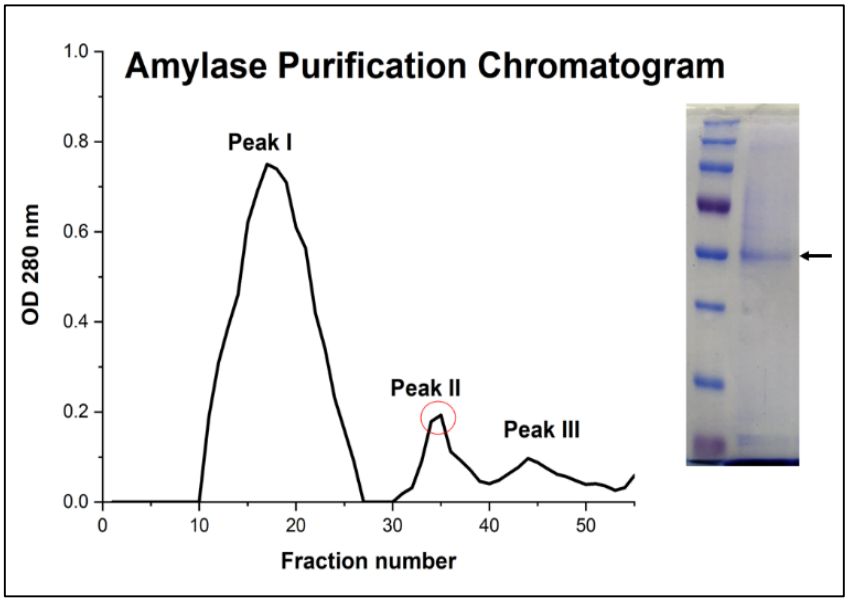



**Fig 1: Purification of Amylases using gel filtration column chromatography. Inset: 12% SDS PAGE with purified amylase enzyme indicated with arrow.**

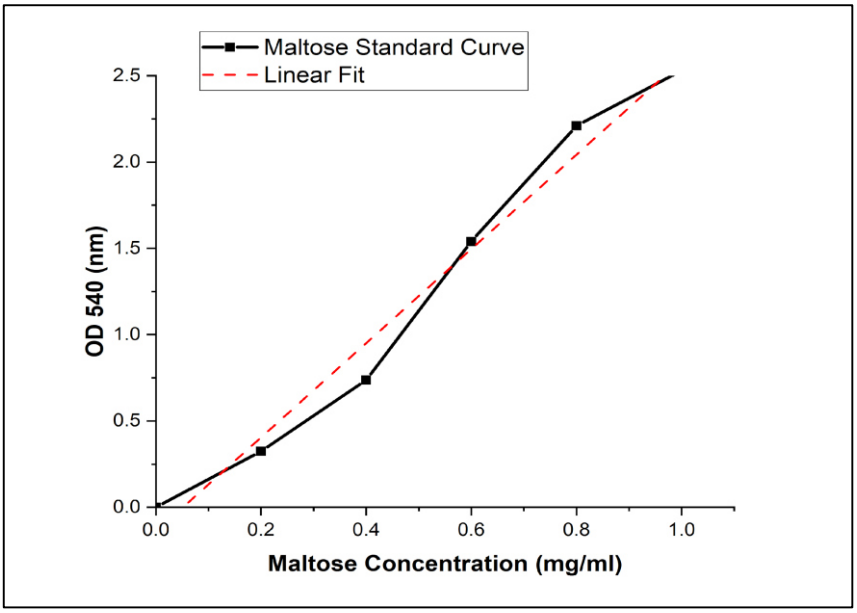



**Fig 2: Maltose standard curve**

### 3.3 *Effect of Initial Substrate Concentration On Amylase Production*

Amylase production from Bacillus species was optimized by varying the growth conditions and parameters. There was no effect observed with initial carbon source concentrations on amylase production. Increase in the substate concentrations of rice starch and wheat starch from 1% to 5% did not show any effect on amylase production. Significantly higher amounts of amylases were produced with rice starch when compared with wheat starch at the same concentration levels (Figure 3).

