WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.453
Volume 14, Issue 5, 1376-1396. Research Article ISSN 2277–7105

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF AMYLASE USING MICROROGANISM

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Article Received on
16 January 2025,

Revised on 06 Feb. 2025,
Accepted on 26 Feb. 2025

DOI: 10.20959/wjpr20255-35804

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ABSTRACT

In this study we investigated the "Production, Purification, and characterization of Amylase using Microorganism, through the utilization of Starch as a Substrate and Microbial Strain' "Bacillus Subtilis". The production of amylase and exposure of the producers to various parameter for the maximum activity of enzyme. Enzyme was produced by the submerged fermentation method. Fermentation experiment conducted at a Controlled temperature range of 35^{o}C to 38^{o}C and orbital shaker speed is 120 rpm. After Fermentation Amylase Activity in the crude enzyme was determined by estimation of the reducing sugar liberated by the action of amylase on soluble starch. Reducing sugar were estimated by 3, 5 Di nitro salicylic acid DNS Method. The production of amylase using microorganism by using factorial design method (2^{3}factorial) when factor affecting production like concentration of starch, temperature and ph. were consider. These factors were conducted in 2 levels resulting to 8 Batches the significance of the media was estimated by ANOVA statistics method and the precision of the data was estimated by fit statistics, and it was found that the 48 hours incubation Fermented media 2.5% starch, 38^{o}C temperature and PH-8 showed maximum activity, (Activity: 495.21Mu). Check the maximum activity of crude enzyme after this media as processing to ammonium sulphate precipitation test. After the precipitation of standard DNS assay in media 2.5% starch, PH-8 and temperature 38^{o}C, it was found that at 60% AS saturation showed maximum activity of enzyme. (Activity: 260.7 Mu). this research provides valuable insights into the production of amylase form starch using Bacillus subtilis as the microbial strain. The formulas used to determine amylase activity are also emphasized in this Assay. Every data item in this document is explained with an

illustration. This technical study might be essential for the work of the amylase enzyme. The optimization of process parameters like pH, temperature and % of starch and statistical analysis contribute to better understanding of amylase enzyme production and offer potential application in various industries.

KEYWORDS: Amylase, fermentation, Starch, DNS, Bacillus subtilis.

INTRODUCTION

Amylase: An amylase is an enzyme that catalyses the hydrolysis of starch into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Amylase is produced by a variety of living organisms, ranging from bacteria to plants & humans. Bacteria & fungi secrete amylases to the outside and inside of their cell to carry out extracellular and intracellular enzyme.

Types of amylases

α- Amylase: Found in plants (Adequately), fungi (Ascomycetes and Basidiomycetes) and bacteria (Bacillus). α- Amylase is used in ethanol production to break starches in grains into fermentable sugars. α-amylase called "Term amyl".

β- Amylase: Beta-amylase is found in bacteria, fungi, and plants; bacteria and cereal sources are the most heat stable. During the ripening of fruit, β-amylase breaks starch into maltose, resulting in the sweet flavour of ripe fruit. The optimum pH for β-amylase is 4.0–5.

γ- Amylase: γ- Amylase cleaves α (1-6) glycosidic linkages, in addition to cleaving the last α (1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose.

Enzyme application in non-food material

- Leather industry.
- Paper industry.
- Animal feed industry**.**

Enzyme application in food material

- Starch processing industry.
- Baking industry.
- Dairy industry.

- Animal and vegetable food.
- Juice and wine industry.

Fermentation: Fermentation is a metabolic process that converts sugar to acids, gases or alcohol. Fermentation is also used more broadly to refer to the bulk growth of Microorganisms on a growth medium. In microorganisms, fermentation is the primary means of producing adenosine triphosphate (ATP) by the degradation of organic nutrients anaerobically.

Industrial fermentation: Industrial fermentation is the intentional use of fermentation by microorganisms such as Bacteria and fungi to make products useful to humans. Fermentation systems may be liquid, also known as submerged or solid state, also known as surface.

Submerged fermentation (SmF)

Submerged fermentation is method of manufacturing biomolecules by submerging enzymes and reactive compounds in liquid like alcohol, oil, or nutrient broth. It is commonly used in industrial manufacturing for its high moisture content, making it suitable for bacteria that require moisture for growth. The main application of submerged fermentation is the extraction of secondary metabolites in liquid form for use.

Principal of submerged fermentation: Submerged fermentation involves microorganisms growing in liquid environments, using molasses and broth as substrates. The high rate of substrate utilization leads to significant depletion of nutrients. A starter culture, such as fungi or bacteria, is inoculated into nutrient-rich broth. The process requires high oxygen levels, as enzymes and products are produced when microorganisms react with broth and nutrients. Bioactive compounds are secreted into the reactant broth/media.

The broth's composition is such that the proportion of broth to nutrients is optimal for the development of antibiotics, industrial enzymes, and other compound.

Solid State Fermentation (SSF): Solid state fermentation (SSF) is a biomolecule manufacturing process used in various industries, including food, pharmaceuticals, cosmetics, fuel, and textiles. It involves microorganisms growing on solid support, producing metabolites like cellulose, hemicellulose, pectin, and proteins. SSF is suitable for producing enzymatic complexes, which are used in sectors requiring digestibility, solubility or viscosity. Origins date back to ancient Egypt and include western foods, Asian foods and beverages.

Advantages of submerged fermentation and solid- state fermentation

1. Increased volumetric output.
2. Typically, less complicated and requiring less energy.
3. Could be simpler to satisfy aeration requirements.
4. Identical to some bacteria and fungi's native habitat.
5. Simpler downstream processing.

MATERIAL AND METHODS

Materials

Table no. 1: List of materials.

