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PRODUCTION AND PURIFICATION OF α-AMYLASE FROM *BACILLUS SPECIES* ISOLATED FROM SOIL USING AGRO WASTE RICE HUSK IN SOLID STATE FERMENTATION

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ABSTRACT
Amylase is an enzyme that catalyzes the breakdown of starch into sugars. The amylases obtained from microorganisms have a broad spectrum of industrial uses as they are more stable than plant and animal amylases. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity, and also microbes are easy to manipulate to derive enzymes of desired nature. The present study focuses on the isolation of amylase producing *Bacillus species* from soil sample and production of bacterial amylase. In this study, we have also compared the amylase produced in normal basal medium and in media in which rice husk has been given as a specific substrate with basal medium. The amylase enzyme produced was estimated by DNS method, in which it was observed that when rice husk was used as a substrate it gave 59% higher production of amylase compared to the normal basal medium. The amylase produced in normal basal medium was 7.27 U/ml whereas amylase produced in basal medium with rice husk as specific substrate was 18.1 U/ml. The amylase enzyme was then extracted from the medium by ammonium sulfate precipitation method and purified by dialysis. For further analysis and characterization, the enzyme was subjected to SDS-PAGE analysis in which molecular weight of amylase was determined to be approximately 50 kDa. Hence it was concluded that agro waste rice husk can be used as an effective and economical substrate for the production of microbial amylase.

KEYWORDS: α-Amylase, *Bacillus subtilis*, Dinitrosalicylic acid, agro waste, rice-husk.

INTRODUCTION
Life is an intricate meshwork involving a perfect coordination of a vast majority of chemicals reactions. Some of these reactions result in synthesizing large molecules, others in cleaving large molecules and all of them either utilize energy or liberate energy. All these reaction occurs very slowly at the low temperature and the atmospheric pressure the condition under which living cell carry on their life processes, yet in the living cells these reactions proceeds at extremely high rate. This is due to the presence of some catalysts produced and synthesized inside the body of the organism. Enzymes are biocatalysts protein in nature; they catalyze the biochemical reaction taking place in the living cell without any overall change (Jain *et al*., 2006).

Enzymes are the large biomolecules that are required for the numerous chemical interconversions that sustain life. They accelerate all the metabolic processes in the body and carry out a specific task. Enzymes are highly efficient, which can increase reaction rates by 100 million to 10 billion times faster than any normal chemical reaction. Due to development in recombinant technology and protein engineering, enzymes have evolved as an important molecule that has been widely used in different industrial and therapeutic purposes. Microbial enzymes are currently acquiring much attention with rapid development of enzyme technology. Microbial enzymes are preferred due to their economic feasibility, high yields, consistency, ease of product modification and optimization, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity. Microbial enzymes play a major role in the diagnosis, treatment, biochemical investigation, and monitoring of various dreaded diseases. Amylases are very important enzymes that have been vastly studied and have great importance in different industries and therapeutic industry (Gurung *et al*., 2013).

Due to their wide range of activities based on their nature of reaction enzymes are being classified according to their enzyme catalyzing reaction. The Enzyme Commission number (EC number) is a numerical

classification scheme for enzymes; based on the chemical reactions they catalyze (Webb 1992). As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme. Except for some of the originally studied enzymes such as pepsin, rennin, and trypsin, most enzyme names end in "ase." The enzyme nomenclature scheme was developed starting in 1955, when the International Congress of Biochemistry in Brussels sets up an Enzyme Commission. The first version was published in 1961. The current sixth edition, published by the International Union of Biochemistry and Molecular Biology in 1992, contains 3196 different enzymes. The International Union of Biochemistry (I. U. B.) initiated standards of enzyme nomenclature which recommend that enzyme names indicate both the substrate acted upon and the type of reaction catalyzed. According to the enzyme commission the enzymes are divided into 6 parts: Oxidoreductase (EC 1), Transferase (EC 2), Hydrolase (EC 3), Lyase (EC 4), Isomerase (EC 5), Ligase (EC 6) (Gurung *et al*., 2013).

