Study on screening, optimization and production of microbial chitinase enzyme from different soil samples

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ABSTRACT

Chitin is the second most abundant polysaccharide widely distributed in the marine and terrestrial environments. Chitinase, the enzyme that breakdowns chitin, has received attention due to their wide applications in medicine, biotechnology, agriculture, waste management and industrial applications such as food quality enhancer and biopesticides. The present study was aimed to screen, optimize and produce microbial chitinase enzyme from different soil samples from Valsad district of South Gujarat. In the present study, chitinase producers were isolated and screened. The obtained chitinase producers were characterized morphologically, biochemically and were further checked for chitinase activity. The isolate with highest activity was optimized for various parameters. Further, chitinase production was carried out and purified by partial purification. In the present study, 16 isolates were obtained and screened for chitinolytic activity and on the basis of chitin hydrolysis zone, on colloidal chitin agar medium, 4 isolates with higher chitinase producing ability were used for chitinase production by fermentation process. The current study revealed that the isolate AS7 showed highest enzymatic activity. Furthermore, culture medium was optimized for the maximum production of chitinase enzyme by isolate AS7. In conclusion, with highest chitinase producing AS7 isolate, the best production was obtained in Luria Bertaini broth with substrate colloidal chitin at optimum pH 7, temperature 37°C and incubation time of 5 days.

Keywords: Chitinase, Chitinolytic bacteria, Colloidal chitin, Biopesticides, Optimization.

INTRODUCTION

Chitinase are a group of enzymes that degrade chitin, produced by diverse range of life forms such as snails, crustaceans, insects, vertebrates, plant and microorganism (Mukharjee and Sen, 2004). Chitin $(C_8H_{13}O_5N)_n$ is a long-chain polymer of N-acetylglucosamine, a derivative of glucose. These enzymes are able to degrade the chitin present in the cell wall of fungi as well as the exoskeletons of insects. Chitin is water insoluble and degraded naturally by microorganism such as chitinolytic bacteria using chitinase as hydrolytic enzyme. Chitinase is secreted to outer cells of bacteria and bind to chitin molecule to break chitin into N-acetylglucosamine monomer.

Chitinolytic bacteria are capable of producing chitinase and hydrolyzing chitin progressively to produce GlNAc (N-acetylglucosamine) monomer through enzymatic reaction. These bacteria are found in soil, marine, lake, or chitinous waste such as industrial shrimp waste (Setia and Suharjono, 2015). Chitinases hydrolyze the β-1, 4 glycosidic bonds between the GlcNAc residues to produce chitooligosaccharides (Paulsen *et al.*, 2016). Chitin can be degraded by chemically and enzymatically. Chemical degradation of chitinous wastes is degraded through demineralization and deproteinization, which causes corrosive problems, and give low yield and high costs. Being more eco-friendly and cost effective method as compare to the chemical method for chitinous degradation, the enzymatic method can be adopted as an alternative (Krithika and Chellaram, 2016).

Chitinase are constituents of several bacterial species. Some of the best known include *Aeromonas, Serratia, Vibrio, Streptomyces and Bacillus* (Kuddus and Ahemad, 2013). Chitinases can be classified as endochitinases or exochitinases. Endochitinases cleave chitin at internal sites to generate multimers of GlcNAc. Exochitinases catalyze the hydrolysis of chitin progressively to produce GlcNAc, chitobiose or chitotriose. Chitinase has a wide-range of applications such as preparation of pharmaceutically important chitooligosaccharides and N-acetyl D-glucosamine, preparation of single-cell protein, isolation of protoplasts from fungi and yeast, control of pathogenic fungi, treatment of chitinous waste and control of malaria transmission (Kuddus and Ahemad, 2013). Chito-oligomers produced by enzymatic hydrolysis of chitin are used in various fields like in medical, agricultural and industrial applications, such as antibacterial, antifungal, antihypertensive and as a food quality enhancer (Kuddus and Ahemad, 2013). The present study was aimed to screen, optimize and produce microbial chitinase enzyme from different soil samples from Valsad district of South Gujarat.

MATERIAL AND METHODS

Sample collection

Soil samples were collected from which were rich in chitin wastes like local fish market, beach sand, river soil, pond soil, agricultural soil from different region of Valsad district of Gujarat, India.

