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Screening of amylase producing microbes from rhizosphere soil and its potential application in baking bread

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Abstract
Improvement of microbial strains for the production of industrial products has been a hallmark of all commercial, microbial fermentation processes. The present investigation deals with the isolation and characterization of microbes for the enhancement of seed germination collected from Coimbatore. Out of 5 isolate were selected by screening test. Based on morphologically, and biochemical characterization the isolates were identified as *Pseudomonas* sp, *Bacillus* sp, *Ecoli* sp, *Proteus* sp, *Shigella* sp. The isolated bacterial strains were subjected to mutation process for strain improvement by sequential treatment with physical agents (UV) and chemical agents (EMS). These mutant strains were subjected for the production of lytic enzymes like protease and amylase. Amylase is an enzymes which breaks the starch into sugar, usually important constituent of the human diet. These amylase enzymes are used for the baking industries for baking bread. Further, it was subjected for the application of baking industries for making bread by using amylase enzymes.

Keywords: mutagenesis, UV, EMS, rhizosphere soil, lytic enzymes, protease, amylase

Introduction
Rhizosphere is a zone between the root surface and soil, and adjacent to the roots. The bacteria, which live in this zone may remain in the soil but adheres to the roots after gentle shaking. (Ferris *et al.,* 1992) [17].

For industrial use, enzyme must be produced at low cost and should be reusable and reproducible. To achieve this, many techniques have been developed for strain improvement. Strain improvement is usually done by mutating the microorganisms that produces the enzymes by techniques such as classical mutagenesis which involves exposing the microbes to physical mutagens such as ultra-violet rays, ethyl Methane Sulfonate (EMS), ethidium Bromide (EtBr).

Physical Mutagenesis
Ultra Violet Radiation
UV irradiation is a disinfection method that uses short wavelength ultraviolet light to kill or inactive microorganisms by destroying nucleic acids and disrupting their DNA. Ultraviolet irradiation (UV) is the best mutagenic agent in prokaryotic organisms. It also characterized as the most proper mutagens to be employed in obtaining high yielding productive strains.

For industrial use enzyme must be produced at low cost and should be reusable and reproducible. To achieve this many techniques have been developed for strain improvement. Strain improvement is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, Ɣ-rays, UV rays, etc., and chemical mutagens such as NTG, EMS, EtBr, etc. (Parekh *et al*.,2004) [39].

Chemical Mutagenesis
Ethyl Methane Sulfonate (EMS)
Ethyl methane sulfonate is a mutagenic, tertagenic, and possibly carcinogenic organic compound with the formula $C_3H_8SO_3$. EMS is often used in genetics as a mutagen. Mutations induced by EMS can be studied in genetics screens or other assays. The mutations may also produce mutant proteins with intersecting properties or enhanced or novel functional that may be of commercial uses.

Lytic Enzymes
Lytic enzymes present a range of applications in industrial biotechnology: in controlling the development of food pathogens (Oliveira *et al*., 2012), in the release of enzymes from lactic acid bacteria to cheese ripening, and in the release of other intracellular products such as polyhydroxybutyrate produced by *Bacillus* sp. (Loessner 2005).

Amylase and its Application
Amylase is an enzymes that breaks down starch into sugar, starch the primary stage polysaccharide is an important constituent of the human diet.

In the food industry amylolytic enzymes have a large scale of

applications, such as the production of glucose syrups, high fructose corn syrups, maltose syrup, reduction of viscosity of sugar syrups, reduction of turbidity to produce clarified fruit juice for longer shelf-life, solubilisation and saccharification of starch in the brewing industry.

The present study was focused on strain improvement of bacterial strain through mutation and to assess the enhancement of the production of amylase enzymes from both mutant strains and wild strains to assess the higher production of amylase enzymes for industrial uses.

Materials and Methods

Sample Collection

Sample was collected from Hindusthan gardens, situated in Coimbatore, Tamil Nadu, India. The soil sample were collected in sterile plastics bags and labelled properly.

