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Screening of Multifaceted Thermophilic Actinomycete AASD15 for Bioactive Molecules and Metal Nanoparticles

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Abstract

Thermophilic Actinomycetes are one of the most widely distributed group of Gram positive, mainly aerobic filamentous bacteria present in compost, peat ,hot spring etc. Thermophilic Actinomycete AASD15 was isolated from soil sample collected from Pashan Lake, Pune, which grew at 55 °C and tolerated 10% of NaCl concentration. Chalky white colony morphology was seen of the isolate AASD15 on modified CSA. The slide culture of the isolate showed non-extensive branching with spores. Activity of extracellular enzymes *viz.* Pectinase, Tyrosinase, Asparginase and Thermolysin like Protease (TLP) were 28.88U/ml, 6.55 U/ml, 3.96 U/ml and 8.85 U/ml respectively. Approximate molecular weights of Pectinase, Tyrosinase, Asparginase and Thermolysin like Protease (TLP) (SDS-PAGE) were 34 kDa, 15 kDa, 135 kDa and 36 kda respectively. A white precipitate was produced by TLP from AASD15 by Biotransformation/Condensation reaction of two amino acids *viz*. Z-L-aspartic acid and L-Phenylalanine methyl ester which might be Aspartame (Artificial sweetener). Zinc nano particle was also produced from Zinc salt solution by AASD15 which shows maximum absorbance at 300nm.

Keywords: Thermophilic Actinomycetes, Asparginase, Pectinase, Tyrosinase, Thermolysin like Protease (TLP), Zinc nanoparticle.

Introduction

Actinomycetes are a group of prokaryotic organisms belonging to subdivision of the Gram-positive bacteria phylum. All members of this order are characterized in part by high G+C content (>55mol %) in their DNA. Actinomycetes are ubiquitous in nature and are well known as saprophytic soil inhabitants. The soil actinomycetes produce a volatile compound called geosmin, which literally translates to “earth smell”. This organic substance contributes to the typical odour one gets when rain falls on soil (Uzel, A., et al 2011). Actinomycetes may be aerobic or anaerobic, although the thermophilic forms are primarily aerobic.

The majority of Actinomycetes are mesophilic, growing at temperatures ranging from 18°C to 40°C. A few Actinomycetes are thermophilic, which means that they have an optimum temperature for growth at 55°C and above. (Gousterovac, A., et al 2014).

Thermophiles are the most primitive organisms which are important biotechnologically for thermostability, less incubation time, early sporulation and immense industrial feasibility. (Gousterova, A., et al. 2014). Habitats of thermophiles are mostly pristine thermal springs and meteorite craters. Thermoactinomycetes

constitute an extensive and diverse group of Gram-positive, aerobic, largely mycelial bacteria, many of which are commercially important in the production of bioactive molecules (Anusuya .D, Geetha .M 2012, Jaralla, E.M. et al 2014). Thermophilic Actinomycetes are especially suitable for industrial application due to their rapid growth rate and high production rate. (Gousterova, A., et al. 2014). With the growth of industrialization, demand of thermostable enzymes has increased tremendously due to its high thermostability and feasibility to the processes involved .One of the obvious advantages of carrying biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. (Gousterova, A., et al. 2014). Synthesis of bioactive molecules at the high temperatures has significant influence on the bioavailability. Thermostable enzymes can also be used as models for understanding the basis of thermostability (K. Salahuddin et al. 2011).

Different types of bioactive molecule can be produced by thermophilic actinomycetes such as commercially viable enzymes *viz.* Thermolysin (EC 3.4.24.27) like protease, a thermostable neutral metalloproteinase enzyme produced by the Thermophilic actinomycete, *Thermoactimyces thalpophilus* requires one zinc ion for enzyme activity and four calcium ions for structural stability. Thermolysin specifically catalyzes the hydrolysis of peptide bonds containing hydrophobic amino acids. However thermolysin is also widely used for peptide bond formation through the reverse reaction of hydrolysis. Thermolysin is the most stable member of a family of metalloproteinases. The enzyme brought about a revolution in industrial enzymology since 1979 when Isowa (Japan) showed the enzymatic peptide synthesis of the artificial sweetener, *viz.* aspartame, through biotransformation/condensation reaction of two amino acids involving Z-L-Aspartic acid and L- Phenyalanine methyl ester by thermolysin (Majumder, D.R. and Kanekar,P.P.2012, Majumder, D.R. et al, 2013).