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper (Pandey *et al*., 2000).

Amylase is an enzyme that catalyzes the breakdown of starch into sugars. Amylase is abundantly present in human saliva, where it begins the mechanical process of digestion. Foods that contain much starch but little sugar, such as rice and potato, taste slightly sweet as they are chewed because amylase turns some of their starch into sugar in the mouth. The pancreas also makes amylase (alpha amylase) to hydrolyze dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. Plants and some bacteria also produce amylase. As diastase, amylase was the first enzyme to be discovered and isolated by Anselme Payen in 1833. All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds. It is widely used in industries and has nearly 25%of the enzyme market (Sidhu *et al*., 1997); Rao *et al*., 1998). Although amylases can be obtained from several sources, like plants and animals, the enzymes from microbial sources generally satisfy industrial demands and had made significant contribution to the foods and beverages industry in the last three decades. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Microbial amylase has almost surpassed the synthetic sources in different industries. (Pandey *et al*., 2000). Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized in to exo-acting, endo-acting and debranching enzymes. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermo acidophilic bacteria (Boyer *et al*., 1972). Nowadays amylase from these sources is vastly used in amylase production under extreme conditions of pH and temperature (Sethi *et al*., 2013).

Amylases have a wide range of application in various industries such as in the food, bread making, paper industries, textiles, sweeteners, glucose and fructose syrups, fruit juices, detergents, fuel ethanol from starches, alcoholic beverages, digestive aid, and spot remover in dry cleaning. Bacterial α-amylases are also being used in clinical, medicinal, and analytical chemistry (Pandey *et al*., 2000). The widely used thermostable enzymes in the starch industry are the amylases (Jensen *et al*., 2002). The starch industry has the most widespread applications of amylases, which are used during starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielsen *et al*., 2000). The application of enzymes in detergents making enhances the detergents ability to remove tough stains and also makes detergent eco-friendly. There is an extensive use of amylase in processed food industry such as baking, brewing, production of cakes, preparation of digestive aids, fruit juices, and starch syrups. Sizing agents like starch are added to yarn before fabric production for fast and secure weaving process (Ahlawat *et al*., 2009). A higher than normal concentration of amylases may predict one of several medical conditions, including acute inflammation of the pancreas, perforated peptic ulcer, strangulation ileus, torsion of an ovarian cyst, macroamylasemia, and mumps. In other body fluids also amylase can be measured, including urine and peritoneal fluid. In various human body fluids the level α- amylase activity is of clinical importance, for example, in diabetes, pancreatitis, and cancer research (Das *et al*., 2011).

In the present study, amylase producing microbe is isolated from garden soil sample and it is identified by microscopic examination and growth on selective media. This study also focuses on use of better economic substrate i.e. rice husk compared to normal Basal medium for amylase production and further purification of enzyme amylase by ammonium sulfate precipitation and dialysis method. The purity and molecular weight of enzyme is also determined by SDS-PAGE analysis.

MATERIALS AND METHODS

Isolation of Amylase Producing Microbes from Soil Sample

Preparation of starch agar plates

Starch agar (peptic digest 5g/l, yeast extract 1.5 g/l, beef extract 1.5 g/l, starch 2 g/l, sodium chloride 5 g/l, agar 15 g/l in 1000ml sterile distilled water) was prepared, sterilized and poured in petriplates and allowed to solidify.

Collection and serial dilution of soil sample

Fertile garden soil sample was collected and one gram was mixed with 10 ml of sterile distilled water. It was mixed well to suspend the microorganisms adhering to the soil particles into water. This was considered as 10^{-1} dilution. Soil was allowed to settle at the bottom of the flask and then the supernatant was further diluted till 10^{-7} dilution in test tubes.

Plating on starch agar plates

Then 0.1ml of the sample from 10^{-5}, 10^{-6} and 10^{-7} dilution were plated on starch agar plates by spread plate method with the help of sterile l-glass rod. The plates were incubated overnight at room temperature.