Strain Isolation and Characterization

Serial dilutions were used for the isolation of bacteria and the sample were plated on nutrient agar and incubated at 37˚C for 24 hours. After successful growth, microorganisms were identified on the basis of colony morphology and named as S1, S2, S3, S4, S5 respectively.

Morphological Characterization

Gram staning were performed to observe the cellular morphology and gram nature of the isolated 5 bacterial strains. (Jahir *et al*., 2011).

Biochemical Characterization

The bacterial isolates were characterized by biochemical tests such as Indole production test, Methyl Red test, Voges Proskauer test, Citrate Utilization test, Nitrate Reduction test,Catalase test, Carabohydrate test, Urease test, Oxidase test, Hydrogen Sulphide test, and Triple Iron Sugar test by standard protocol by Sherman *et al.,* (2005).

Strain Improvement by Mutagenesis

UV Mutagenesis

The isolates of 5 bacterial strains were subjected to UV mutagenesis at the range of 260nm for 10 minutes. After 10 minutes 0.1 ml of UV treated cultures were transferred to nutrient agar plates to observe growth strain improvement.

EMS Mutagenesis

The mutagenesis of the bacterial strains were subjected to EMS mutagenesis. The pellet were washed thrice with sterile buffer of 0.1 ml of tris- Hcl buffer (p^H 7.5) and resuspended in 1.5ml of same buffer, the microfuge tube is kept on ice bags for few minutes. And 1.5µl of EMS was added to the strains incubated on water bath for 30˚C for different time intervals. The cells were washed with same buffer. The mutagenesis samples were serially diluted and plated on nutrient agar plates.

Production of Amylase

The mutagenesis of the bacterial strains were subjected for the production of amylase production. Broth was prepared and inoculated with loopful of culture and it was incubated for 24-48 hours at 37°C and starch hydrolysis agar plates were prepared and the culture were streaked in plates. And 2-3 drops of iodine solution were added to the culture plates and zone of incubation was observed.

Quantitative Method

Spectrophotometric Analysis

Broth was prepared and inoculated with loopful of cultures and incubated for 24-48 hours at 37° C. And the bacterial strains were observed for amylase production. Enzyme activity was read at UV-VIS spectrophotometric at range 640 nm.

Partial Purification of Amylase Enzymes

Amylase production medium was prepared and sterilized inoculated with isolated strains of amylase production in bacteria and the culture was inoculated in shaker at 37°C for 48hrs. After the time of incubation solution was centrifuged and supernatant was collected which is used for the source of crude enzymes and the crude enzymes was inoculated and used for the further purification process.

Ammonium Sulfate Precipitation

20ml of the crude enzymes was prepared and the mixture was left overnight at 4°C by using a magnetic stirrer and the solution was centrifuged and pellet was collected and the pellet was mixed with the 10ml of 50_mM sodium acetate buffer.

Dialysis

Dialysis membrane of 110 KDa was selected. One end was tighten with the help of thread and the sample was loaded leaving a sufficient head space and other tighten and immersed in sodium acetate buffer (10mm. P^H5.5)and it was kept in magnetic stirrer and the buffer was changed in an interval of 3.7 and for 24 hours.

Molecular Determination of SDS-page

Glass plates was washed and coated with petroleum jelly. Spacer strips was arranged properly to avoid leakage and the volume of the gel solution required for making separating gel was calculated for the number of protein samples. APS and TEMED were added for pouring the gel between two plates. Allow the el to polymerize for 30-60 minutes.

Application for the Enzymes Amylase

Baking Bread

The enzyme activity was done and the application was done for the amylase for baking bread.

Results and Discussion

Sample Collection

Rhizosphere soil sample was collect from Hindusthan gardens, Coimbatore.

Fig 1: soil sample collected from rhizosphere region of plant root.

Isolation of bacteria from rhizosphere soil

The 5 bacterial strains were isolated from rhizosphere soil, on the basis of morphology and appearance by nutrient agar plates it is followed by serial dilution. The 5 bacterial isolates named as RS1, RS2, RS3, RS4, RS5.(Ebtesam EL- bestawy *et al*., 2003) It was reported that, the bacterial isolates was done from plates with separate colonies through culturing and re-culturing using streaking method followed by serial dilution.