### 3.4 *Effect of Inoculum Size On Amylase Production*

Increase in inoculum size of *Bacillus subtilis and Bacillus megaterium from 1% to 5%* showed increase in the amylase production however further increase in inoculum size from 5% to 10% did not show any significant changes in amylase production (Figure 4).

### 3.5 *Effect of Nitrogen Source (Yeast Extract) On Amylase Production*

Addition of nitrogen sources (yeast extract) to the fermentation broth showed substantial effect of amylase production. We observed substantially higher levels of amylase production in *Bacillus megaterium* when compared with *Bacillus subtilis* at similar initial substrate concentration (Figure 5).

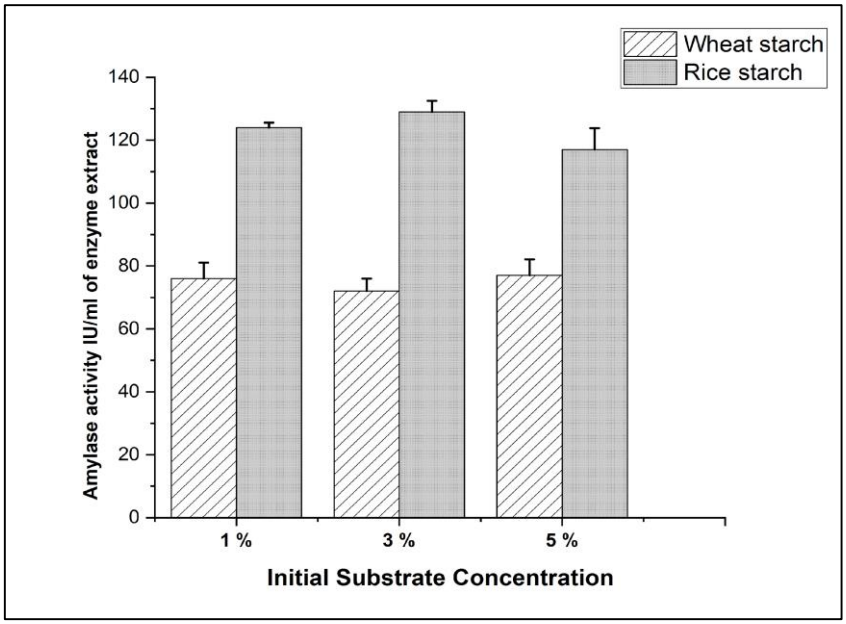



**Fig 3: Effect of initial substrate concentration on amylase production. Values shown as mean±SEM, (n=5). All the values obtained were found significant (P<0.05)**

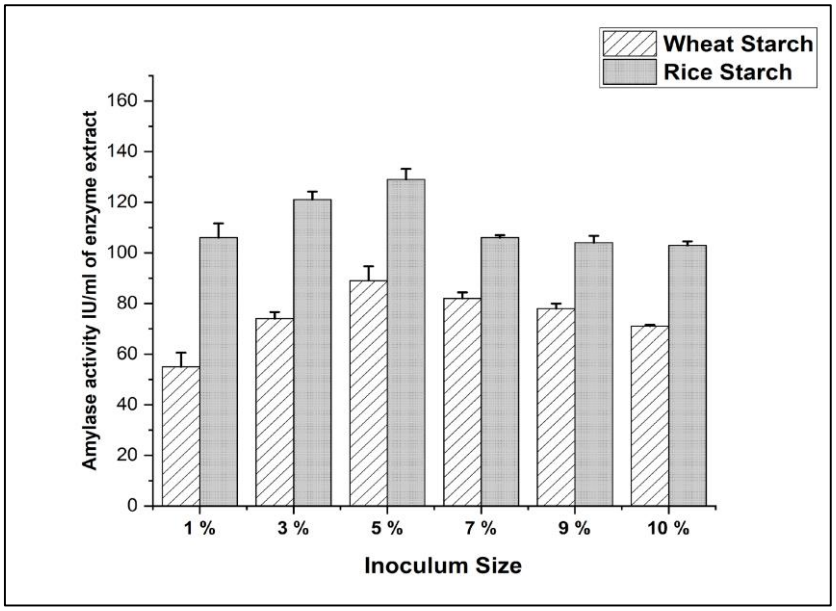



**Fig 4: Effect of inoculum size on amylase production. Values shown as mean±SEM, (n=5). All the values obtained were found significant (P<0.05)**