Sr. No	Ingredient	Company Name
1	Bacillus Subtilis MTCC- 441	CSIR INSTITUTE.
2	Starch	Modern Industries, Nashik
3	Nutrient Broth	HI MEDIA
4	3, 5, Dinitro salicylic Acid	Research-lab fine chem industries, Mumbai.
5	Maltose	Sunstar Biopolymers ltd, Gujrat, India
6	Sodium potassium tartrate hydrate tetra	Sisco Research Laboratory. Navi Mumbai
7	2 (N). Sodium Hydroxide	Bean Town Chemical, Mumbai.
8	Iodine	Eskay Iodine Pvt. Ltd. (Factory), Gujarat
9	Ammonium Sulphate	Merck Specialties Private Limited, Worli, Mumbai.
10	Sodium Phosphate (Dibasic)	Complete lab solution, Malegaon, Nashik.
11	Sodium Phosphate (Monobasic)	Research-lab fine chem industries, Mumbai
12	Phosphate Buffer	Merck life science private limited, Mumbai.

Methods (Detailed methodology) select the microorganism

A Suitable microbial strain (e, g. Bacillus Subtilis). Bacillus subtilis is an aerobic, Grampositive soil bacterium, which has been widely used for the production of Enzymes. B. subtilis has become an ideal expression host for the production of various industrial enzymes.

Preparation of inoculum

A Suitable Microbial Strain Bacillus Subtilis MTCC NO- 441 obtained from, CSIR-INSTITUTE OF MICROBIALTECHNOLOGY, Sector 39-A, Chandigarh 160036 India. Was selected which is obtained from and a pure culture was obtained. The culture was propagated

on a suitable growth medium to increase the Biomass. After the Biomass had grown, it was harvested, and an inoculum was prepared by suspending the cells in a sterile medium.

Methods

1. Prepare 100 ml of nutrient broth. 1.3 g in 100 ml of water.
2. Clean the 250-ml Erlenmeyer flasks. Autoclave it along with the flasks.
3. After autoclaving the media, cool it to room temperature. And mark the flask.
4. After cooling the media.
5. Take 1-2 loops of Bacillus subtilis added to nutrient broth inside the laminar air flow.
6. Incubate the media at 37 °C for 24 hours.

5.2.4 Precautions

1. Always autoclave media.
2. Use the mask, hand gloves.

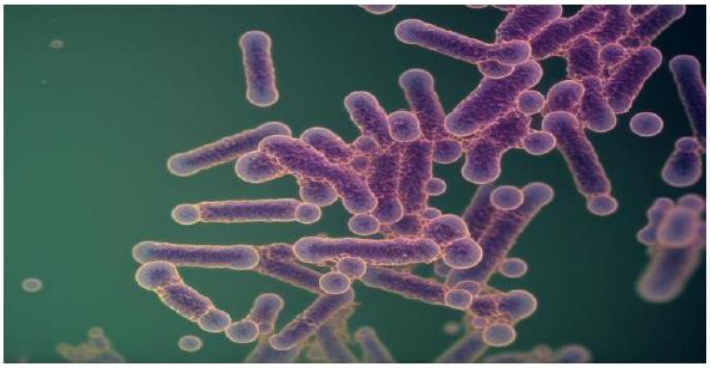


Fig. no. 1: (Bacillus subtilis bacterial microbes.)

Media preparation

The necessary components were dissolved in water to prepare the medium. The medium was sterilized. The ph. of the medium was adjusted to the optimal range for the microorganism used.

Starch used as a substrate

Starch is known as a carbon source and the main substrate of amylase, which is comprised of two parts, amylose (25–30%) and amylopectin (70–75%)

Composition of media including different % of starch, different temperature and different ph

Methods

1. To prepare eight fermentation media with different pH, temperature, and percentages of starch.
2. Take eight 250-ml Erlenmeyer flasks and clean them.
3. Add 1.3 g of nutrient broth to 100 ml of water in each flask and autoclave.
4. After the autoclave, cool the media to room temperature.
5. Then add % wise in each flask (e.g., 1.5 % starch = 1.5 g of starch, 2.5 % of starch = 2.5 g of starch) and mix well.
6. Then add 5 ml of Bacillus subtilis growth culture to each flask and mix well.
7. Adjust the PH at the PH Meter.
8. Then this medium is processed for fermentation for 48 hours of incubation for amylase production.

Fermentation setup

The sterile substrate and media were inoculating with 5ml of inoculum solution of bacillus subtilis in each flask. After inoculating the substrate and media, the content present in 250 ml Erlenmeyer flasks was mixed properly and incubated at 35^0c and 38^0c Temperature at 48 hours 120 rpm in incubator. The temperature and speed of agitation were maintained at optimal levels to promote the growth and metabolism of the microbial strain.[70] Finally, percentage of amylase produced through the submerged fermentation method was calculated.

Biochemical test

The ability of microorganism to utilize certain biomolecules, resulting in useful organic compounds for themselves from the basis of various biochemical tests. Biochemical tests are of different types, where the identification or distinction between different microorganisms made on various bases.[72]

Iodine test

Iodine test is a test of detecting the presence of starch. The sample turns blue black color when a few drops of potassium iodide solution are placed on the sample.

Methods

1. Mix well and properly, and the results Make a 1% starch solution with 1 gram of starch weighed and transferred to 100 ml of water.
2. Make an iodine solution by adding 10 g of potassium iodide to 30 ml of water, then 5 g of iodine crystals slowly, and then making up the volume with 100 ml of water.
3. Then take one test tube and add 2 ml of starch solution, 2 ml of sample, and 1 ml of iodine solution.
4. The blue-black color shows the amylase is not present, but this solution is clear after adding the 1 ml of iodine solution.
5. The blue-black color is not shown for this test. So, amylase is present in my sample, which conforms.

Yield comparison with standard

Maltose standard curve

Constructing a standard curve / graph for maltose helps us to estimate concentration of reducing sugars present in an unknown sample and for determining the activity of amylase enzyme in for the coming experiment.