Fig 2: isolation of bacterial colonies from rhizosphere soil by serial dilution method.

Morphology Characterization

Gram's staining

The results of morphology characterization of gram's staining, it was observed that 1 isolates were identified as gram negative bacteria, and 4 isolates were identified as gram positive. Bacterial isolates resulted in table

Table 1: Gram's staining of isolated bacterial strains

List of bacteria	Gram nature	Shape
RS1	+	Rod
RS2	-	Rod
RS3	-	Rod
RS4	-	Rod
RS5	-	Rod

RS= Rhizosphere soil, += positive,-= negative

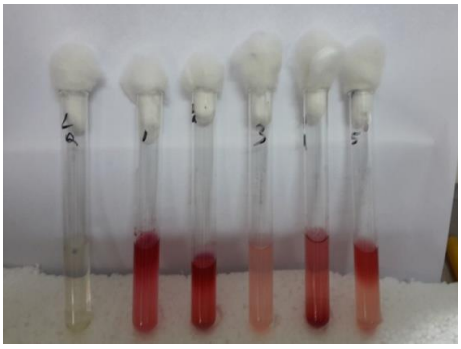
Biochemical Characterization

Table 2: biochemical characterization of isolated bacterial strains.

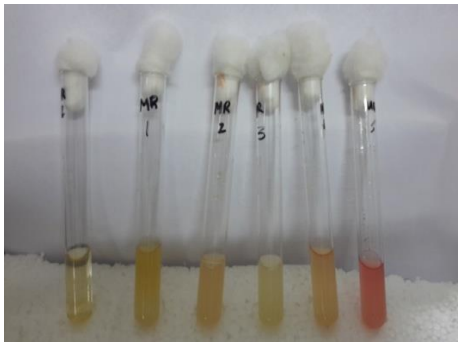
Paramaters	S1	S2	S3	S4	S5
Gram staining	-	+	-	-	-
Morphology	rod	Rod	rod	rod	Rod
Indole production test	+	-	-	-	+
MR test	-	-	-	-	+
VP test	-	-	+	-	-
Citrate utilization test	+	+	+	-	-
Carbohydrate fermentation test	-	-	-	-	+
TSI test	-	+	+	-	-
Oxidase test	-	-	+	-	+
Urease test	+	-	+	-	+
Catalase test	-	-	-	+	-
Nitrate reduction test	-	+	-	-	-
Hydrogen sulphide test	-	-	-	-	+

MR = Methyl Red, VP = Voges- Proskauer, TSI = Triple Sugar Iron. + = Positive, - = Negative

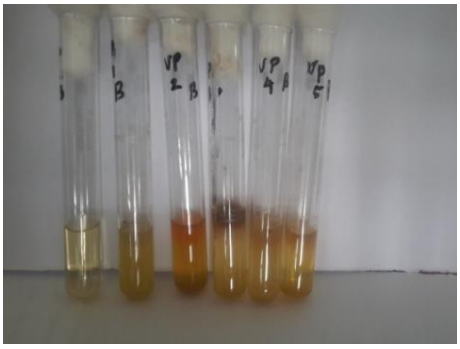
Biochemical Test for the Bacterial Strains