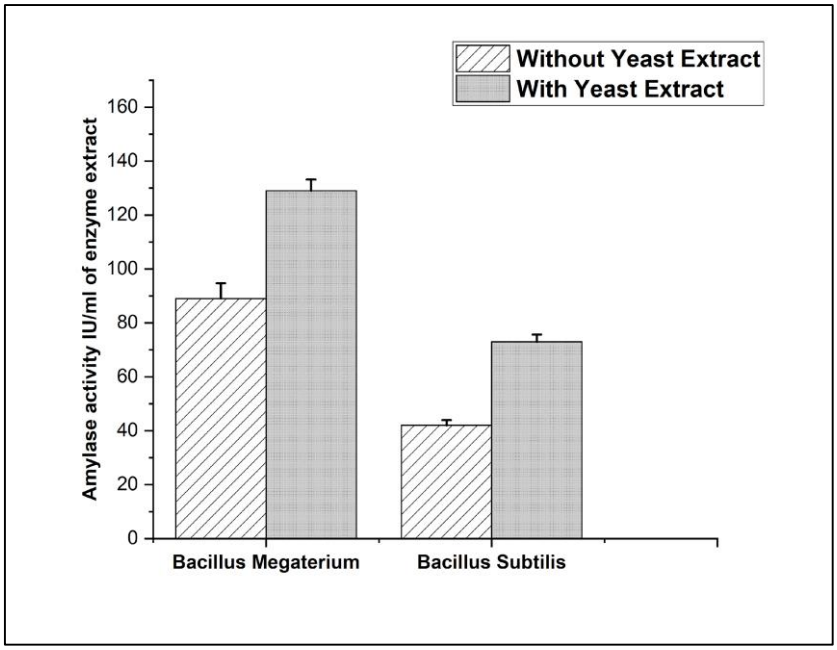



**Fig 5: Effect of nitrogen source (Yeast extract) on amylase production. Values shown as mean±SEM, (n=5). All the values obtained were found significant (P<0.05)**

The study observed significantly higher levels of amylase production using rice starch when compared with wheat starch also higher levels of amylase production observed with *B. megaterium* when compared with *B. subtilis* [23-25]. The study observed 1% rice starch and 5% *B. megaterium* inoculum and 1% yeast extract can be used for cost effective production of amylases[26,27]. In the current study cost effective amylase production was perfromed and optimized the yield of extracellular amylases from *Bacillus megaterium* to a concentration level of 120 IU/ml of fermentation broth. Although the amylase production levels from the current study are not very high when compared to other studies we were able to achieve these concentrations with significantly lower cost of production [28-30].

### *3.6 Effect of Temperature On Amylase Activity*

The study observed a profound effect of temperature on amylase activity. With increase in temperature from 25$^0$C to 50$^0$C there was a rapid increase in amylase activity. Maximum amylase activity was observed at 50$^0$C. Drastic reduction in amylase activity was observed exposing the enzyme beyond 70$^0$C temperature. (Figure 6)