Construction of maltose standard curve by dns method

Preparation of 3, 5-dinitrosalicylic acid [DNS] - About 1g of DNS is dissolved in 50ml of distilled water. To this solution add about 30g of sodium potassium tartrate tetra hydrate in small lots, the solution turns milky yellow in color. Then add 20ml of 2N NaOH, which turns the solution to transparent orange yellow color. The final volume is made to 100 ml with the distilled water. This solution is stored in an amber colored bottle.[77]

Methods

1) Maltose working solution: 0.18 g of maltose is weighed and made up to 100 ml with distilled water.
2) Pipette standard maltose solution in the range of 0.2, 0.4, 0.6, 0.8, and 1 ml into 5 separate test tubes.
3) A test tube containing a blank solution is also prepared.
4) Using distilled water, bring the volume up to 2ml in each test tube, including the test tube containing the blank solution.
5) Add 1 mL of DNS reagent to each tube and cover the test tubes with aluminum foil.
6) Heat the contents in the test tubes in a boiling water bath for 5 minutes.

7) Cool the test tubes to room temperature.
8) Then add 9ml distilled water to each test tube and mix well.
9) Take 1ml from each test tube into different cuvettes and place each cuvette in a colorimeter and record the intensity of dark orange red color at 540 nm as the 'absorbance' or OD.

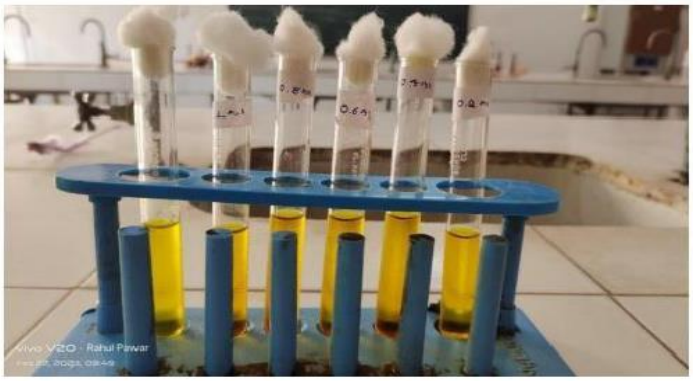


Fig. no. 2: Maltose solution in the range of 0.2, 0.4, 0.6, 0.8 and 1 ml)

Amylase assay

This intensity change in color is measured using a MICROPLATE READER as the absorbance at 540nm wavelength. Estimation of amylase activity was determined as using 3, 5- dinitro salicylic acid (DNS) to estimate the reduced sugar formed due to enzymatic hydrolysis of the soluble starch. Suitable volume of sample withdrawn from the fermented broth was centrifuged in a refrigerated centrifuge at 10,000 rpm at 40 C. To 0.5 ml of the supernatant containing the crude enzyme, 0.5 ml of 1% (w/v) soluble starch in citrate buffer (pH 7.0) was added and incubated at 37C for 30 min. The reaction was stopped by adding DNS (0.5 ml). The mixture was then heated in a boiling water bath for 5 min and cooled down.

Enzyme Activity Formula

$$\text{Unit/ ml of enzyme extract} = \frac{mg\ of\ maltose\ released}{Volume\ of\ enzyme\ use} \times 30$$

Methods

1) Perform the amylase assay using 3, 5-dinitrosalicylic acid (DNS).
2) Clean the 4 test tubes, add the 1st test tube to 0.5 ml of broth media, the 2nd test tube to 0.5 ml of DNS reagent, and the 3rd test tube as a blank (0.5 ml of water + 0.5 ml of DNS).

3) In the 4th test tube, add the (0.5 ml broth media + 0.5 ml DNS).
4) Mix well and incubate at 25o C for 3 minutes.
5) After the incubation, check the amylase activity using the 96-well plate method.
6) Take a 96-well plate and add the 0.2 microliter solution to each well.
7) Then add the 0.2 ml of blank solution.
8) Take the absorbance at 540 nm in the microplate reader.
9) To check the crude enzyme activity.

Ammonium sulphate precipitation

Ammonium sulfate precipitation can be used to separate proteins by altering their solubility in the presence of a high salt concentration.

Methods

1) Take five test tubes and add 5 ml of media to each. And mark the test tubes at 20%, 30%, 40%, 50%, and 60%.
2) Then add the solid ammonium sulphate in percentages to each test tube.
3) 20% =0.53g, 30% = 0.82g, 40% = 1.13g, 50% = 1.45g, 60% = 1.80g.
4) Then mix well properly, incubate for 30 minutes, 0 0C.
5) Centrifuge the whole test tube at 10,000 rpm for 10 minutes.
6) After centrifuging, remove the AS solution, add the phosphate buffer (pH-7), and check the amylase activity.

RESULTS

Maltose calibration curve

0.18 g maltose is dissolved in 100 ml DI water as stock solution.

Table no. 2: Maltose calibration curve.

Maltose (ml)	DI Water (ml)	DNS (ml)	DI Water (ml)	Absorbance Maltose(540nm)
0.2	1.8	1	9	0.081
0.4	1.6	1	9	0.15
0.6	1.4	1	9	0.185
0.8	1.2	1	9	0.23
1	0	1	9	0.273

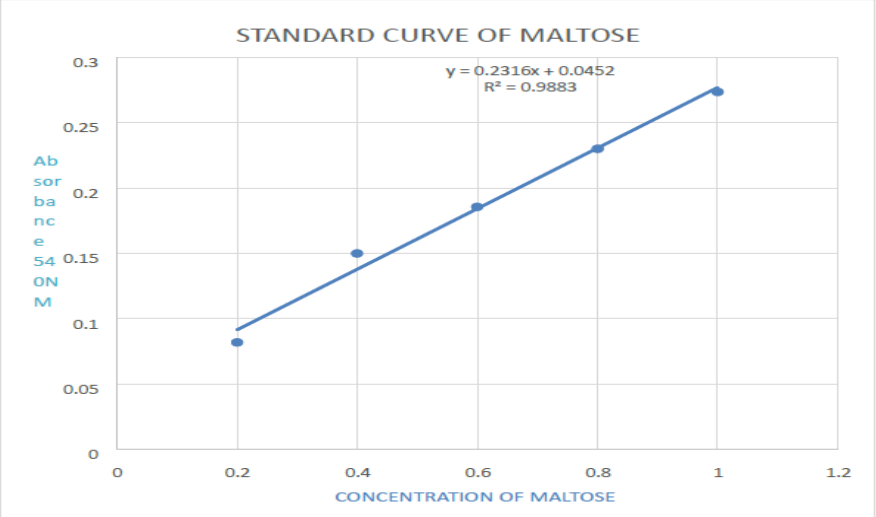


Fig. no. 3: Calibration curve of maltose.