Isolation of chitinolytic bacteria

For screening of chitinase producing bacteria, the agar medium amended with colloidal chitin was used. The medium consists of (g/L^{-1}): Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; yeast extract, 0.05; Agar, 24 and colloidal chitin 1% (w/v). The colonies showing clearance zone on a creamish background were considered as chitinase-producing bacteria (Setia and Suharjono, 2015).

Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin (Hi Media) by the modified method of Hsu and Lockwood. In brief, chitin powder (40g) was added with 600ml of concentrated HCl and kept for 60 minutes at 30°C with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 2L of water. The suspension was collected by filtration with suction on a coarse filter paper and washed by suspending it in about 5L of DW. Washing was repeated 3 times until the pH of the suspension was 7. After the above treatment, the loose colloidal chitin was used as a substrate (Divatar *et al*., 2016).

Screening of chitin degradation

Screening was performed with bacterial isolates on the colloidal chitin agar medium by agar well diffusion method and incubated at 37°C. Bacterial isolates were selected on the basis of a larger hydrolysis zone around the well after 96 hour of incubation was measured by zone meter (Kuddus and Ahemad, 2013).

Morphological and Biochemical characterization of isolates

The potent producers of chitin degrading isolate AS5, AS-6, AS-7 and RS-2 were further studied for its morphological and biochemical properties. The strain was identified according to the Bergey's Manual of systematic Bacteriology (Williams *et al*., 1989).

Chitinase Production

For chitinase production, colloidal chitin broth (100 ml) in 250ml capacity Erlenmeyer flasks was inoculated with 1ml (OD_{600}=1.0) of potent strain AS5, AS-6, AS-7 and RS-2. It was incubate at room temperature on a rotary incubator (150 rpm) for 72 hour. Culture supernatant was collected for 2 days by centrifugation at 12,000 rpm for 20 minute. From the supernatant than perform the enzyme activity and protein estimation and highest enzyme activity showing use for the further studies.

Assay of chitinase activity and protein estimation

Chitinases production was measured in terms of chitinase activity exhibited by the culture supernatant in the enzyme assay. Chitinases activity was assayed by DNSA method. Chitinase activity was assayed in a reaction mixture containing 1 ml of suitably diluted enzyme extract and 1.0 ml of 1% of colloidal chitin dissolved in 0.05 M phosphate buffer (pH 7) and incubated at 40°C for1 hour. The reaction mixture was then centrifuged at 8000 rpm for 20 minutes. 1.0 ml of supernatant was transferred to a clean test tube to which 1.0 ml of DNSA reagent and 2-3 drops of NaOH (1%) was added, vortexes and boiled for 5 minute. The solution was then cooled to room temperature and 5 ml of distilled water was added and absorbance was read at 540 nm. Concentration of N-acetylglucosamine was quantified using standard curve already prepared. One unit of chitinases was defined as the amount of enzyme that liberated one micro mole of N-acetylglucosamine per ml minute under the assay conditions (Lestari, 2017).

Optimization of culture condition

- **Effects of medium on chitinase production**

Two different medium namely Nutrient Broth and Luria Bertaini Broth amended with 1.0% colloidal chitin was used to determine the growth of bacteria and chitinase production. The culture was inoculated (1%) and incubated at 37°C for 24 hour in a rotary shaker (120 rpm). After two day of incubation, the cultures were harvested, centrifuged at 10,000 rpm for 15 minute and the supernatant used for chitinase assay (Shanmugaiah *et al*., 2008). The chitinase production was observed in spectrophotometer at 540 nm.

- **Effects of Incubation period**

For optimum incubation time, the bacterial culture was grown in LB broth with optimized media up to 7 days. The chitinase enzyme assay was performed for each pH to check chitinase production.

- **Effect of substrate on chitinase production**

To find out the best substrate for enzyme production, the chitinase production was carried out by using different substrates (1%) in medium viz. chitin powder (CP) and colloidal chitin (CC) at previously optimized conditions. The chitinase production was observed at 540 nm.

- **Effect of pH on chitinase production**

To find out the optimum pH for enzyme production, the chitinase production was carried out by using different pH in medium 5, 6, 7 and 8 at previously optimized conditions. The chitinase enzyme assay was performed for each pH to check chitinase production.