Indole production test

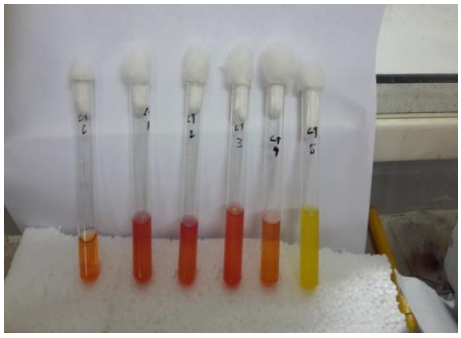


methyl- red test

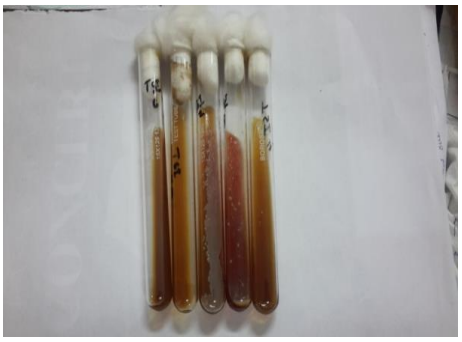


Voges proskauer

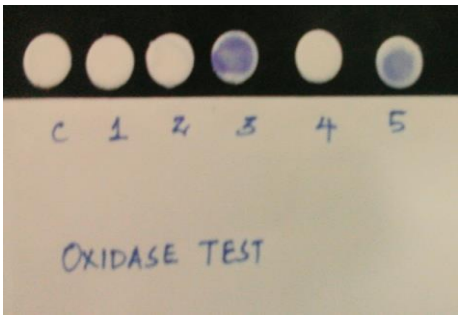
Citrate utilization test



Carbohydrate test

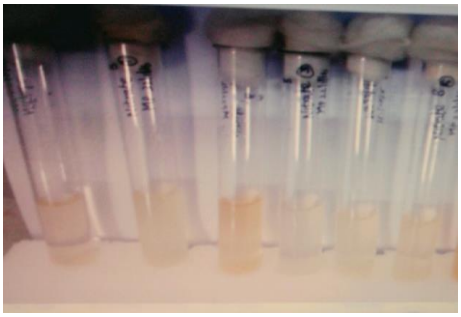


Triple iron sugar



Oxidase test

Urease test



Nitrate reduction test

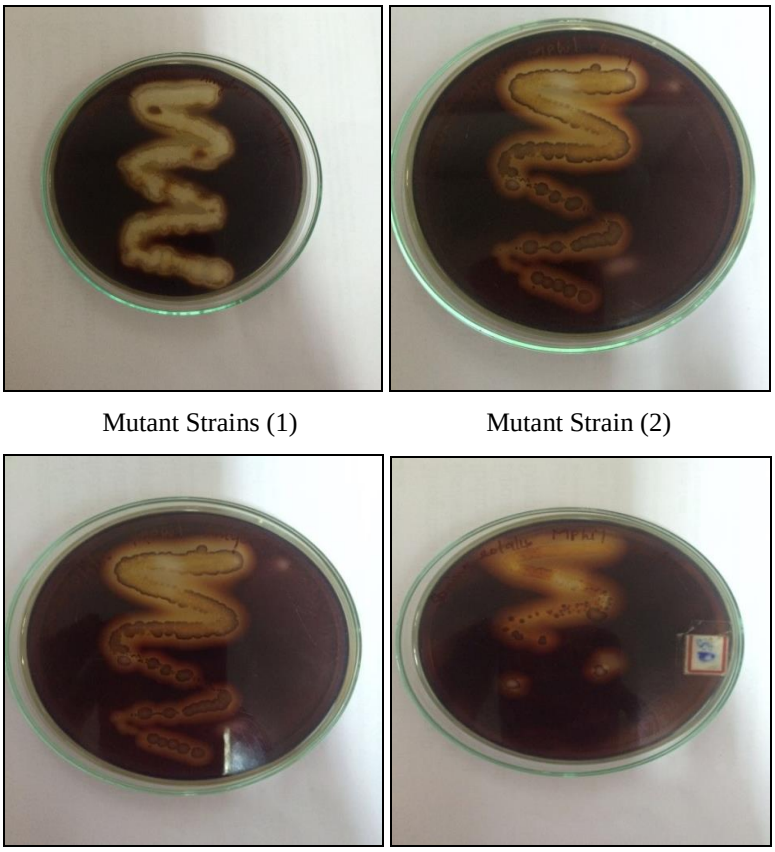
Fig 3

Screening of Lytic Enzymes Producing Bacteria.