Assay of amylase

Procedure

1. Use DNS method described by the reaction mixture is composed of cell-free supernatant:0.5 ml fermented broth media + 0.5 ml of DNS; blank: 0.5 ml water + 0.5 ml DNS.
2. The reaction is completed at 25 °C for 10 minutes, followed by the addition of 0.5 ml of 3, 5-dinitrosalicylic acid (DNS) to the contents to eliminate the reaction, and then boiled in a water bath for 10 minutes and cooled at room temperature.

Fig. no. 4: (Fermentation media).

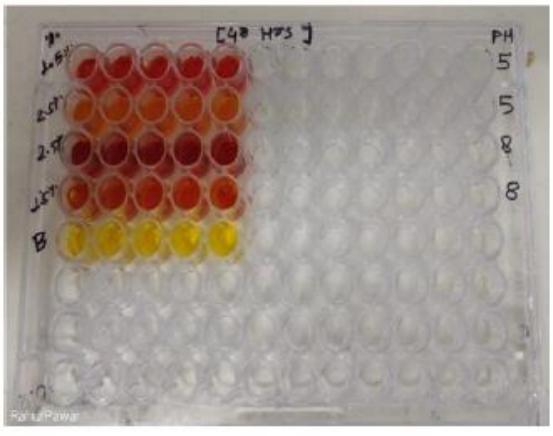


Fig. no. 5: (After 48 hours fermentation to check amylase activity in 96 well plate).

Anova

The study examined amylase production using microorganisms using a 2^3-factorial design method, considering factors like starch concentration, temperature, and pH. Eight batches

were created, with ANOVA statistics and fit statistics estimating media significance and data precision.

Anova for selected factorial model

Response 1: Activity

Factor coding is **Actual**.

Sum of squares is **Type III - Partial**

The model's F-value of 116426.82 indicates significance, with P-values less than 0.0500 indicating significant terms (B, C). Values greater than 0.1000 indicate non-significant terms. If insignificant terms exist, model reduction may improve the model.

Fit statistics

The **Predicted R^2** of 0.9999 is in reasonable agreement with the **Adjusted R^2** of 1.0000; i.e., the difference is less than 0.2. **Adeq. Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 986.766 indicates an adequate signal. This model can be used to navigate the design space.

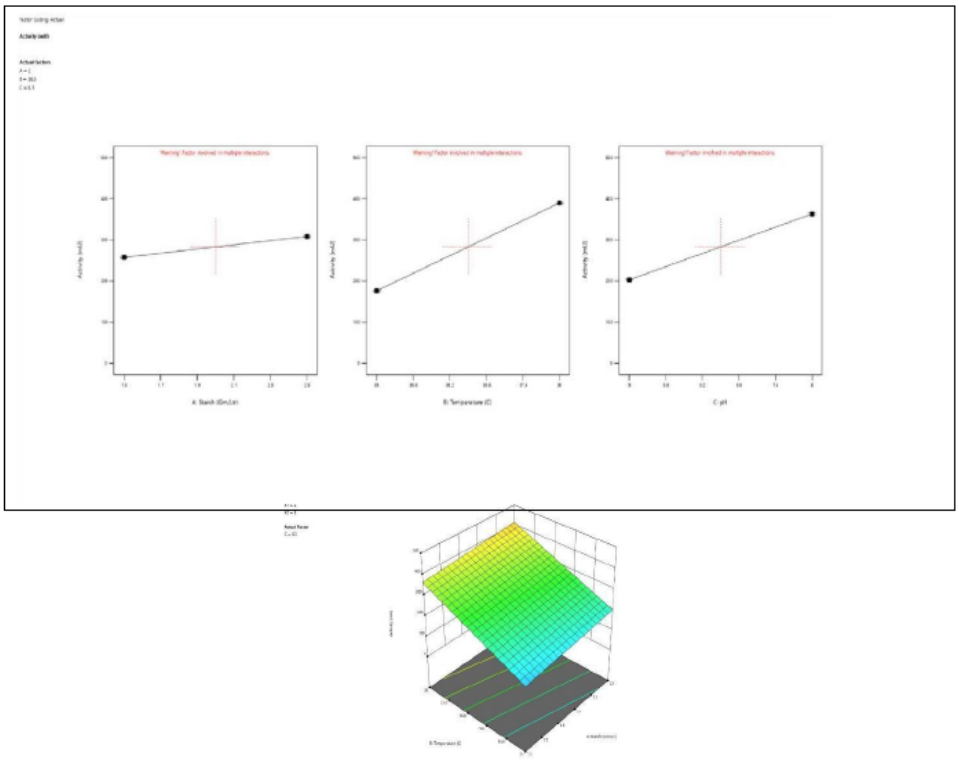
Coefficients in terms of actual factors

The coefficient estimate represents the expected change in response per unit change in factor value, while the intercept in an orthogonal design is the overall average response. VIFs indicate multi-collinearity, with VIFs less than 10 tolerable.

Final equation in terms of actual factors

Activity	=
-2781.91111	
+44.58500	Starch
+71.81889	Temperature
+57.33722	pH
+0.110000	Starch * Temperature
+0.320000	Starch * pH
-0.127778	Temperature * pH

The equation in terms of actual factors can predict response levels for each factor but should not be used to determine relative impact due to scaled coefficients and the intercept not at the design space centre.



Amylase activity

Table no. 3: (48 hours incubation assay activity).