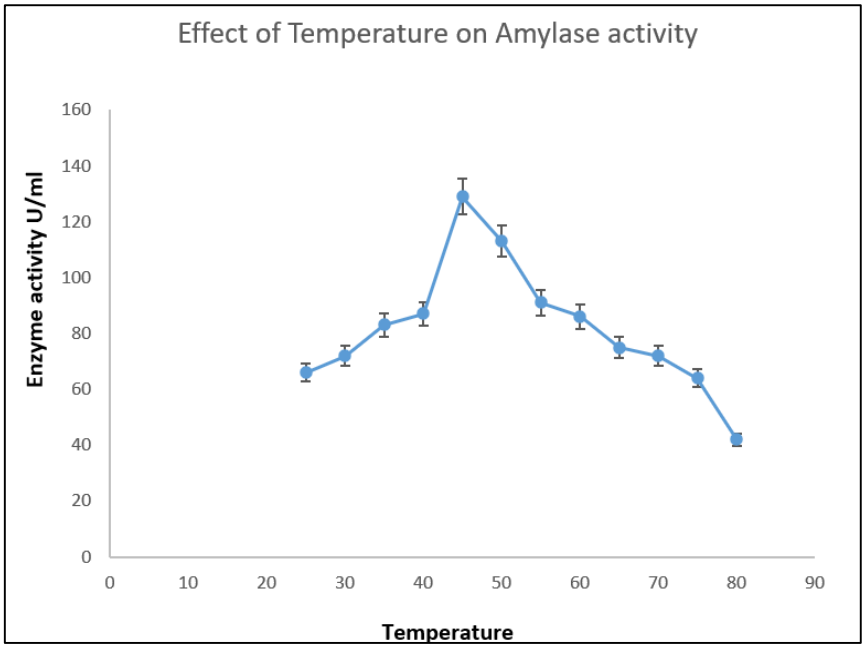



**Fig 6: Effect of temperature on amylase activity. Values shown as mean±SEM, (n=3). All the values obtained were found significant (P<0.05)**

### *3.7 Effect of pH On Amylase Activity*

Exposing purified amylase to varied pH solution showed drastic effects on amylase activity. The study observed maximum activity of purified enzyme at pH 7. (Figure 7)

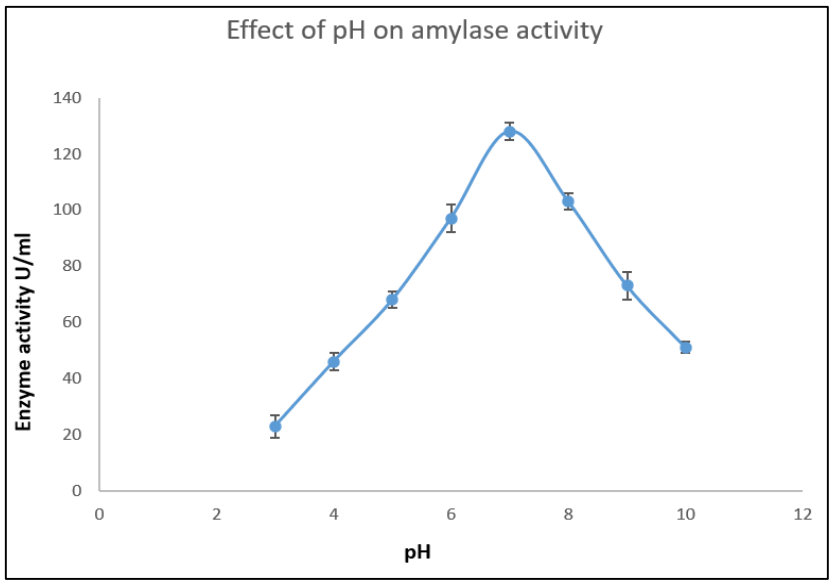



**Fig 7: Effect of temperature on amylase activity. Values shown as mean±SEM, (n=3). All the values obtained were found significant (P<0.05)**

### *3.8 Effect of Various Chemical On Amylase Activity*

The study observed a drastic reduction in amylase activity by 90% treating purified enzyme with Methanol, isopropanol, and acetonitrile. Similar kind of effect was observed treating amylase enyme with detergents like SDS and tween20 however the extent of reduction in amylase activity was lower i.e., 60% when compared with organic solvents. No effect on amylase activity was observed treating purified enzyme with 10 mM EDTA and 2 mM PMSF solutions.