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase catalyzing the o-hydroxylation of monophenols to the corresponding catechols (monophenolase or cresolase activity) and the oxidation of monophenols to the corresponding o-quinones (diphenolase or catecholase activity). The tyrosinases are produced by actinomycetes. It is involved in the biosynthesis of melanin and catalyses the ortho-hydroxylation of tyrosine (monophenol) to 3,4-dihydroxyphenylalanine or DOPA (o-diphenol) and the oxidation of DOPA to dopaquinone (o-quinone). This o-quinone can then be transformed into melanin pigments through a series of enzymatic and non-enzymatic reaction. Thus, tyrosinase catalyses both the ortho-hydroxylation of monophenols (cresolase or monophenolase activity) and the two-electron oxidation of o-diphenols to o-quinones (catecholase or diphenolase activity) (Popa, C. and Bahrim, G., 2011, Raval, K. M. et al, 2012).

L-Asparaginases (EC 3.5.1.1) catalyse the hydrolysis of L-asparagine into L-aspartate and ammonia. The asparaginases are produced by actinomycetes. These are useful anti-leukaemic agent. Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine (Swain et al. 1993; Manna et al. 1995). Hence, they are not capable of producing L-asparagine and mainly depend on the L-asparagine from the circulating plasma pools. Supplementation of L-asparaginase results in continuous depletion of L-asparagine. Under such environment, cancerous cells do not survive. This phenomenal behavior of cancerous cells was exploited by the scientific community to treat neoplasias using L-asparaginase. This enzyme is also a choice for acute lymphoblastic leukemia, lymphosarcoma and in many other clinical relating to tumor therapy in combination with chemotherapy (Dange, V.U. and Peshwe, S.A., 2013, Deshpande, N.M., 2014).

Pectinases are very important in vegetable and fruit processing industries. Pectinase (EC 3.2.1.15) is an enzyme that breaks down pectin, a polysaccharide found in plant cell walls. Commonly referred to as pectic enzymes, they include pectolyase, pectozyme and polygalacturonase. One of the most studied and widely used commercial pectinases is polygalacturonase. It is useful because pectin is the jelly-like matrix which helps to cement plant cells together and in which other cell wall components, such as cellulose fibrils, are embedded. Therefore pectinase enzymes are commonly used in processes involving the degradation of plant materials like clarification of fruit juices. Pectic substance is a polysaccharide composed of α-1, 4-linked D-galacturonic acid (Praveen Kumar G, and Suneetha V., 2015).

Physical incineration and chemical processes using strong acids are hazardous as well as expensive for treatment of e-waste which contains heavy metals such as silicon, arsenic, iron, copper, aluminum, lead, zinc, chromium, cadmium, mercury and barium. These heavy metals are viable option for recycling.

Nanoscience has revolutionized nanotechnology especially in the field of medicine. Biological processes using microorganisms and plant extracts for synthesis of metal nanoparticles have been suggested as valuable alternatives to traditional methods. (Majumder, D. R., 2012). Metal nanoparticles such as gold, silver, copper, titanium and zinc synthesized by actinomycetes have found many applications in the fields of diagnostics and therapy. (Waghmare et al. 2011, Majumder, D. R., 2013).

Materials and Methods

Sample Collection: Soil sample was collected from Pashan Lake Pune.

Isolation of Thermophilic Actinomycete: The soil sample 1 gm was subjected to serial dilutions 10^{-1} to 10^{-5}. Dilutions were cultured 10^{-5} and 10^{-4} on modified casein starch agar plates. The plates were incubated at 55° C for 3 days. Isolate AASD15 was characterized by Morphological (Gram staining, Slide culture) physiological (optimization by Temperature and NaCl Concentration).

Screening for Extracellular Enzymes:

L-Asparaginase Activity: The L-Asparaginase plate assay method was adopted to screen isolate AASD15 for L-asparaginase activity on modified Czapek Dox's (MCD) agar medium.

Composition of Modified Czapek Dox's (MCD) agar medium (g/L):

Glucose—2.0, L-asparagine—10, KH_2PO_4—1.52, KCl —0.52, $MgSO_4 \cdot 7H_2O$— 0.52, copper nitrate ($CuNO_3 \cdot 3H_2O$)—0.001, $ZnSO_4 \cdot 7H_2O$—0.001, and $FeSO_4 \cdot 7H_2O$—0.001, pH-6.2.

Phenol red indicator (0.009%) was added from a stock solution of 2.5% of the dye in ethanol. The plates were incubated at 55° C for 3 days. The phenol red indicator was used to check the ability of test isolate AASD15 for production of L-Asparaginase.