		Factor 1	Factor 2	Factor	Response
Std	Run	A: starch	B: Temperature	C: pH	Activity
		GM/LTR			mU
3	1	1.5	38	5	285
6	2	2.5	35	8	282.32
5	3	1.5	35	8	231
4	4	2.5	38	5	335.69
1	5	1.5	35	5	71.29
8	6	2.5	38	8	495.21
2	7	2.5	35	5	121
7	8	1.5	38	8	444.21

RESULTS

Amylase production using Bacillus subtilis was carried out, and it was found that the 48-hour incubation period was effective. Fermented media with 2.5% starch, a 38^{o}C temperature, and PH-8 showed maximum activity.

Ammonium sulphate precipitation results

25 ml crude enzyme was saturated with ammonium sulphate, stirred overnight, and centrifuged. The enzyme's maximum activity in 2.5% starch was 495 mU at 38oC and Ph 8.

Procedure

1. To prepare amylase, mix 5ml of media in test tube at different concentrations.
2. Add solid ammonium sulphate in proportions, mix, incubate, centrifuge at 10,000 rpm for 10min, remove AS solution, and add phosphate buffer pH 7 for purification.
3. Pellet was dissolved in 5ml of phosphate buffer (pH-7) for further purification. Amount of ammonium sulphate for 1 litre cell free extract was calculated from table.

After precipitation std dns assay in media. 2.5 %starch, ph-8 tempreture-38^{0}C.

Table no. 4: Precipitation activity results.

Sr no	% of as saturation	Average	Activity
1	20 %	0.3422	76.92
2	30 %	0.1806	35.04
4	40 %	0.223	46.02
5	50 %	0.2444	51.6
6	60 %	1.0516	260.7
BLANK		0.1142	

Results: After the precipitation of the standard DNS assay in media with 2.5% starch, PH: 8, and temperature of 38^{o}C, it was found that at 60% AS saturation, activity was higher at 260.7.

SUMMARY AND CONCLUSION

Summary

The thesis explores the production, purification and characterization of amylase using bacillus subtilis and starch as carbon source. Amylases are essential enzymes produced by bacteria, plants and people and their classification, mechanism of action, application and production technologies like submerge fermentation are discussed. The study focuses on increasing amylase activity with results showing maximum activity in fermented media with 2.5% starch, pH-8 and 38° c temperature.

CONCLUSION

Amylase, a bacterial enzyme is produced by bacillus subtilis and has high enzyme activity. Starch is a sustainable carbon source for amylase production. Optimization of fermentation parameters including pH, temperature and starch significantly influences production. The maximum amylase activity is achieved at 38° c with pH-8 being the optimum. The proteins activity increases after 60% ammonium sulphate precipitation.

REFERENCES

1. Paul D, Bengal W. Microorganisms and A-Amylase: A Concise Review. *Innovare Journal of Science*, 2016; 4(4).
2. Sindhu R, Binod P, Madhavan A, et al. Molecular improvements in microbial A-amylases for enhanced stability and catalytic efficiency. *Bioresour Technol*, 2017; 245. Doi: 10.1016/j.biortech.2017.04.098
3. Kumari N, Rani B, Malik K, Avtar R, Nisha Kumari C. Microbial amylases: An overview on recent advancement. *~ 198 ~ Journal of Entomology and Zoology Studies*, 2019; 7(1).
4. Mondal S, Mondal K, Halder SK, Thakur N, Mondal KC. Microbial Amylase: Old but still at the forefront of all major industrial enzymes. *Biocatal Agric Biotechnol*, 2022; 45. doi: 10.1016/j.bcab.2022.102509
5. Mehta D, Satyanarayana T. Bacterial and archaeal α-amylases: Diversity and amelioration of the desirable characteristics for industrial applications. *Front Microbiol*, 2016; 7(JUL). doi:10.3389/fmicb.2016.01129
6. Kim JS, Cha SS, Kim HJ, et al. Crystal structure of a maltogenic amylase provides insights into a catalytic versatility. *Journal of Biological Chemistry*, 1999; 274(37). doi:10.1074/jbc.274.37.26279
7. Zhao Y, Wang M, Huang G. Structure-activity relationship and interaction mechanism of nine structurally similar flavonoids and α-amylase. *J Funct Foods*, 2021; 86. doi: 10.1016/j.jff.2021.104739
8. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. Microbial αamylases: A biotechnological perspective. *Process Biochemistry*, 2003; 38(11). doi:10.1016/S0032-9592(03)00053-0
9. Benjamin S, Smitha RB, Jisha VN, et al. A monograph on amylases from < i>Bacillus</i> spp. *Advances in Bioscience and Biotechnology*, 2013; 04(02). doi:10.4236/abb.2013.42032
10. Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A. 10α-Amylases from microbial sources - An overview on recent developments. *Food Technol Biotechnol*, 2006; 44(2).
11. Kusunoki M. A retrospect of the structure determination of Taka-amylase A. *J Biochem*, 2022; 171(1). doi:10.1093/jb/mvab137
12. Nikolic S, Rakin M, Vukasinovic M, Siler-Marinkovic S, Mojovic L. Bioethanol from corn meal hydrolyzates. *Chemical Industry and Chemical Engineering Quarterly*, 2005; 11(4). doi:10.2298/ciceq0504189n