- **Effect of temperature on chitinase production**

To find out the optimum temperature for enzyme production, the chitinase production was carried out at different temperature such as 4°C, room temperature (28°C), 37°C and 45°C at previously optimized conditions. The chitinase enzyme assay was performed for every temperature to check the optimum temperature for chitinase production.

Chitinase production

Chitinase production was carried out by submerge fermentation technique using optimum media condition. The medium was heated to homogenize 100ml was distributed in 250ml flask and then sterilized by autoclaving. Medium was inoculated with a loop full of the propagated selected isolate and was placed at optimum temperature and for specific time. The culture supernatant was collected by centrifugation at 12,000 rpm for 20 minutes. The supernatant was the concentrated by ammonium sulphate precipitation.

Partial purification by Dialysis

The partial purification of enzyme was carried out by ammonium sulphate precipitation method. It was added to the culture supernatant in small quantities with constant stirring in order to achieve saturation. Then the supernatant was concentrated by precipitation with ammonium sulphate to 60%-100% levels. The precipitates were dissolved in 50mM phosphate buffer (pH-7) and centrifuged and dialyzed against the same buffer which then partially purified.

RESULTS AND DISCUSSION

Isolation and screening of Chitinase Producing Organisms

A total of 16 different chitinolytic isolates were isolated form 5 soil samples collected from different sites of Valsad district of Gujarat state, India. Soil samples were collected from five different sites of Valsad district which includes Fish market soil, Beach sand, River field soil, Agriculture field soil and pond field soil. Out of 16 isolates, 10 were isolated from agricultural soil, 2 from Sea beach sand, 1 from pond soil, 2 from river soil and 1 from fish market soil. All the 16 isolates were shown the zone of clearance based on the chitin degradation on colloidal chitin agar (CCA) plate (Figure 1). They were further purified, screened by secondary screening and their morphological and biochemical characteristic were studied.

In 2013, Kuddus and Ahmad have also isolated the 58 morphologically different chitinolytic bacterial isolates form 15 different soil samples of Lucknow, India. In 2016, Divatar *et al*., 2016 have also isolated the 75 morphologically different chitinolytic microbes from 54 different soil samples of Gulbarga, Karnataka, India.

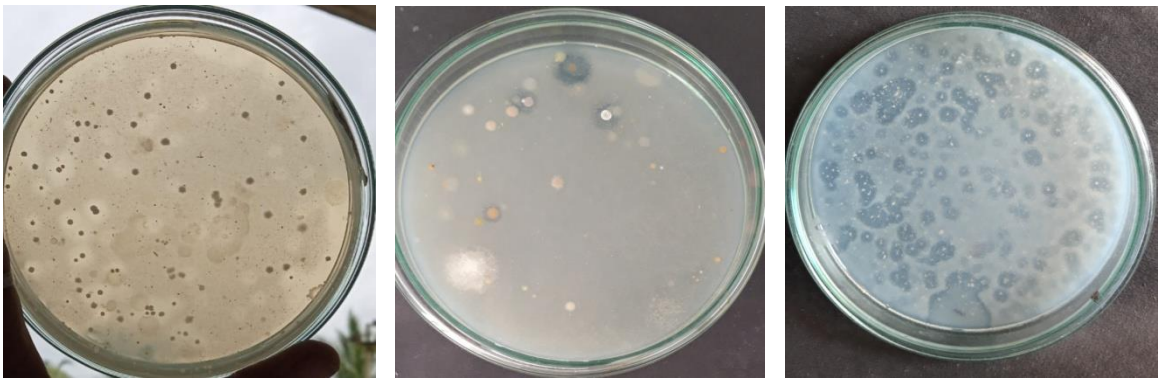

Figure 1: Chitinase producing organisms on Colloidal chitin agar plate

Secondary Screening of Chitinase Producing Isolates

All the 16 isolates were screened and shown the clear zone of chitin hydrolysis. Zone size were measured for each isolate where in isolate AS7 was reported to have the highest zone size of 22 mm and isolate RS1 was reported to have least zone of hydrolysis 12 mm (Figure 2).

The higher zone of chitin hydrolysis producing isolates were further studied for their morphological and biochemical characteristic. In 2008, Shanmugaiah *et al*., in their study, have

also performed secondary screening of chitinase producing *Bacillus laterosporous* MML2270 from rice rhizosphere soil which is similar to our study.