Starch-Iodine test

After incubation, specific 4 suspected colonies were picked using sterile inoculation loop and preserved in fresh sterile nutrient broth tubes. Then whole plate was flooded with iodine solution. Amylase-positive colonies that formed clear zone of starch hydrolysis were revived from previously preserved nutrient broth.

Identification of Various Amylase Producing Microbes

Microscopic Examination

Simple staining: Amylase-positive colonies from nutrient broth were subjected to simple staining after overnight incubation at room temperature. A drop of culture was smeared on a microscopic slide. The smear was heat fixed and flooded with methylene blue. It was kept for a minute to get stained. Then it was washed, air dried and observed under microscope.

Gram staining: The culture was also subjected to Gram staining. A drop of culture from amylase-positive nutrient broth was smeared on a microscopic slide and heat-fixed. The smear was flooded with crystal violet. It was kept for a minute and then washed gently. Iodine solution was flooded over the smear, kept for a minute and then gently washed away. The smear was washed with 95% ethyl alcohol for 30 seconds. Then the smear was counterstained with 0.25% safranin for 30 seconds. The slide was then washed, drained, air-dried and observed under microscope.

Biochemical Tests

Indole test: The culture is inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at 37°C. Following incubation few drops of Kovac's reagent are added. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and con. HCl. Formation of a red or pink coloured ring at the top is taken as positive.

Methyl-Red test: The culture is inoculated into glucose phosphate broth, which contains glucose and a phosphate buffer and incubated at 37°C for 48 hours. Over the 48 hours the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acid. The pH of the medium is tested by the addition of 5 drops of MR reagent. Development of red color is taken as positive. MR negative organisms produce yellow color.

Voges Proskauer test: The culture is inoculated into glucose phosphate broth and incubated for at least 48 hours. 0.6 ml of alpha-naphthol is added to the test broth and shaken. 0.2 ml of 40% KOH is added to the broth and shaken. The tube was allowed to stand for 15 minutes. Appearance of red color is taken as a positive test. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents.

Citrate utilization test: The culture was inoculated into slope of Simmon's citrate agar slants and incubated overnight at 37°C. If the organism has the ability to utilize citrate, the medium changes its color from green to blue.

Growth on Selective Media

MYP (Mannitol-Yolk-Polymixin B) selective agar (peptic digest 5g/l, yeast extract 1.5g/l, beef extract 1.5g/l, starch 2g/l, sodium chloride 5g/l, agar 15g/l in 1000 ml sterile distilled water) was prepared, sterilized, poured in petriplates and allowed to solidify. After solidification, 0.1ml of the amylase-positive culture was plated on MYP-agar plates by spread plate method with the help of sterile L-glass rod. The plates were then incubated overnight at room temperature.

Production of Enzyme Amylase in Basal Medium and Comparison Using Rice Husk as a Specific Substrate

Preparation of basal medium

The basal medium (soluble starch 5g/l, peptone 20g/l, $MgSO_4H_2O$ 1g/l, K_2HPO_4 3g/l in 1000ml of sterile distilled water) was prepared, sterilized and taken in fermentation bottle of 500ml capacity.

Collection and Preparation of specific substrate

The rice-husk sample was collected from local region in Srivilliputtur. The rice-husk was powdered prior to the addition into the basal medium to facilitate easy uptake by microorganisms.

Preparation of basal medium with rice-husk substrate

The basal medium along with rice husk as a specific substrate (soluble starch 5g/l, peptone 20g/l, $MgSO_4H_2O$ 1g/l, K_2HPO_4 3g/l, powdered rice-husk 100g/l in 1000ml ofsterile distilled water) was prepared, sterilized and taken in fermentation bottle of 500ml capacity.

Preparation of inoculum

Nutrient broth (peptone 5g/l, yeast extract 3g/l, beef extract 5g/l, sodium chloride 2g/l in 1000ml of sterile distilled water) was prepared and sterilized. The amylase producing culture from glycerol stock was inoculated in freshly prepared nutrient broth and incubated at room temperature for 24 hours. This was considered as starter culture.