13. Jin W, Wang Z, Peng D, et al. Effect of pulsed electric field on assembly structure of α-amylase and pectin electrostatic complexes. *Food Hydrocoll*, 2020; 101. doi: 10.1016/j.foodhyd.2019.105547
14. Kumari A, Singh K, Kayastha AM. α-Amylase: General Properties, Mechanism and Biotechnological Applications - A Review. *Curr Biotechnol*, 2011; 1(1). doi:10.2174/2211551x11201010098
15. Rani K, Rana R, Datt S. Review on characteristics and application of amylases. *International Journal of Microbiology and Bioinformatics*. Published online, 2015.
16. Kumari A, Singh K, M. Kayastha A. α-Amylase: General Properties, Mechanism and Biotechnological Applications - A Review. *Current Biotechnology e*, 2012; 1(1). doi:10.2174/2211550111201010098
17. Moradi M, Shariati P, Tabandeh F, Yakhchali B, Khaniki GB. Screening and isolation of powerful amylolytic bacterial strains. *Int J Curr Microbiol Appl Sci*, 2014; 3(2).
18. Gouda S, Das G, Sen SK, et al. *Endophytes: Biology and Biotechnology*, 2018; *1:* 10.
19. Shinmyo A, Kimura H, Okada H. Physiology of α-amylase production by immobilized Bacillus amyloliquefaciens. *European Journal of Applied Microbiology and Biotechnology*, 1982; 14(1). doi:10.1007/BF00507996
20. Savaner, SP. Sohani S. Review on Microbial A -Amylase, Types & Their Industrial. *Kala Sarovar (UGC Care Group-1 Journal)*, 2020; 23(2020).
21. Boehlke C, Zierau O, Hannig C. Salivary amylase - The enzyme of unspecialized euryphagous animals. *Arch Oral Biol*, 2015; 60(8). doi: 10.1016/j.archoralbio.2015.05.008
22. Farooq MA, Ali S, Hassan A, Tahir HM, Mumtaz S, Mumtaz S. Biosynthesis and industrial applications of α-amylase: a review. *Arch Microbiol*, 2021; 203(4). doi:10.1007/s00203-020-02128-y
23. de Souza PM, e Magalhães P de O. Application of microbial α-amylase in industry - a review. *Brazilian Journal of Microbiology*, 2010; 41(4). doi:10.1590/s1517-83822010000400004
24. Sundarram A, Murthy TPK. α -Amylase Production and Applications: A Review. *J Appl Environ Microbiol*, 2014; 2(4). doi:10.12691/jaem-2-4-10
25. Thacker SP, Ramamurthy V, Kothari RM. Characterization of Barley βAmylase for Application in Maltose Production. *Starch - Stärke*, 1992; 44(9). doi:10.1002/star.19920440906

26. Ray RR, Nanda G. Microbial β-amylases: Biosynthesis, characteristics, and industrial applications. *Crit Rev Microbiol*, 1996; 22(3). doi:10.3109/10408419609106459
27. Thacker SP, Ramamurthy V, Kothari RM. Characterization of Barley βAmylase for Application in Maltose Production. *Starch - Stärke*, 1992; 44(9). doi:10.1002/star.19920440906 Vaidya S, Srivastava P, Rathore P, Pandey A. Amylases: A Prospective Enzyme in the Field of Biotechnology. *J Appl Biosci*, 2015; 41(1).
28. ZHAO CH, HUO NB, FENG H, LI ZQ. An Overview and Application of Amylase. *DEStech Transactions on Environment, Energy and Earth Sciences*, 2018; (epme). doi:10.12783/dteees/epme2018/24657
29. Vaidya S, Srivastava P, Rathore P, Pandey A. Amylases: A Prospective Enzyme in the Field of Biotechnology. *J Appl Biosci*, 2015; 41(1).
30. Nasir G, Zaidi S, Tabassum N, Asfaq. A review on nutritional composition, health benefits and potential applications of by-products from pea processing. *Biomass Convers Biorefin*. Published online, 2022. doi:10.1007/s1339902203324-0
31. Yu Y, Feng M, Wang Q, Liu M, Gao F, Lin S. Effect of electron beam irradiation on physicochemical properties of corn starch and improvement of enzymatic saccharification of corn starch at high concentration (45%). *J Food Process Eng*, 2021; 44(7). doi:10.1111/jfpe.13699
32. Widiana DR, Phon S, Ningrum A, Witasari LD. Purification and characterization of thermostable alpha-amylase from Geobacillus sp. DS3 from Sikidang Crater, Central Java, Indonesia. *Indones J Biotechnol*, 2022; 27(4). doi:10.22146/ijbiotech.71643
33. Dahiya S, Bajaj BK, Kumar A, Tiwari SK, Singh B. A review on biotechnological potential of multifarious enzymes in bread making. *Process Biochemistry*, 2020; 99. doi:10.1016/j.procbio.2020.09.002
34. Madhu A, Chakraborty JN. Developments in application of enzymes for textile processing. *J Clean Prod*, 2017; 145. doi:10.1016/j.jclepro.2017.01.013
35. Shahid M, Mohammad F, Chen G, Tang RC, Xing T. Enzymatic processing of natural fibres: White biotechnology for sustainable development. *Green Chemistry*, 2016; 18(8). doi:10.1039/c6gc00201c
36. Kakkar P, Wadhwa N. Extremozymes used in textile industry. *Journal of the Textile Institute*, 2022; 113(9). doi:10.1080/00405000.2021.1948251
37. Kalia S, Bhattacharya A, Prajapati SK, Malik A. Utilization of starch effluent from a textile industry as a fungal growth supplement for enhanced α-amylase production for industrial application. *Chemosphere*, 2021; 279. doi:10.1016/j.chemosphere.2021.130554