Asparaginase Estimation by Nesslerization: L-asparaginase activity was measured by the modified method of Imada et al. (1973). The isolate AASD15 was grown for 3 days at 55°C in liquid modified Czapek Dox (CD) medium. A reaction mixture containing 0.5 mL of 0.5 M tris HCl buffer (pH 8.2), 0.1 mL of 40 mM L- asparagine, 1.0 mL of suitably diluted enzyme source (culture filtrate of Isolate AASD15) and 0.4 mL of distilled water (total volume of 2.0 mL) was incubated at 55°C for 30 min. The reaction was terminated by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). Blank tubes were prepared by adding the enzyme source after the addition of TCA. After termination of the reaction, 3.7 ml of distilled water and 0.2 mL of Nessler's reagent were added to 0.1 mL of the above reaction mixture and incubated for 20 min. The amount of ammonia released during the reaction was determined by measuring the absorbance at 450 nm. One international unit (IU) of L-asparaginase is the amount of enzyme needed to liberate 1 µmol of ammonia in 1 min at 27°C (Imada et al. 1973).

$$\text{Units/mL enzyme} = \frac{(\mu\text{mole of NH3 liberated})\ (2.5)}{(0.1)\ (30)\ (1)}$$

2.5 = Initial volume of enzyme mixture (mL)
0.1 = Volume of enzyme mixture used in final reaction (mL)
30 = Incubation time (minutes)
1 = Volume of enzyme used (mL)

Tyrosinase Activity: The Tyrosinase broth and plate assay method was adapted to screen isolate AASD15 for Tyrosinase activity on tyrosine broth and tyrosine agar. Composition of tyrosine broth (g/l): 0.1gm Tyrosine dissolved in 100 ml distilled water and a few drops of chloroform to prevent the loss of tyrosinase if produced by the isolate and having pH 7.In broth assay method a loopful of the isolate AASD15 were inoculated in 0.1% tyrosine water Incubation was done at 55°C for 24 to 48 hrs. When tyrosinase is produced, broth colour changes from colourless to pink to brown and ultimately black due to melanin production.

Composition of tyrosine agar (g/l): Peptone 0.5%, Beef extract 0.3%, Agar 2%, L-tyrosine 0.5%, Distilled water 100 ml pH 7.The isolate AASD15 were spot inoculated on tyrosine agar plate. Incubation was done at 55°C for 24-48 hrs. When tyrosinase is produced, colour of the medium around the growth changes from colourless to pink to brown and ultimately black due to melanin production.

Tyrosinase Assay: Spectrophotometer was adjusted to 280 nm and 55°C. Following solutions were pipetted into test tube in the following order:

Solution to be added	Amount (ml)
0.5 M phosphate buffer, pH 6.5	1.0
0.001 M L-tyrosine	1.0
Reagent grade water (MR)	0.9

The reaction mixture was oxygenated by bubbling oxygen through a capillary tube for 4-5 minutes. Absorbance was recorded at 280 nm for 4-5 minutes to achieve temperature equilibration and to establish blank. 0.1 ml of the supernatant of the enriched culture in tyrosine broth (presumably expected to be producing tyrosinase) was added to the reaction mixture and absorbance was recorded for 10-12 minutes. A non-linear "lag" of 2-3 minutes can be expected. Calculation of enzyme activity was done using the following formula:

$$\text{Units of enzyme/ ml} = \frac{(\Delta \text{ A 280 nm/min Test} - \Delta \text{ A 280 nm/min Blank}) \text{ X (df)}}{(0.001)\ (0.1)}$$

Pectinase Activity: The Pectinolytic plate assay method was adapted to screen isolate AASD15 for Pectinase activity on Congo red pectin agar medium.

Composition of Congo red pectin agar medium (g/L): Pectin– 1.0g, Yeast extract – 0.5 g, Agar –1.5 g, Congo red –0.0012 g, D/W – 100 ml.

The isolate AASD15 were spot inoculated on Congo red pectin agar plate. Incubation was done at 55 ° C for 3 days A clear zone around the isolate AASD15 were indicater of pectinase activity.

Pectinase Assay: Polygalacturonase activity was determined by quantifying the amount of reducing groups expressed as galacturonic acid units, liberated during the incubation of 1 ml of 1% (w/v) pectin, prepared in 0.2 M phosphate buffer (pH 7.2) with 500 µl of the enzyme at 55 °C for 30 min, by DNSA method. One unit of polygalacturonase activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute under standard assay conditions and expressed as units per litre (U/l). Specific activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute per milligram of total enzyme protein and expressed as units per milligram (U/mg).