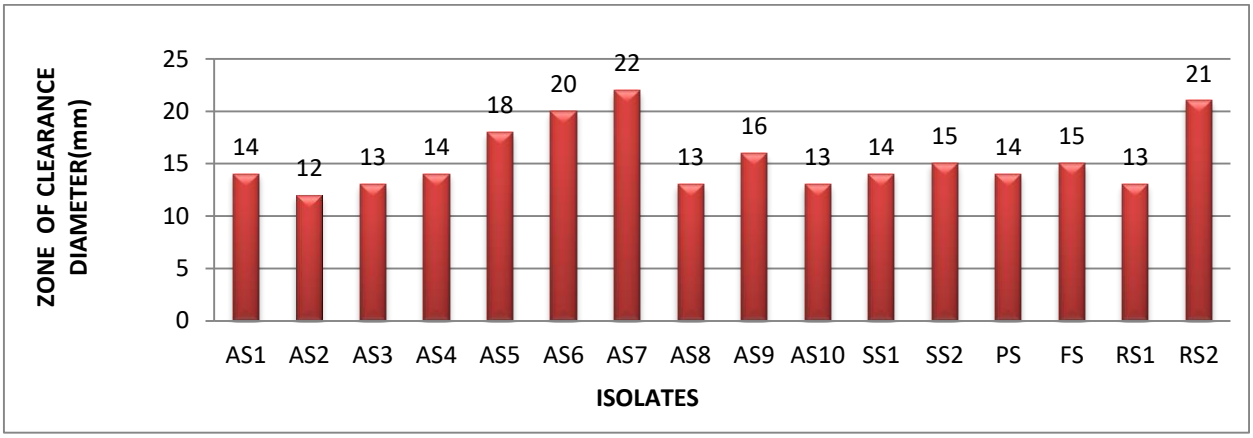

Figure 2: Zone of chitin hydrolysis by obtained isolates

Morphological and Biochemical characterization of chitinase producing isolates

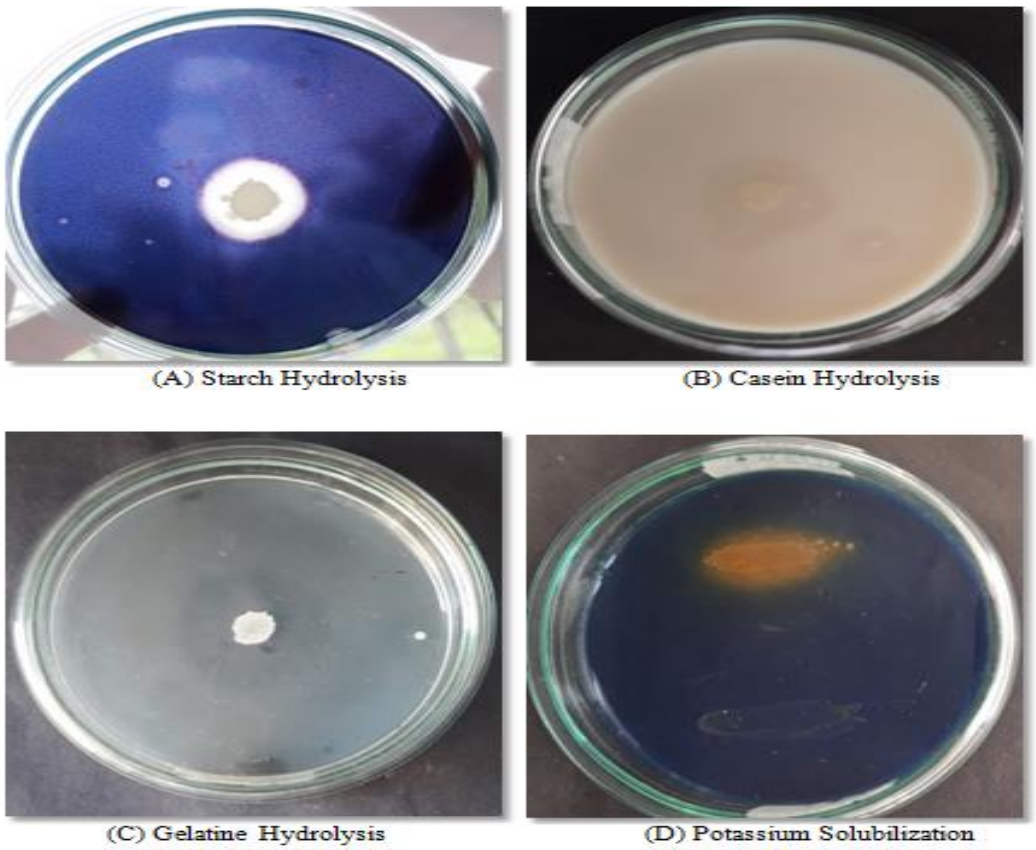

Figure 3: Screening for various enzymes production by AS7

Figure 4: Biochemical tests results of bacterial isolate RS2

Table 1: Results of various biochemical tests of isolates AS5, AS6, AS7, RS2

TESTS		**ISOLATES**			
		AS5	**AS6**	**AS7**	**RS2**
Indole Production Test		-	-	-	-
Methyl Red Test		-	-	-	-
Vogues - Proskauer Test		-	-	-	+
Citrate Utilization Test		-	-	-	+
Nitrate Reduction Test		+	+	+	+
Oxidase Test		+	+	+	+
Catalase Test		+	-	+	+
Hydrogen Sulphide(H_2S) Test		-	-	-	-
Urea Hydrolysis Test		-	-	-	-
Starch Hydrolysis Test		+	+	+	+
Casein Hydrolysis Test		-	-	+	-
Gelatin Hydrolysis Test		+	+	+	-
Potassium Solubilization Test		-	-	-	+
TSI	Butt	Acid	Acid	Acid	Acid
	Slant	Acid	Acid	Alkaline	Alkaline
	Gas	-	-	-	+
Sugar Utilization Test	Glucose	-	+	++	++
	Sucrose	+	+	++	++
	Maltose	+	-	++	++
	Mannitol	+	+	+	++
	Lactose	-	-	+	+
	Xylose	+	+	++	++