### *3.9 Kinetic Parameters*

Purified amylase kinetic studies were performed using starch as substrate. Purified amylase enzyme showed typical michelis menten enzyme rate kinetics. $K_m$ and $V_{max}$ calculate from lineweaver burk plot was found to be 0.37 mg/ml and 104 U/mg respectively. (Figure 8)

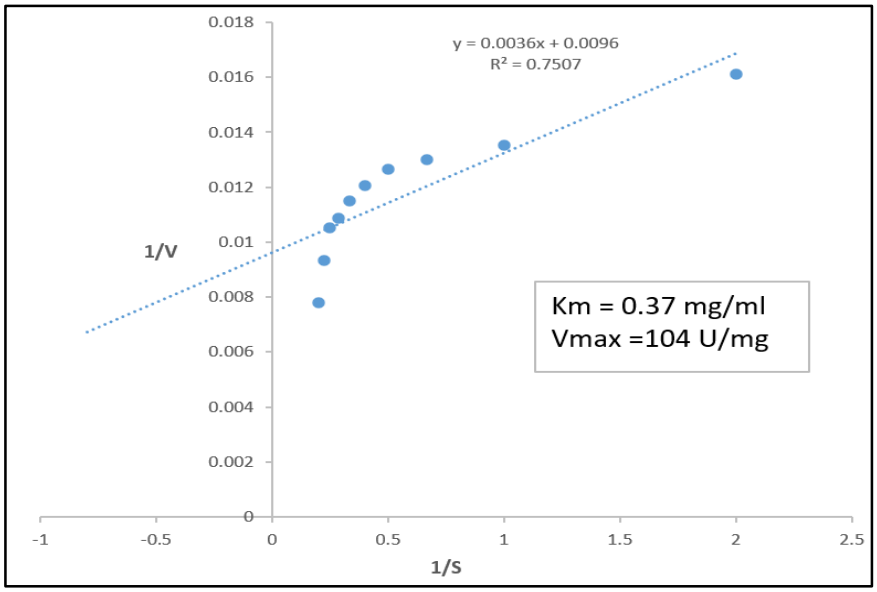



**Fig 8: Lineweaver burk plot to determine $K_m$ and $V_{max}$ of purified amylase enzyme**

## 4. CONCLUSIONS

Amylases are one of the important hydrolytic enzymes used in saccharification by several industries. To meet the industrial demand of amylases a cost-effective optimized production is essential. The current study focused on purification and production of extracellular amylases from *Bacillus subtilis* and *Bacillus megaterium* using submerged fermentation. Enzyme stability studies and enzyme kinetic studies were performed with purified amylases. The Km and Vmax of purified amylase enzyme was found to 0.37 mg/ml and 104 U/mg respectively. Optimization studies were performed with two different carbon sources i.e., rice starch and wheat starch at 1%,3%,5% initial substrate concentrations. Significantly higher amounts of amylases were produced with rice starch when compared with wheat starch at the same concentration levels. Increase in inoculum size from 1% to 5% has increased the production of extracellular amylases, however further increase in inoculum size beyond 5% there is no significant change in the production levels of amylases. In the current study production of extracellular amylase from *Bacillus megaterium* were optimized up to a concentration of 120 IU/ml of fermentation broth. However, optimization of micronutrients and other essential elements is required to further enhance the production levels of extracellular amylases.

## 5. ACKNOWLEDGEMENT

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## 6. AUTHORS CONTRIBUTION STATEMENT

Chandrasekhar Chanda conceived the idea and supervised the finding of this work. Pradyumna Iragavarapu, Aayush Sasi Kumar Nair, Ravi Kumar Reddy Kasireddy, Bala Naga Ganesh Doddipatla analyzed the data and necessary inputs were given towards the designing of the manuscript. Ranganadha Reddy Aluru involved in manuscript writing and statistical analysis. Chandrasekhar Chanda involved in manuscript corrections. All authors discussed the methodology and results and contributed to the final manuscript.

## 7. CONFLICT OF INTEREST

Conflict of interest declared none.

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