Culture inoculation and solid state fermentation

1ml of the 24 hours old starter culture was inoculated into each of the fermentation bottles containing basal medium and rice-husk with basal medium. Then the fermentation bottles were incubated at room temperature.

Estimation of Produced Amylase by Dns Method

Amylase produced in both the fermentation bottles was estimated by employing the 3, 5-dinitrosalicylic acid (DNS) method of Bernfeld.

DNS Reagents

- 1% DNS (3, 5-dinitrosalicylic acid)
- 1M Potassium sodium tartarate (Rochelle salt)
- 0.4M NaOH

1g of DNS was dissolved in 50ml distilled water by stirring at room temperature (RT), then 20mlof 2M NaOH and 28.2g of Rochelle salt was added and finally the volume was made up to 100ml adding distilled water. The reagent was stored at room temperature.

Other solutions

Starch solution: 1.0% starch solution was prepared fresh by dissolving 1g soluble starch in 100ml of distilled water.

Maltose stock solution: 50µg of maltose was dissolved in 50ml distilled water in a standard flask and stored at 4^oC.

Crude Amylase Extraction from Fermentation Media

2 ml of enzyme extract was taken separately from both the fermentation bottles in 24 hours interval for estimation. It was centrifuged separately at 3000 rpm for 20 minutes and the supernatant was collected and kept in ice cold condition. This enzyme solution was further used for enzyme estimation.

Amylase activity assay

0.5ml of enzyme solution was pipette out in a test-tube and incubated the tubes at 25^oC for 3 minutes. Then 0.5ml of starch solution was added and incubated for 5 minutes at room temperature. The reaction was stopped by adding 1ml DNS reagent. The solution was heated in a boiling water bath for 5 minutes. The solution was cooled in running tap water. The volume was made up to 10ml by the addition of distilled water. The absorbance was read at 540 nm using UV-Visible Spectrophotometer. This assay was done separately for both the enzyme solutions and noted down the difference for comparison. Blank was prepared without enzyme.

Purification of Enzyme Amylase and Characterization

Amylase was purified by fractionation by ammonium sulphate followed by dialysis the purity was checked by running the purified enzyme in SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis).

Ammonium sulfate Precipitation

About 9ml of crude amylase solution from both the samples was brought to 80% saturation by the addition of 5g powdered ammonium sulfate in each enzyme solution sample. The mixture was allowed to precipitate for 30 minutes at room temperature in a magnetic-stirrer. The mixture was then centrifuged at 5000 rpm for 20minutes. The pellet was then dissolved in 10ml of 50mM sodium acetate buffer (pH-5.5) for further purification.

Purification by dialysis method

Activation of dialysis membrane: The dialysis membrane was handled with the help of forceps. For activation of the membrane, it was kept immersed overnight in the 50mM sodium acetate buffer (pH-5.5) at 4^o C.

Dialysis of amylase enzyme: The precipitate obtained after ammonium sulphate fractionation dissolved in 10ml of 50mM sodium acetate buffer (pH-5.5) was placed in dialysis membrane bags tied on both side to prevent leakage. It was immersed in a large volume of sterile distilled water for 24 h at 4^o C with continuous stirring. The membrane has pores that permit small molecules such as ammonium and sulphate ions to cross, and hence equilibrates in the larger volume of water outside, while not permitting large protein molecules to cross. Dialysis increased the volume of the enzyme solution.

Collection of purified amylase: The purified amylase was then carefully pipette out in eppendorf tubes and stored in 4^o C. This was further analyzed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis).

SDS-PAGE Analysis of amylase enzyme

The electrophoresis unit was assembled such that the glass plates are clamped to the unit along with the spacers placed in-between them at two vertical edges. 1% agarose (0.05g in 5ml of distilled water) was prepared and boiled to dissolve the agarose. Then it was poured in a thin horizontal layer at the lower edge of the plates to seal the assembly. It was allowed to solidify by cooling down for 5-10 minutes.