In the present study, all the 16 chitinase producing isolates were morphologically characterized where two isolates were reported Gram negative and fourteen were reported Gram positive. In biochemical characterization, various biochemical tests were performed for characterization of higher chitinase producing AS5, AS6, AS7, RS2 isolates. All the characteristics were studied as described in Bergey's Manual of Systematic Bacteriology (Table 1).

Production of chitinase

Submerged fermentation was performed with the selected four isolate AS5, AS6, AS7 and RS2 to analyze the chitinase productivity. Isolate AS7 found good enzyme yield with 35 U/ml as compared to other bacterial isolates. The enzyme activities shown by AS5, AS6 and RS2 were found to have 19.16 U/ml, 28.33 U/ml and 21.66 U/ml respectively (Figure 5).

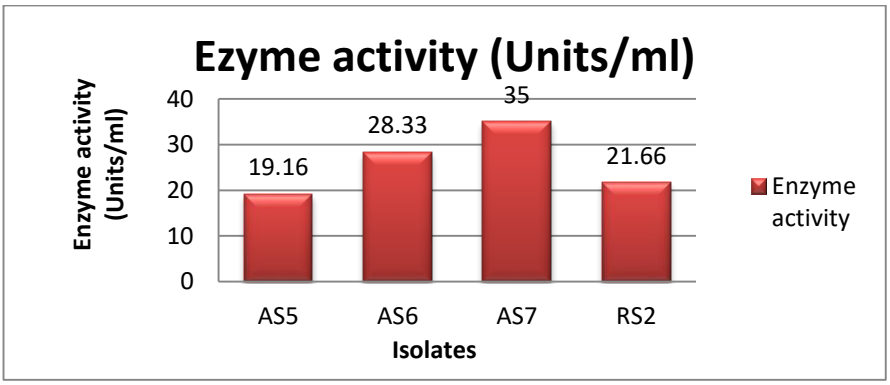

Figure 5: Chitinase activities of the obtained isolates

Optimization of culture condition

- **Effects of medium on chitinase production:**

  Optimization of medium for chitinase production was carried out using two different media such as Nutrient broth and Luria Bertaini broth with 1.0% colloidal chitin as substrate. In the current study, Luria Bertaini broth was found to support the highest chitinase production that was 53.33 U/ml. On the contrary, chitinase production with Nutrient broth was achieved 30.83 U/ml (Figure 6).