In order to isolate the positive producing of lytic enzymes from 5 bacterial isolates was streaked in two different media. To observe clear zone of incubation for both amylase and protease activity. The present study revealed the production of lytic enzymes viz. amylase, protease, activity for both wild strains and mutant strains. These enzymes produced by microorganism for the biocontrol purposes in laboratory. This type of production is studied by (Geetha *et al*., 2014).

Comparsion Studies in Amylase for Both Wild Strains and Mutant Strains.

The five bacterial isolates produces amylase for both the wild strain and mutant strain. The clear zone of incubation observed in starch hydrolysis agar. Among all the five bacterial wild strains, two strains (RS1, RS2) showed moderate intensity of clear zone of incubation. Three strains (RS3, RS4, RS5) showed less intensity of clear zone of incubation. As compared to wild strains, three mutant strains produce clear zone of incubation (RS1, RS2) produced from high intensity of amylase activity and other three strains (RS3,RS4 RS5) had showed low intensity of amylase activity. This means wild strain of two isolates (RS1, RS2) produce amylase activity, as compared to the wild strains, mutant strains of three isolates (RS1, RS2) produced high production of amylase activity.



Mutant Strains (1) Mutant Strain (2)

Wild Strains (1) Wild Strains (2)

Fig 4

Quantitative Analysis for Amylase

Among five bacterial strains, wild strains and mutant strains where compared for UV spectrophotometer analysis at 540 nm. Wild strains showed in (ws1, ws2) moderate intensity of growth whereas in mutant strains (ms1, ms2) showed high intensity of growth but there is no growth observed in (ms3, ms4, ms5).