Protease activity: The protease plate assay method was adopted to screen isolate AASD15 for protease activity on CGYP agar medium according to the modified Cowan's method (Cowan et al. 1982).

Composition of CGYP agar medium (g/L): Casein – 1g, Glucose – 1g, Yeast extract – 0.5 g , Peptone – 0.5 g, NaCl – 0.5 g , $CaCl_2$ – 0.2,D/W – 100 ml.

The isolate AASD15 were spot inoculated on CGYP agar plate. Incubation was done at 55 °C for 3 days. The zone of clearance around the colonies of the isolate AASD15 were observed due to casein hydrolysis on CGYP agar plates.

Protease Assay: The reaction mixture was composed of –30 µl culture filtrate, 270 µl Phosphate buffer (50 mmol/1 pH -6). 250 µl Hammerstem Casein (2% in phosphate buffer 50 mmol/1) and Incubate at 55 °C 10 min. Reaction was terminated by addition of 2 .5 ml of 10 % Trichloroacetic acid (w/v). Mixture then centrifuged at 10000 rpm for 10 min at 4° C. Then 0.5 ml supernatant was taken as enzyme source and the activity was determined.

Determination of protein content by Folin Lowry method for asparaginase, tyrosinase, pectinase and protease (TLP): Protein content was measured with Bovine Serum Albumin (BSA) as standard protein by Folin Lowry method. 1 ml of sample was mixed with 5 ml of reagent C (Reagent I: 2% Na_2CO_3 in 0.1 N NaOH, Reagent II: 1% $CuSO_4$ in distilled water,

Reagent III: 2% Sodium Potassium Tartarate in distilled water) and incubated for 10 min. Then 0.5 ml of reagent D (commercial Folin Ciocalteau reagent was diluted 1:1 with Distilled water) was added and again incubated at room temperature in dark for 30 minutes. Absorbance was measured at 660 nm. Protein content was expressed as milligrams of protein per millilitre of sample. Following respective assay for determination of carbohydrate content for each enzyme and Folin Lowry method, specific activity of enzymes were calculated using the following formula-

$$\text{Specific activity} = \frac{\text{Units of enzyme / min / ml}}{\text{mg of protein / ml}}$$

SDS-PAGE of partially purified enzymes:

SDS-PAGE was carried out in a 12% polyacrylamide gel using Tris-glycine buffer (pH 8.3) by the method of Laemmli, U.K., 1970. The ammonium sulphate precipitated protein post dialysis sample obtained from AASD15 was loaded on to a denaturing polyacrylamide gel and compared with mid-range protein marker. Silver staining was performed to visualize the protein bands. The gel results were documented in Gel Doc EZ Imager, BioRad; Software: Image Lab 3.0.

Synthesis of metal nano-particles

Preparation of biomass: The isolates AASD15 was grown in 100ml modified CSA broth and incubated at 55˚C for 15 days .Centrifugation was done at 10,000rpm for 15min. then supernatent was discarded and pellet were washed with sterile distilled water (3 times). Mycellial mass was separated from sterile distilled water by centrifugation at 1500 rpm for 10min. Mycellial mass was weighed and used for synthesis of Zinc nanoparticle.

Exposure of biomass to metal solution: 0.1g of mycelial mass was exposed to 5ml of sterilized aqueous solution of $ZnSO_4$ at various dilutions (10^{-2}, 10^{-3}, 10^{-4}mM). Reaction mixture in a tube were observed for visual color change after 0, 24, 48,72hrs.

UV-Visible Spectroscopic analysis: Biosynthesis of metal ion was studied by taking 2 ml of reaction mixture at different time intervals (0, 24.48,72hrs) and centrifuging it at 8000 rpm for 10 min. Supernatant was collected (2ml) and absorbance was taken at different wavelength (200 – 800 nm) on UV – visible spectrophotometer.