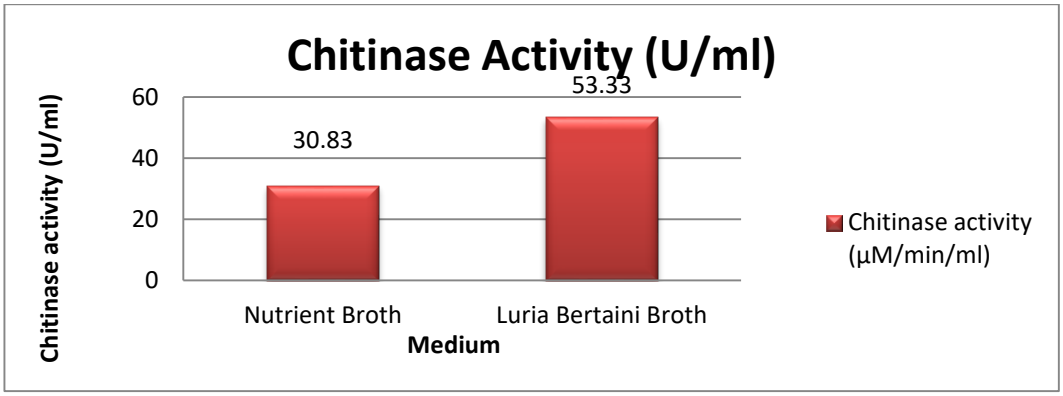

Figure 6: Effect of media on chitinase production

In 2011, Karunya *et al*. they have also studied on chitinase production by using *Bacillus subtilis* and reported the highest enzyme activity with Luria Bertaini broth which shows agreement with our study. In 2013, similar work was also carried out by Kuddus and Ahmad. In their study, they have studied six different types of media for two bacterial strains *A. hydrophila* as HS4 and *A. punctata* as HS6. Amongst all the tested media, LB broth with colloidal chitin was found to be more productive for both the strains.

- **Optimization of Incubation period**

Optimization of incubation period in chitinase production, the medium was inoculated with AS7 isolate and incubated at different time intervals. The enzyme activity gradually increases with increase in incubation time and showed the highest enzymatic activity on 5^{th} day of incubation. The enzymatic activity started decreasing as with increase in incubation time during fermentation (Figure 7). In 2008, Shanmugaiah *et al*., in their study, have also optimized the conditions for chitinase production and activity like incubation time wherein they have found the best chitinase enzyme activity at 4^{th} day of incubation period. In 2016, Santhi has performed the similar study and reported the best chitinase activity at 5^{th} day of incubation time which was similar with our findings. In 2011, Karunya *et al*., in their studies, have reported the highest chitinase activity at 4^{th} day of incubation and then decreases as time period was increased. The finding was nearly similar with our findings.

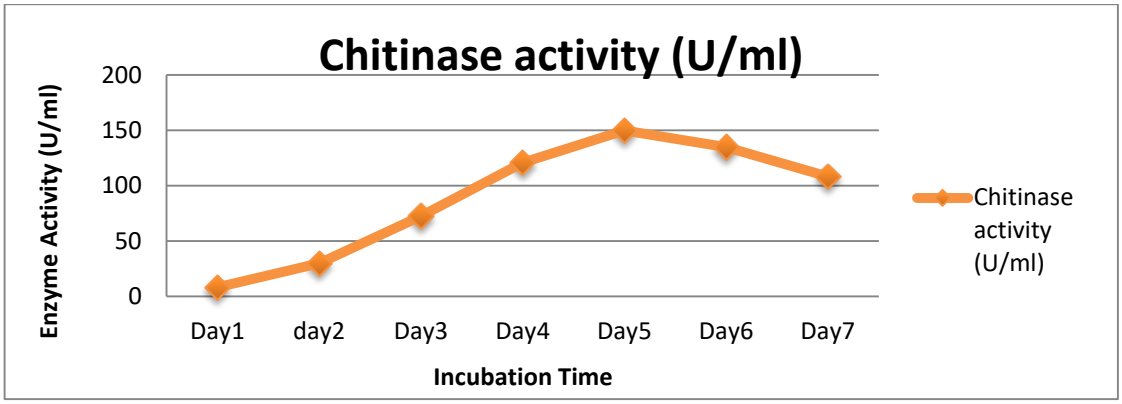

Figure 7: Optimization of Incubation time

- **Optimization of Substrate**

Optimization of substrate for chitinase production was carried out using two different substrates chitin powder and colloidal chitin with concentration of 1% in the Luria Bertaini broth. Out of both, colloidal chitin was found to be best substrate for chitinase production using AS7. With colloidal chitin, the activity obtained was 87.5 U/ml which was lesser than 72.5 U/ml obtained with chitin power (Figure 8). In 2013, Kuddus and Ahmad, in their study, have used various substrates like fish shell (FS), chitin powder (CP), and colloidal chitin (CC). They have also reported colloidal chitin as a best substrate for chitinase production which was similar with our results.