38. Md. Mamun Kabir S, Koh J. Sustainable Textile Processing by Enzyme Applications. In: *Biodegradation Technology of Organic and Inorganic Pollutants*, 2022. doi:10.5772/intechopen.97198
39. Mobini-Dehkordi M, Afzal Javan F. Application of alpha-amylase in biotechnology. *Journal of Biology and Today's World*, 2012; 1(1). doi:10.15412/j.jbtw.01010104
40. Paul JS, Gupta N, Beliya E, Tiwari S, Jadhav SK. Aspects and Recent Trends in Microbial α-Amylase: a Review. *Appl Biochem Biotechnol*, 2021; 193(8). doi:10.1007/s12010-021-03546-4
41. Ziegler P. Cereal beta-amylases. *J Cereal Sci*, 1999; 29(3). doi:10.1006/jcrs.1998.0238
42. Shrivastava S. Screening and Identification of Thermophilic Glycosyl Hydrolases Producing Microorganisms. *Journal of Biotechnology & Bioresearch*, 2018; 1(2). doi:10.31031/jbb.2018.01.000508
43. Samanta S. Structural and Catalytical Features of Different Amylases and their Potential Applications. *Jordan J Biol Sci*, 2022; 15(2). doi:10.54319/jjbs/150220
44. Wang Y chuan, Hu H fang, Ma J wen, Yan Q juan, Liu H jie, Jiang Z qiang. A novel high maltose-forming α-amylase from Rhizomucor miehei and its application in the food industry. *Food Chem*, 2020; 305. doi:10.1016/j.foodchem.2019.125447
45. Shinde VK, Vamkudoth KR. Maltooligosaccharide forming amylases and their applications in food and pharma industry. *J Food Sci Technol*, 2022; 59(10). doi:10.1007/s13197-021-05262-7
46. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. Advances in microbial amylases. *Biotechnol Appl Biochem*, 2000; 31(2). doi:10.1042/ba19990073
47. Technology Strategies, Production and Purification of Amylases: An overview. *The Internet Journal of Genomics and Proteomics*, 2012; 2(2). doi:10.5580/eb0
48. Chandrasekar V, Nambi EV, Sultan S, John SG. Effect of operating conditions of a solid-state fermenter on structural and pasting properties of finger millet flour as applied to bread quality. *J Food Process Eng*, 2022; 45(5). doi:10.1111/jfpe.14017
49. Ahmad MA, Isah U, Raubilu IA, Muhammad SI, Ibrahim D. An overview of the enzyme: Amylase and its industrial potentials. *Bayero Journal of Pure and Applied Sciences*, 2020; 12(1). doi:10.4314/bajopas.v12i1.53s
50. Jujjavarapu SE, Dhagat S. Evolutionary Trends in Industrial Production of αamylase. *Recent Pat Biotechnol*, 2018; 13(1). doi:10.2174/2211550107666180816093436
51. Szwiec K, Goncharova K, Valverde-Piedra JL, et al. Novel potential of pancreatic-like enzymes of microbial origin in exocrine pancreatic insufficiency – study on a pig model.

Journal of Pre-Clinical and Clinical Research, 2015; 9(1). doi:10.5604/18982395.1157568

52. Maicas S. Advances in wine fermentation. *Fermentation*, 2021; 7(3). doi:10.3390/fermentation7030187
53. Augustin MA, Hartley CJ, Maloney G, Tyndall S. Innovation in precision fermentation for food ingredients. *Crit Rev Food Sci Nutr*. Published online, 2023. doi:10.1080/10408398.2023.2166014
54. Stanbury PF, Whitaker A, Hall SJ. Design of a fermenter. In: *Principles of Fermentation Technology*, 2017. doi:10.1016/b978-0-08-099953-1.00007-7
55. Stanbury PF, Whitaker A, Hall SJ. Chapter 7 - Design of a fermenter. *Principles of Fermentation Technology*. Published online, 2017.
56. Sharma R, Garg P, Kumar P, Bhatia SK, Kulshrestha S. Microbial fermentation and its role in quality improvement of fermented foods. *Fermentation*, 2020; 6(4). doi:10.3390/fermentation6040106
57. Karthikeyan A, Joseph A, Subramanian R, Nair BG. Fermenter Design. In: *Industrial Microbiology and Biotechnology*, 2022. doi:10.1007/978-981-165214-1_5
58. Indrastuti YE, Estiasih T, Christanti RA, Pulungan MH, Zubaedah E, Harijono. Microbial and some chemical constituent changes of high cyanide cassava during simultant spontaneous submerged and solid state fermentation of "gadungan pohung." *Int Food Res J*, 2018; 25(2).
59. Matpan Bekler F, Güven K, Gül Güven R. Purification and characterization of novel α-amylase from Anoxybacillus ayderensis FMB1. *Biocatal Biotransformation*, 2021; 39(4). doi:10.1080/10242422.2020.1856097
60. Yahya S, Sohail M, Khan SA. Characterization, thermal stabilization and desizing potential of amylase from A. tubingensis SY 1. *Journal of the Textile Institute*, 2022; 113(6). doi:10.1080/00405000.2021.1914393
61. Costa S, Summa D, Zappaterra F, Blo R, Tamburini E. Aspergillus oryzae grown on rice hulls used as an additive for pretreatment of starch-containing wastewater from the pulp and paper industry. *Fermentation*, 2021; 7(4). doi:10.3390/fermentation7040317
62. Couto SR, Sanromán MÁ. Application of solid-state fermentation to food industry-A review. *J Food Eng*, 2006; 76(3). doi:10.1016/j.jfoodeng.2005.05.022
63. Fruehauf HM, Enzmann F, Harnisch F, Ulber R, Holtmann D. Microbial Electrosynthesis—An Inventory on Technology Readiness Level and Performance of Different Process Variants. *Biotechnol J*, 2020; 15(10). doi:10.1002/biot.202000066