Preparation of 12% Separating Gel-
To prepare separating gel, the given components were added as follows:

- 30% Acrylamide-bisacrylamide Solution - 6ml
- Distilled water - 3ml
- 2.5X Tris-SDS Buffer (pH 8.8) - 6ml
- 10% APS Solution - 125µl
- TEMED - 7.5µl

The gel was poured in-between the plates and allowed to solidify for an hour. Immediately after the gel is poured, distilled water was added to level the gel. After an hour the water was poured off by inverting the casting assembly.

Preparation of 5% Stacking Gel-
To prepare stacking gel, the components were added as follows:

- 30% Acrylamide-bisacrylamide Solution - 1.3ml.
- Distilled water - 5.1ml.
- 5X Tris-SDS Buffer (pH 6.8) - 1.6ml.
- 10% APS Solution - 75µl.
- TEMED - 15µl.

After addition of TEMED, all the components were gently mixed by swirling the beaker. The stacking gel was poured on top of the separating gel and immediately placed the comb avoiding air bubbles. It was allowed to solidify for 30 minutes. 1X Tris-Glycine-SDS Gel Running Buffer was poured in the unit such that the buffer connects the two electrodes and hence completes the flow of current. The comb was removed from the Stacking Gel carefully.

Sample Preparation: 2 tubes were taken for enzyme samples and labeled them respectively. 20µl of each sample was taken in the respective tube and 5µl of 5X Sample Loading Buffer was added to it. The tubes containing Protein Samples were boiled at 100^{o}C in a boiling water bath. 5µl of Prestained Protein Ladder (marker) and 20µl of the samples were loaded immediately after the heat treatment in the wells created by the comb in the Stacking Gel. The power cord was connected to the electrophoretic power supply according to the conventions: Red-Anode and Black-Cathode. Electrophoresis was performed at 100 volts and 10 mA until dye front reaches 0.5 cm above the sealing gel. The gel from in-between the plates was carefully removed using spatula into the plastic tray containing distilled water. The gel was washed for a minute. The water was discarded and staining destaining procedure was performed.

Staining and Destaining of Gel: After removing water, 50 ml of Staining Solution was added in the tray containing gel, till the bands were visible. The gel was removed from Staining Solution. The gel was washed by rinsing with distilled water till a considerable amount of stain leaches out from the gel. The distilled water was changed for 3-4 times. 50 ml of Destaining Solution was added to the gel. Destaining was carried out with constant moderate shaking till distinct bands are observed. The gel was removed from Destaining Solution.

RESULTS

Isolation of Amylase Producing Microbes from Soil Sample
When the starch agar plates were subjected to starch-iodine test, some specific colonies produced clear zone of starch hydrolysis in contrast to the dark blue background where iodine reacts with the starch present in the medium. These specific colonies produce amylase and hence they were revived from the previously preserved nutrient broth for further use. The starch agar plates and the result of starch-iodine test are shown in plate-1.

Identification of various amylase producing microbes
Microscopic examination
Simple staining: The amylase positive microbes were observed to be rod shaped under the microscope when simple staining was performed.

Gram staining: Purple coloured chains of rods were observed under the microscope when gram staining was performed. Hence the organism was confirmed as gram positive.

The results of microscopic examination are shown in plate-2.

Biochemical Tests
The isolated amylase positive culture was characterized by biochemical tests, the results of which are tabulated below-

Table 1: Results of Biochemical Tests.

S. No.	Tests	Result
1	Indole	Negative
2	Methyl-Red	Positive
3	Voges Proskauer	Positive
4	Citrate utilization	Positive

Growth on Selective Media
Yellow coloured colonies were observed on Mannitol-Yolk-Polymixin B plates. Hence the organism was confirmed as *Bacillus Species*. The MYP plates are shown in plate-2.

Amylase Production in Solid State Fermentation
0.4% of culture was inoculated into both the basal medium and basal medium with 10% dry weight of rice husk. It was incubated and amylase production was estimated by DNS method in 24 hours interval for 3 days. The fermentation bottles are shown in plate-3.

Estimation of Produced Amylase by Dns Method
The results for estimation of amylase by DNS method is tabulated below-

Table 2: Estimation of Amylase by Dns Method.