Table 3: Quantitative Analysis for Amylase

Sample (wild strains)	OD value (640nm)
wS1	0.714
wS2	O.317

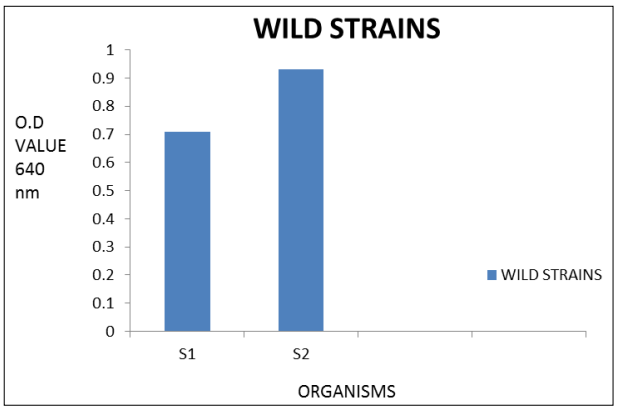
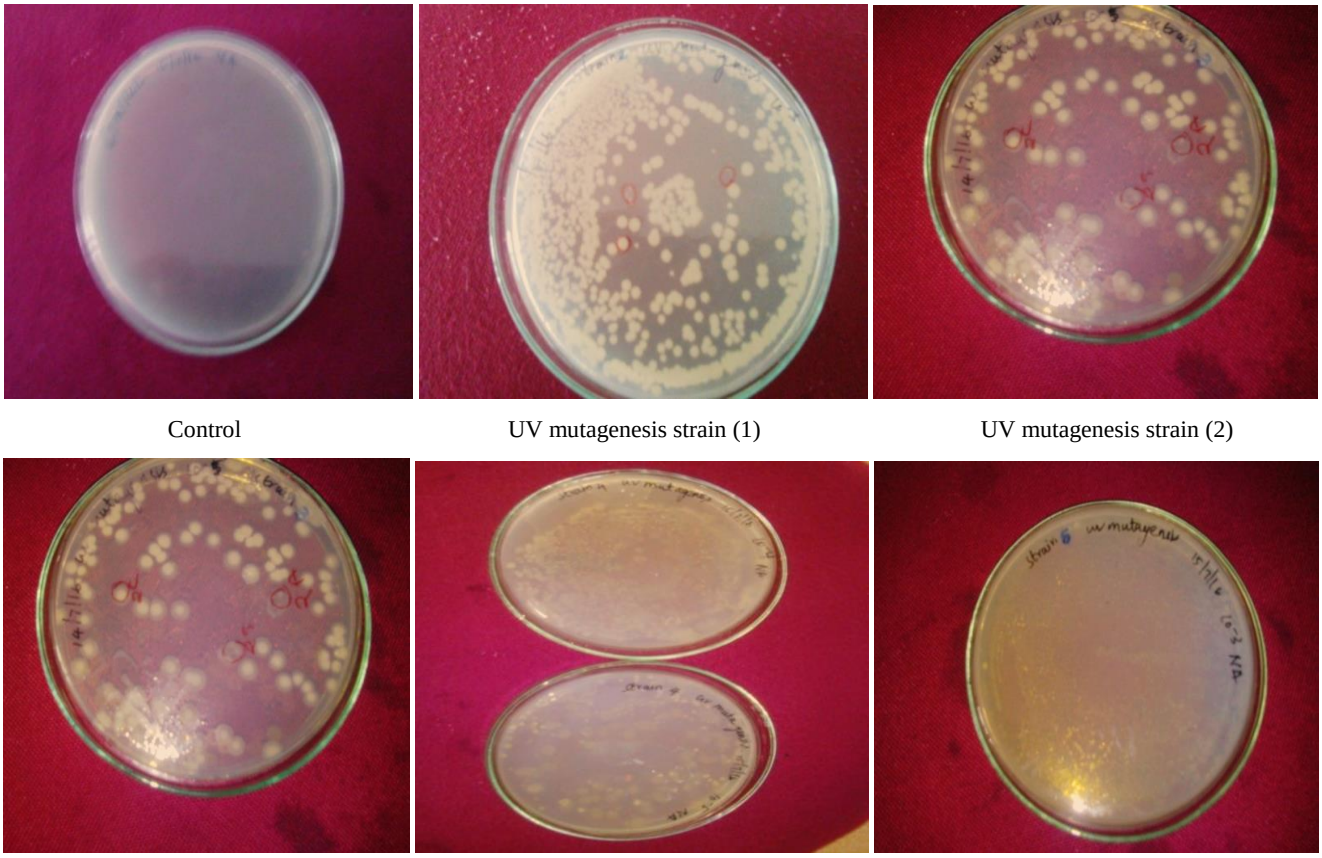


Fig 5: Quantitative analysis for amylase

Strain Improvement

UV Mutagenesis

The UV isolates were plated on nutrient agar. In the present investigations Potent UV strains which showed more growth in UVs1, UVs2, UVs3, UVs5 and no growth was observed.



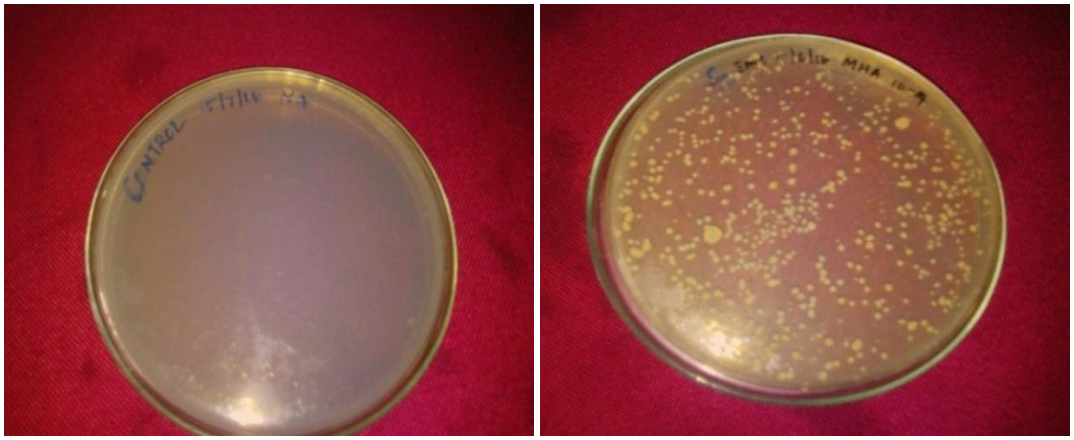
Control | UV mutagenesis strain (1) | UV mutagenesis strain (2)