Results

Isolation and Identification of AASD15

Fig1. Isolate AASD15 Showing Chalky White Growth

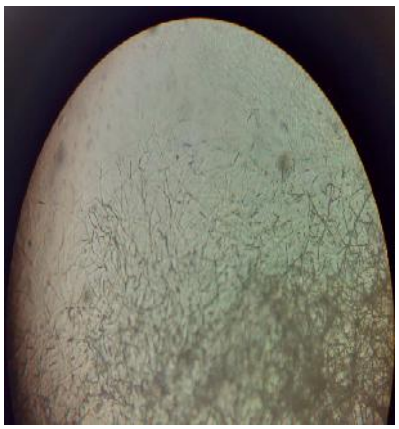

Fig 2. Slide Culture of Isolate AASD15

Growth of isolate AASD15 on different concentrations of NaCl

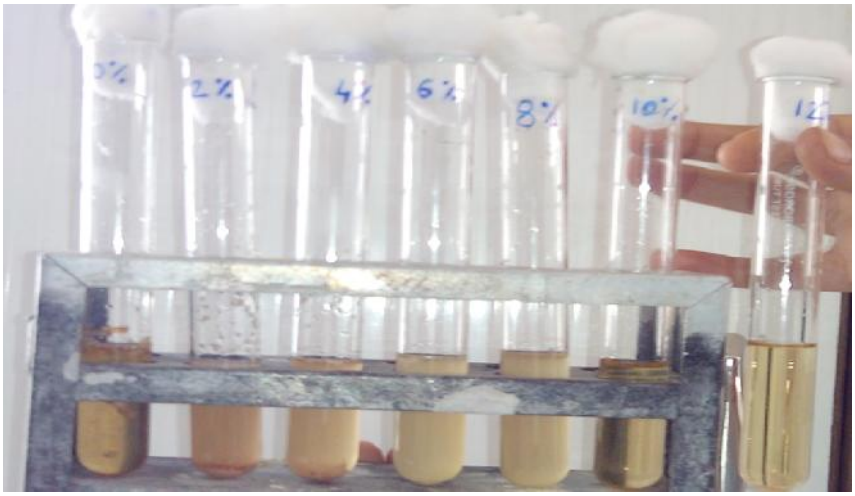

Fig 3. Growth of AASD15 in NaCl concentration

Asparaginase Activity:

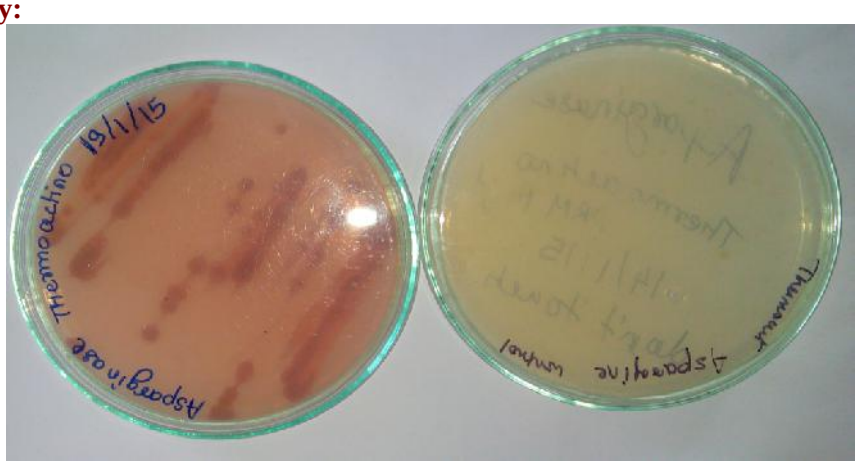

Fig 4. Asparaginase Production by AASD15
Isolate AASD15 show change in media color from yellow to pink.

Tyrosinase Activity:

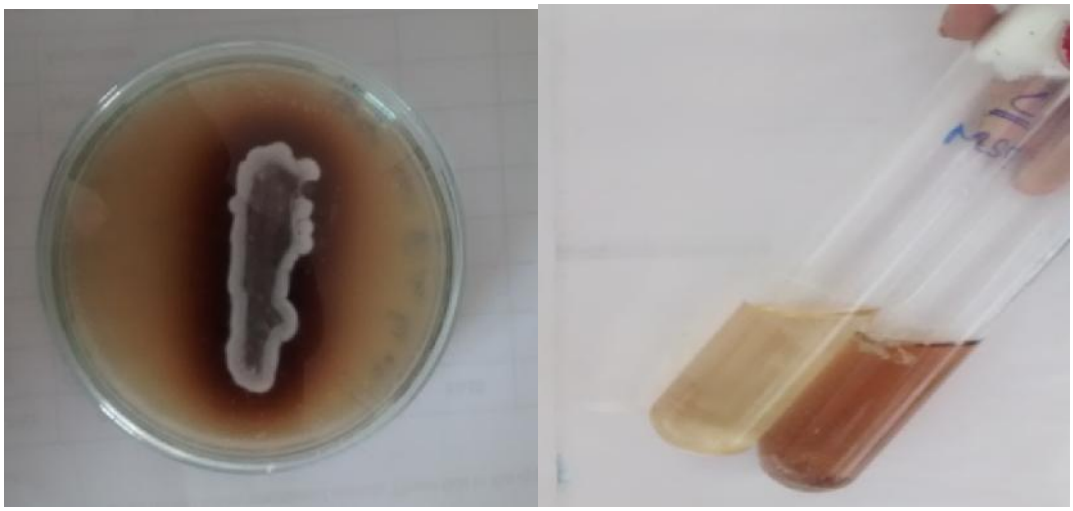

Fig 5. Tyrosinase Production by AASD15
Brown color zone was observed around the colonies of AASD15.