S. No.	Incubation time (hours)	Absorbance at 540nm	
		Basal medium (BM)	BM with rice husk
1	24	0.2	0.1
2	48	0.3	0.15
3	72	0.09	0.08

Graph was plotted for absorbance at 540 nm against time of incubation, which is shown in plate-6. The maximum amylase production and activity was observed in 48 hours of incubation that is calculated to be 7.27 U/ml in normal basal medium and 18.1 U/ml in medium containing rice husk.

Purification of Enzyme Amylase and Characterization

Ammonium sulfate Precipitation: The crude amylase was precipitated and then centrifuged at 3000 rpm for 20 minutes. Supernatant was discarded and the pellet was suspended in 50 mM sodium acetate buffer. The precipitated enzyme is shown in plate-4.

Purification by dialysis method: The precipitated enzyme suspended in sodium acetate buffer was dialyzed against large volume of distilled water and approximately 0.5ml of purified amylase was collected. Dialysis of enzyme is shown in plate-4.

SDS-PAGE Analysis of amylase enzyme: After 2µl of both samples was run on SDS-PAGE, enzyme bands on gel were observed. It was confirmed that approximately 50 kDa enzyme amylase was produced. SDS-PAGE gel is shown in plate-5.

DISCUSSION

Amylase is one of the most widely used industrially significant enzymes which can be produced from various plants and microorganisms. Microbial sources of amylase are of great importance because of the easy manipulation and large scale production. Bacterial amylases have advantages over fungal amylases. Various types of starchy raw substrates are used for the production of amylase. Rice husk is an agro based waste product that can be used as substrate for amylase production in large scale.

Microbial enzymes are widely used in industrial processes due to their low cost, large productivity, chemical stability, environmental protection, plasticity and vast availability (Burhan *et al.*, 2003 and Mishra *et al.*, 2008). In our study we have focused on production of amylase enzyme using economical substrate like agro waste rice husk.

Another study describes the identification and isolation of amylase producing bacteria from soil. They have also studied the influence of different production parameters such as pH, temperature, carbon source and incubation period required for the maximum production of amylase in liquid culture fermentation process. The bacteria isolated from soil were screened for amylase production on starch agar medium. From the soil samples out of 7 bacterial strains only 4 strains showed amylase activity. The four potential isolates were identified by standard morphological and biochemical characterization as *Pseudomonas fluorescens, Bacillus subtilus, E.coli* and *Serratia marscens* (Sethi *et al*., 2013). As concerning to this work, in our study we have isolated *Bacillus Species* from garden soil sample on starch agar plates. We have identified the organism by various biochemical tests and by using Mannitol Yolk Polymixin B selective media.

The bacterial isolate from waste potato dumpsite showing maximum diameter of enzyme activity was propagated in broth supplemented with 1% (w/v) starch medium at incubator shaker at 150 rpm, 370c for 24hrs. After incubation time resultant Broth was centrifuged at 10000, rpm for 10 min. and the supernatant was collected as the source of crude enzyme. The assay was carried out by using soluble starch as substrate few drops of DNS 3-5, Dinitrosalicylic Acid reagent were added and the absorbance was measured at wavelength of 540 nm (Khushwaha *et al*., 2011). Our report has concerned with A. Khushwaha's work. *Bacillus subtilis* isolated from soil sample was propagated in two setups viz. basal medium and 10% (w/v) rice husk containing basal medium. After incubation at room temperature, resultant broth was centrifuged at 3000 rpm for 20 minutes and supernatant was collected and used as crude enzyme. Amylase assay was also carried out by DNS method. Amylase production in rice husk comprising medium was estimated to be 18.1 U/ml in 48 hours of incubation.