UV mutagenesis strain (3) | UV mutagenesis strain (4) | UV mutagenesis strain (5)

Fig 6

EMS Mutagenesis

The EMS isolates were plated on nutrient agar. In the present investigations out of five potent EMS strains which showed moderate growth in (ES2, ES3) and where as in strains (ES1, ES4, ES5) showed average growth. When compared to the EMS and Wild strains, UV mutant strains showed higher growth.



Control | EMS Mutagenesis

Fig 7

Partial Purification of Lipase

Crude enzyme preparation

The culture was grown in the optimized medium described in above for a period of 48 h. At the end of incubation, the bacterial cells in the fermentation broth were removed by centrifugation at 10,000 rpm for 20 min at 4˚C. The cell-free supernatant was used as a crude enzyme for precipitation and purification process.

Ammonium sulfate precipitation

The total protein was taken from the cell free culture filtrate (400 ml) of 48 h old of all 5 bacterial cultures. The protein was precipitated using ammonium sulfate fractions from 20-80% (w/v). The 40–60% (w/v) of ammonium sulphate fractions showed higher lipase precipitation activity than the rest of the fractions. The activity was not observed from 80% (w/v) saturation.

Dialysis

Purification of the precipitated enzyme was subjected to purification process. Purification was done with the help of 0.2 µm dialysis membrane.

Molecular Weight Determination by SDS-page

The purity of lipase was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% of gel. On SDS-PAGE, the purified lipase were showed a different molecular weight, because it was produced from different bacterial strains (Figure 9). Middle molecular weight of standard protein marker used as ladder. Molecular weight of lipase from different bacterial strains, were determined.

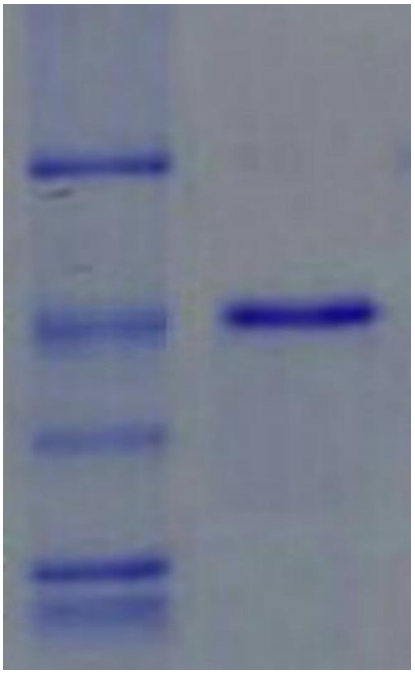
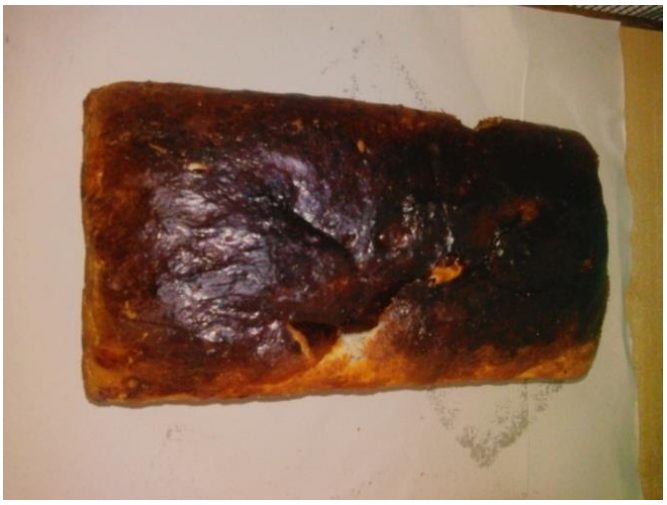