Pectinase Activity:

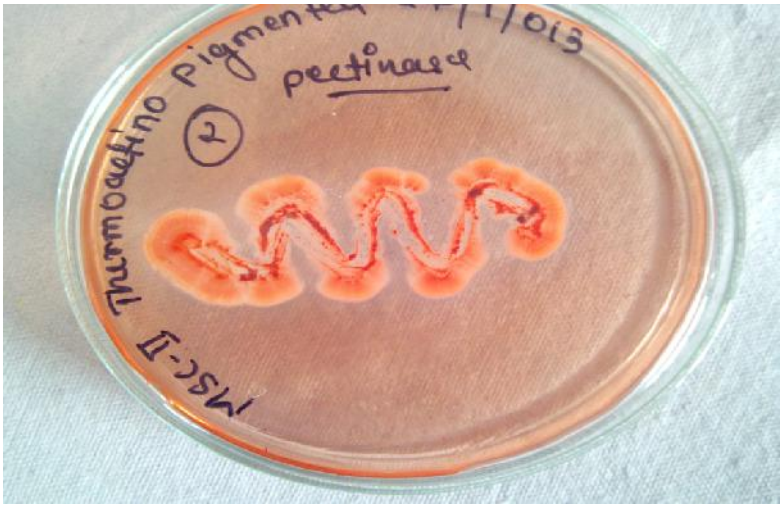

Fig 6. Pectinase Production by AASD15

Isolate AASD15was showing zone of clearance around the colonies.

Protease Activity:

(a) (b)

Fig 7. (a) Protease Production by AASD15 (b) White precipitate Produced by AASD15

(a) zone of hydrolysis was observed on milk agar plate after incubation at 55 °C for 3-4 days
(b) White precipitate was observed after condensation reaction of two amino acids by TLP.

Table 1. Purification chart for enzymes

Name of the Enzyme	Purity of the Enzyme	Percentage of Ammonium Sulphate	Enzyme Activity (U/ml)	Total Protein (mg/ml)	Specific Activity	Fold Purification	Percentage Yield
Asparaginase	crude		3.125	0.0502	62.4	1	100
	purified	55	3.965	0.0165	396	6.34	125.80
Pectinase	crude		1.032	0.0321	34.33	1	100
	purified	60	5.158	0.0251	257.5	7.50	510
		70	28.88	0.0241	1444	42.47	2803.88
Tyrosinase	crude		5.41	0.0519	108.2	1	100
	purified	60	5.90	0.0291	295	2.72	109.25
		70	6.55	0.0262	327.5	3.02	121.07
Protease (TLP)	crude		3.78	0.0406	94.5	1	100
	purified	70	3.92	0.0168	392	4.14	103.7
		80	8.85	0.0107	880	9.31	234.1

SDS-PAGE for different enzymes:

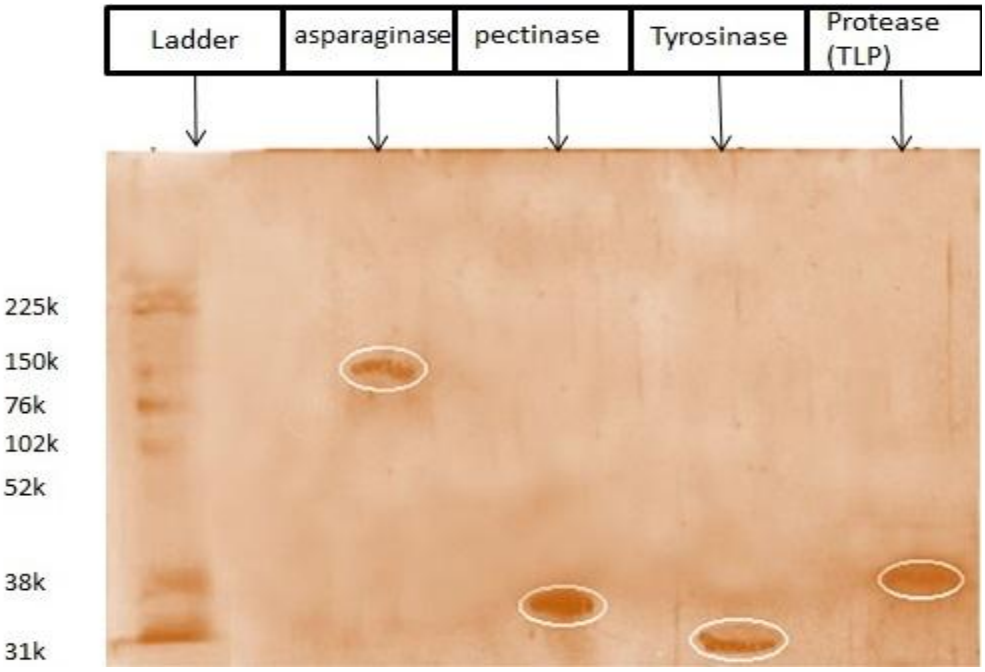

Fig 8 . SDS-PAGE showing approximate molecular weight of four enzymes.

Spectrophotometric analysis of metal nano-particles:

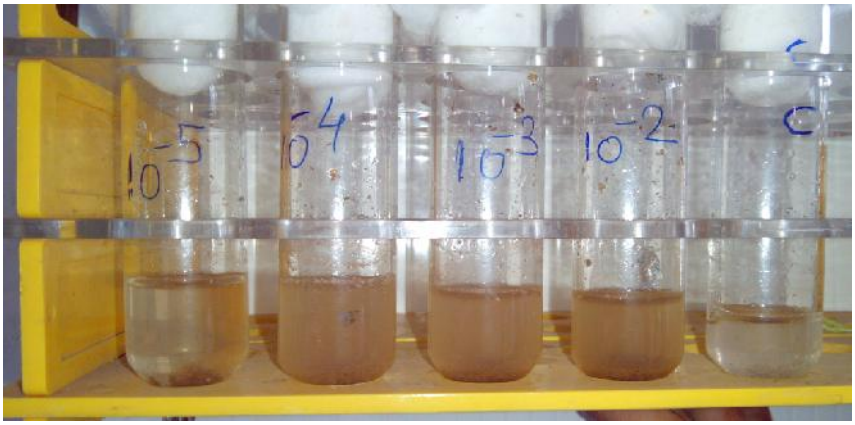

Fig 9. Production of Zinc Nanoparticle by AASD15

Isolate AASD15 shows colour change from light yellowish to Brownish indicating the production of Zinc nanoparticle

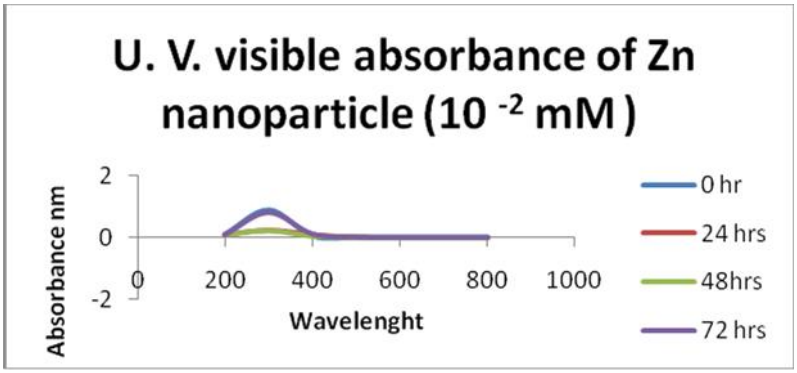

Fig10 a. UV-Visible spectral analysis of $ZnSO_4$ (10^{-2}) synthesized from AASD15

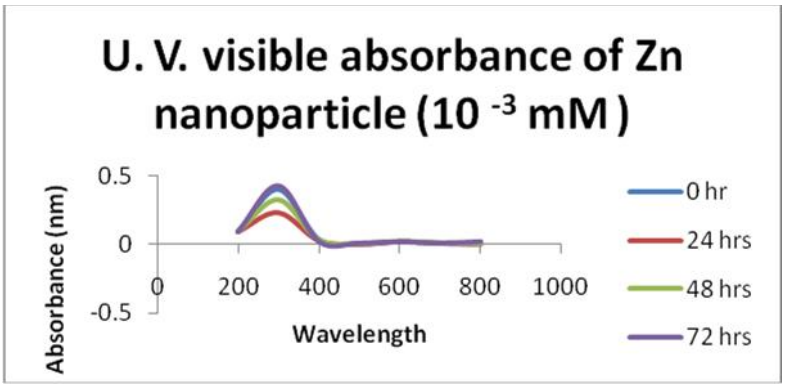

Fig10 b. UV-Visible spectral analysis of $ZnSO_4$ (10^{-3}) synthesized from AASD15