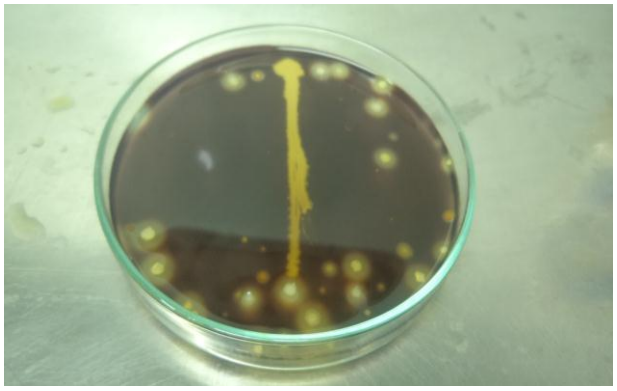
The cost of enzyme production in submerged fermentation is high which necessitates reduction in production cost by alternative methods. The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural byproducts for the reduction of cost of the medium (Anto *et al*., 2006). As concerning to the work of H. Anto, we have used rice husk as a substrate for amylase production. Rice husk is an agro based waste product which is not only economical and easily available but it is also very effective as a substrate for amylase production when given as in 10% w/v along with basal medium. It produces 59% higher concentration of amylase compared to the normal basal medium and hence can be used in large scale production of amylase.

Alpha amylase obtained from 48 hours of fermented extract was characterized in 8% native gel. The observation of the gel profile it was evident that, relative to the crude extract, 30–70% $(NH_4)_2SO_4$ fraction was enriched in protein. This also corroborates with quantitative determination of amylase activity. It was observed that 0–30% $(NH_4)_2SO_4$ fraction had relatively poor amylase activity with respect to its protein content (Raul *et al*., 2014). Our report has concerned with the

work of D. Raul. We have purified the fermented crude amylase enzyme extract by ammonium sulfate precipitation method and dialysis method. Then SDS-PAGE was also performed for characterization of the enzyme which determined the molecular weight of the enzyme as 50 kDa. As concerned to the work done by (Shinde *et al*., 2014) in our study the enzyme purification and characterization was done by ammonium sulfate precipitation, dialysis and SDS-PAGE analysis.

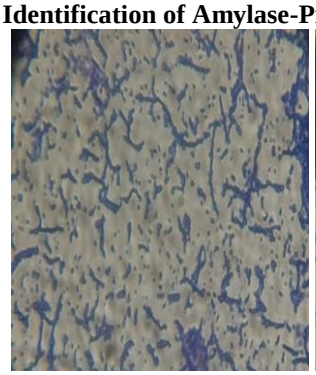
Plate 1
Isolation of Amylase Producing Microbes from Soil Sample

Plating On Starch Agar Plates

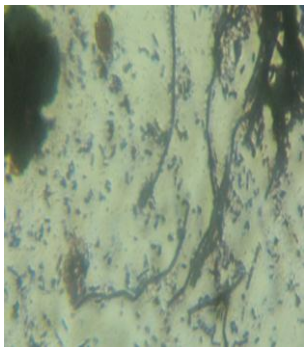


Starch-Iodine Test

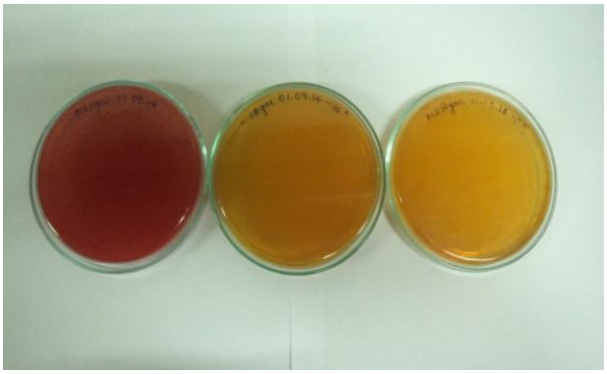
Plate 2
Identification of Amylase-Producers