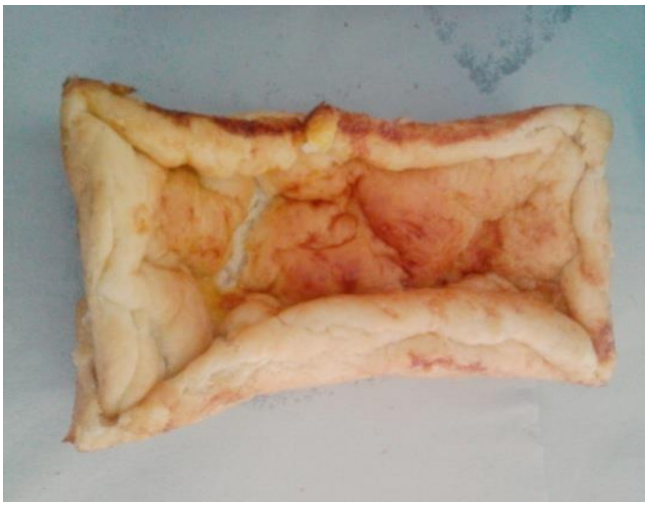
Fig 8

Application of Amylase

Amylase showed high activity in *Bacillus* sp, this strain used for the baking bread and used as the amylase as a source. When compared to the yeast bread, amylase enzymes showed good results. By using amylase as an enzymes it showed softened, easy to cut by knife, spongy, and life span of the amylase enzymes is about 3-5 days.



Yeast bread



Amylase bread

Fig 9

Discussion

Isolation of Bacteria from Rhizosphere Soil

Totally five bacterial strains were isolated from rhizosphere soil. Individual colonies were identified by serial dilution and nutrient agar platting method. Earlier study it was reported that 10 bacterial strains were isolated from rhizosphere soil from rice field by serial dilution technique (Chulwuma Simon *et al*., 2014)

Morphology Characterization

Gram staining were done totally 5 bacterial strains were isolated; it was observed one isolates were identified as Gram positive and four isolates were identified as Gram negative.

Biochemical Test

Through biochemical test it was identified as RS1 (*Escherichia coli* sp), RS2 (*Proteus* sp), RS3 (*Bacillus* sp), RS4 (*Pseudomonas* sp), RS5 (*Klebsiella* sp).

Screening of Lytic Enzymes Producing from Bacteria

To observe the zone of incubation for both amylase and protease enzymes activity. (Moses and Cape *et al*., 1991).

Production of Amylase (Qualitative Analysis)

Among five bacterial isolates, plating method was analyzed for both wild and mutant strains. At wild strains zone of incubation were observed for strain (RS1, RS2) showed moderate growth when compared to wild strains mutant strain (RS1, RS2) showed high growth.

It was reported that that wild strain and mutant strains are used for the production of amylase activity. Mutant strain have showed highest increased production of amylase for the industrial laboratory where as in the wild strain showed only minimum of growth activity in amylase production (M. Prabakaran *et al*., 2009) [37].

Strain Improvement

The isolates UV treated bacterial strains were plated on nutrient agar, when compared to the EMS, wild strains showed an increased growth rate. UV treated four strains S1, S2, S3, S5 showed high growth and no growth was observed in S4. Of five strains that were EMS treated, S2, S3 showed minimum growth, and growth observation was not found in strains S2, S4, S5. Earlier it was reported that EMS treated bacterial strains showed higher growth when compared to yeast strains (Sivasakthi *et al*., 2013) [51]. This is related to my current investigations.

Partial Purification of the Produced Amylase Enzyme

Crude enzyme was purified by ammonium sulfate precipitation method followed by dialysis. Reported that purification of the lipase enzyme, was done by ammonium sulfate precipitation and dialysis method.

Application for Baking Bread

Amylase enzymes were used as a source for baking bread, it was reported that, amylase producing enzymes produce good texture, softened, weighted than the yeast bread. Earlier study reported that, amylase enzymes produce good results when compared to the baker's yeast. (Prabakaran *et al*., 2009) [37].

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