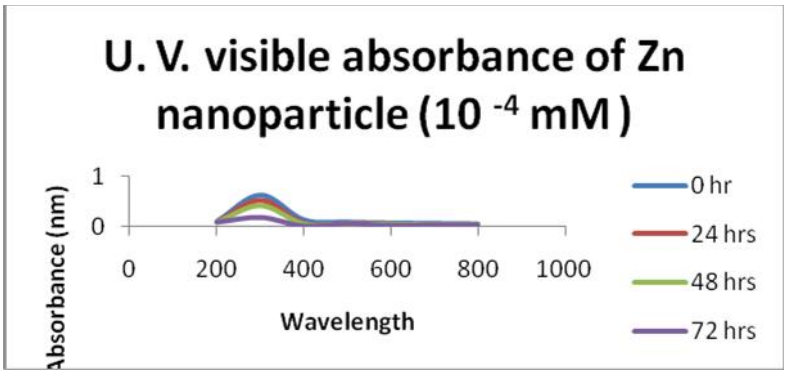

Fig10 c. UV-Visible spectral analysis of $ZnSO_4$ (10^{-4}) synthesized from AASD15

The stability of nano-particles in cell filtrate was confirmed by UV-Vis spectrophotometric analysis for incubation time of 0 – 144 hours. When cell filtrate was analyzed in wavelength 200-800 nm range, the maximum absorbance was observed at 300 nm for Zinc nano-particles produced from both isolates AASD15.

Discussion

Thermophilic Actinomycetes are known to secrete higher amounts of different bioactive molecules such as enzymes and antibiotics, thereby significantly increasing the productivity of this biosynthetic approach. Besides enzymes and antibiotics they are a good source for the synthesis of nanoparticles too. (Waghmare, S., et al. 2011).

The isolation of Thermophilic actinonycete (AASD15) was carried out using modified CSA at 55 ° C which shows a chalky white colony which resembles Thermophilic actionmyctes (Krupp, V. P., et al, 1975, Majumder, D.R. and Kanekar, P.P.2012).

Optimal growth was seen at temperature 55°C and in pH 7.0. The strain AASD15 studied grew at 55 ^{0} C but failed to grow at 37 °C, similar to that of *Thermoactinomyces thalpophilus* (Majumder, D.R. and Kanekar, P.P.2012).

The enzyme activity of asparaginase by the isolate AASD15 was 3.965 U/ml; which was more than the enzyme activity of asparaginase by *Streptomyces ginsengisol* 2.412 U/ml. (Deshpande, N. M., et al, 2014) which is a mesophilic strain. This is a first of its kind report for the isolate AASD15.The yield obtained for the asparaginase from isolate AASD15 is 125.80 %. The molecular weight determined for the asparaginase from AASD15 was 138 kDa.

The enzyme activity of pectinase by isolate AASD15 was 28.88 U/ml, and the yield obtained was 2803.88 % .This is a first of its kind report for the isolate AASD15. The molecular weight determined for the purified pectinase was 34 kDa.

Isolate AASD15, has shown enzyme activity of tyrosinase 6.55U/ml, and yield obtained is 121.07 %. This is a first of its kind report for the isolate AASD15. The molecular weight determined for the purified tyrosinase was 15 kDa.

Our isolate AASD15 shows enzyme activity of protease 3.92U/ml, which seems to be less as compared to the enzyme activity of isolate *Thermoactinomyces thalpophilus* was 105.55 U/ml. (Majumder, D.R. and Kanekar, P.P.2012) and yield obtained of protease by AASD15 is 234.1%. The molecular weight determined for the purified protease (TLP) was 36kD which is comparable (Majumder, D.R. et al, 2013). From the above data it is obivious that thermophilic actinomycete produces better yield compared to the mesophilic strains.

From the present study it was observed that zinc metal nano-particles produced by AASD15 shows highest absorbance at 300 nm compared to previous study it was found that zinc nanoparticle produced by *Streptomyces* spp. which is a mesophilic strain shows highest absorbance at 350 nm, which is confirmed by spectrophotometric analysis in the range of UV-Visible spectrum (200 – 800 nm) (Waghmare, S. et al, 2011).

Future line of work would entail the identification of isolate AASD15 up to species level by 16 S rRNA, scale up studies, characterization of enzymes, SEM analysis of zinc nanoparticle and application studies which will add more effect to the application of these value added products or bioactive molecules.

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