Simple Staining



Gram Staining



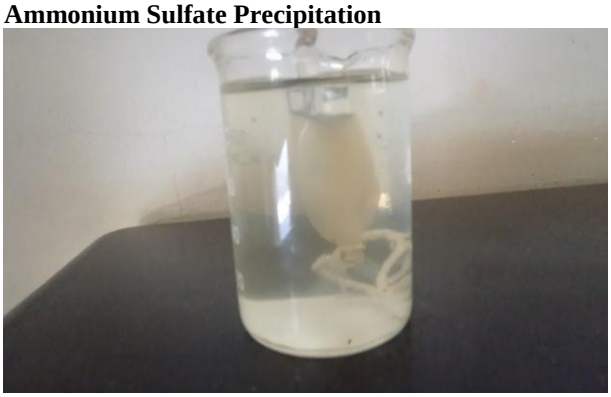
Growth in Selective Medium, MYP - Plates

Plate 3
Enzyme Production

Collection and Preparation of Rice Husk Substrate

Solid State Fermentation in Basal Medium and Rice Husk Substrate in Basal Medium

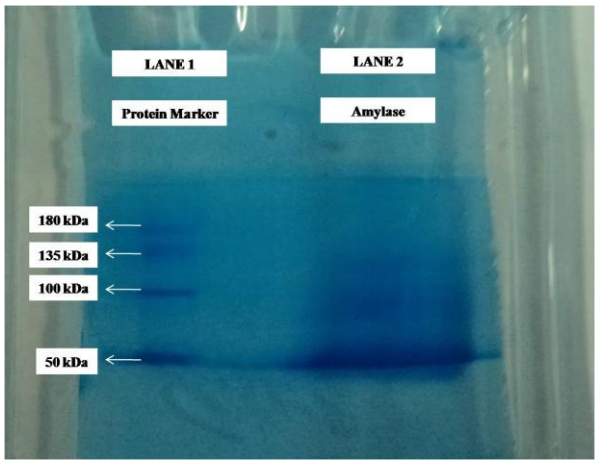
Plate 4
Extraction and Purification
Ammonium Sulfate Precipitation



Dialysis

Plate 5

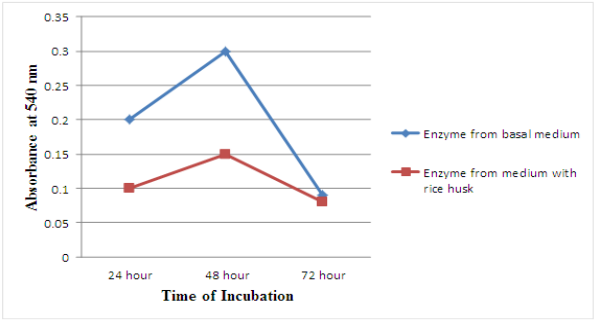
Purified Amylase Enzyme



SDS-Page Analysis

Plate 6

Graph for Estimation of Amylase by DNS Method



CONCLUSION

The amylase producing microorganism was isolated from garden soil sample, characterized and subjected to fermentation for amylase production. Optimum time for production of bacterial population was observed to be 48 hours. Rice husk substrate concentration given was 10% w/v. *Bacillus species was* found to be most frequently occurring amylolytic bacteria in soil sample. Though some preliminary enzymatic parameters like pH optimum and temperature optimum have been determined for alpha amylase from 48 hours of SSF at 37^0C, complete purification as well as molecular weight determination is also performed. The activity of the enzyme was estimated by DNS method for both the basal medium and rice husk as substrate and it was found to be 59% higher in the medium with rice husk as a substrate that is 18.1 U/ml and 7.27 U/ml in normal basal medium. At present, with the goal to purify alpha amylase, partial concentration of enzyme was achieved through 80% ammonium sulfate precipitation. Further work for complete purification of alpha amylase was conducted with the aid of other purification techniques like dialysis method and SDS-PAGE to check purity and approximate molecular weight which was approximately 50 kDa.

Alpha-amylases are one of the most widely used enzymes 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. With increase in its application spectrum, the demand is for the enzyme with specificity. From the present study it can be concluded that production of α-amylase carried out in solid state fermentation with rice husk as a specific substrate is being looked at as a economical potential tool for large scale amylase production. Rice husk is an agro-waste product hence it can be used as an effective and economical substrate for the commercial production of amylase enzyme.

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