64. Khan JA, Priya R. A study on partial purification and characterization of extracellular amylases from Bacillus subtilis. *Advances in applied Science Research*, 2011; 2(3).
65. Zusfahair Z, Ningsih DR, Kartika D, Fatoni A. Amylase from bacillus thuringiensis isolated from tapioca waste: isolation, partial purification and characterization. *Malaysian Journal of Fundamental and Applied Sciences*, 2016; 12(1). doi:10.11113/mjfas.v12n1.404
66. Arunkumar M, Divya SK, Mahesh N, Balakumar S. Development of Improved Strategies for the Survival of Lactobacillus Plantarum MTCC 1407 in Probioticated Custard Apple Juice. *Proceedings of the National Academy of Sciences India Section B - Biological Sciences*, 2021; 91(1). doi:10.1007/s40011-020-01196-w
67. Nandi S, Bose T, Mahato S, Chatterjee S, Chatterjee A. Isolation and partial characterization of amylase produced by fungal isolates from the agroindustrial waste source. *J Appl Biol Biotechnol*, 2022; 10(4). doi:10.7324/JABB.2022.100406
68. Sheela JM, Divya K, Premina S. Amylase production by aspergillus Niger and penicillium species by solid-state and submerged cultivation using two food industrial wastes. *Nature Environment and Pollution Technology*, 2021; 20(3). doi:10.46488/NEPT.2021.V20I03.020
69. Vidyalakshmi R, Paranthaman R, Indhumathi J. Amylase Production on Submerged Fermentation by Bacillus spp. *World Journal of Chemistry*, 2009; 4(1).
70. Lolita J. Optimization of Cultural Conditions for Production of Alpha-amylase fromb Bacillus sp. under Submerged Fermentation (SmF). *Int J Curr Microbiol Appl Sci*, 2022; 11(1). doi:10.20546/ijcmas.2022.1101.016
71. Senthilkumar PK, Uma C, Saranraj P. *Amylase Production by Bacillus Sp. Using Cassava as Substrate*, 2012; 3.
72. Sana S, Anjum AA, Ahmad MUD, Nawaz M, Ahmad A, Farooq HR. Dynamics of amylases production potential by Aspergillus niger isolates from soil. *Pak J Bot*, 2021; 53(2). doi:10.30848/PJB2021-2(17)
73. SA, Abdella MAA, El-Sherbiny GM, Ibrahim AM, El-Shamy AR, Atalla SMM. Application of one –factor- at-a-time and statistical designs to enhance α-amylase production by a newly isolate Bacillus subtilis strain-MK1. *Biocatal Agric Biotechnol*, 2019; 22. doi:10.1016/j.bcab.2019.101397
74. Sajjad M, Choudhry S. Effect of starch containing organic substrates on alpha amylase production in Bacillus strains. *Afr J Microbiol Res*, 2012; 6(45).

75. Abdul-Wahed HE, Mohammed WH, Ali MA, Noori RS. Stimulation of amylase activity by UV exposure and potato peels. *Plant Cell Biotechnol Mol Biol*, 2020; 21(59-60).
76. Yang Y, Wang Y, Wu Y, et al. Screening of Optimal Conditions for Piglet Diet Predigested by Compound Enzyme Preparation in Vitro and Effects on Its Quality. *Chinese Journal of Animal Nutrition*, 2022; 34(5). doi:10.3969/j.issn.1006267x.2022.05.058
77. Chen X, Zhang C, Yan B, Guo ZQ, Gao HX, Ma HX. Study on Preparation, Thermal Behavior and Enthalpy of Formation of 3, 6-Dihydrazine-1, 2, 4, 5tetrazine-3, 5-dinitrosalicylic Acid Salt. *Huozhayao Xuebao/Chinese Journal of Explosives and Propellants*, 2017; 40(6). doi:10.14077/j.issn.10077812.2017.06.007
78. Lakshmi SS, Mahesh CH, Gayatri K, Manisha P, Aishwarya K. Statistical optimization of amylase production and its purification from a palm wine isolate Bacillus sp., Q-164. *Biocatal Agric Biotechnol*, 2020; 29. doi:10.1016/j.bcab.2020.101820
79. Rajesh R, Gummadi SN. α-Amylase and cellulase production by novel halotolerant Bacillus sp.PM06 isolated from sugarcane pressmud. *Biotechnol Appl Biochem*, 2022; 69(1). doi:10.1002/bab.2091
80. Hassan BA, Alsalami NY, Jebor MA. Amylase production, purification and characterization from Escherichia coli. *Journal of Pharmaceutical Sciences and Research*, 2018; 10(7).
81. Paul JS, Beliya E, Tiwari S, Patel K, Gupta N, Jadhav SK. Production of biocatalyst α-amylase from agro-waste 'rice bran' by using Bacillus tequilensis TB5 and standardizing its production process. *Biocatal Agric Biotechnol*, 2020; 26. doi:10.1016/j.bcab.2020.101648
82. Ahmed NE, El Shamy AR, Awad HM. Optimization and immobilization of amylase produced by Aspergillus terreus using pomegranate peel waste. *Bull Natl Res Cent*, 2020; 44(1). doi:10.1186/s42269-020-00363-3
83. Shinde S, Soni R. "Production and Partial Purification of α-Amylase from Bacterial Strains ". *International Journal of Genetic Engineering and Biotechnology ISSN 0974*, 2014; 3073(1).
84. T RK, Ramkumar J, Karthikeyan S, G RBN, Manivasagan V. ISOLATION, OPTIMIZATION, PRODUCTION AND PURIFICATION OF ALPHA AMYLASE FROM SOIL BACTERIA. *International Research Journal of Engineering and Technology(IRJET)*, 2017; 4(7).

85. Kalyani G, Rajesh EM. Production and Purification of Amylase from Bacillus subtilis Isolated from Soil. *International Journal of Engineering and Management Research*, 2018; 8(3). doi:10.31033/ijemr.8.3.33
86. Chimata MK, Chetty CS, Suresh C. Fermentative Production and Thermostability Characterization of Amylase from Aspergillus Species and Its Application Potential Evaluation in Desizing of Cotton Cloth. *Biotechnol Res Int*, 2011; 2011. doi:10.4061/2011/323891
87. Obafemi YD, Ajayi AA, Olasehinde GI, Atolagbe OM, Onibokun EA. Screening and partial purification of amylase from *Aspergillus Niger* isolated from deteriorated tomato (*Lycopersicon Esculentum* mill.) fruits. *African Journal of Clinical and Experimental Microbiology*, 2017; 19(1). doi:10.4314/ajcem.v19i1.7
88. Abirami G, Ramprasath C, Suganthi M, Abinaya Devi E, Mathivanan N. Partial Purification of Extracellular Amylase from Halotolerant Actinomycetes Streptomyces brasiliensis Mml2028. *Journal of Experimental Biology and Agricultural Sciences*, 2022; 10(5). doi:10.18006/202210(5).1053.1068