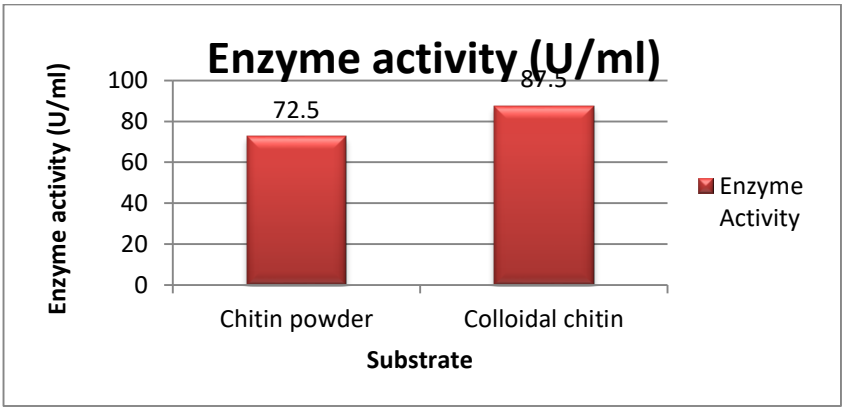

Figure 8: Optimization of substrate

- **Optimization of pH**

The production medium was optimized for pH ranging from 5-8. The enzyme activity was assayed after 72 hours of incubation at room temperature. Isolate AS7 showed highest activity at pH 7 that was 102.5 U/ml. Chitinase activity was found to be decreased at basic pH 8 which was 64.32 U/ml. The medium with pH 5 and pH 6, the activities obtained were 44.16 U/ml and 62.5 U/ml respectively (Figure 9).

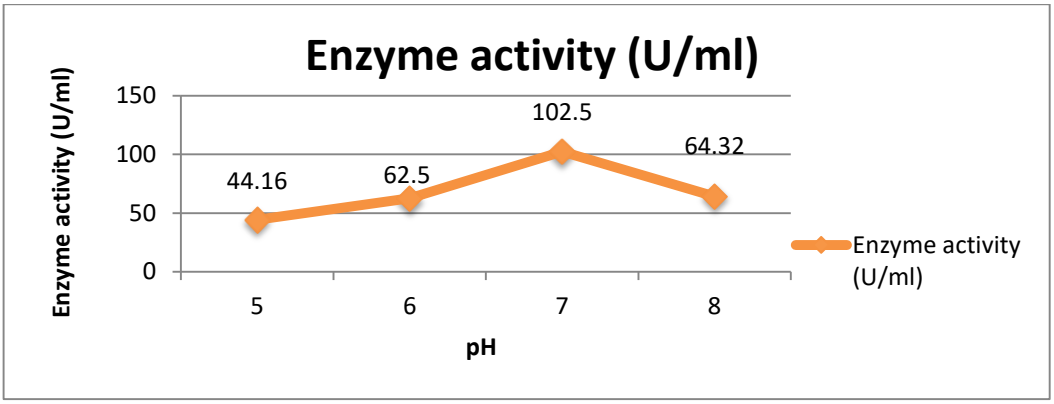

Figure 9: Optimization of pH

- **Optimization of Temperature**

The production medium was optimized at various temperatures i.e. 4°C, 28°C, 37°C and at 40°C for bacterial isolates AS7. Isolate AS7 showed highest enzyme activity at 37°C which was found 139.16 U/ml followed by at 40°C which was found to be 120 U/ml. The lowest enzymatic activity was obtained at low temperature 4°C which was only 1.66 U/ml followed by at 28°C which was found to be 29.16U/ml (Figure 10). In present study isolate AS7 showed highest enzyme activity at 37°C.

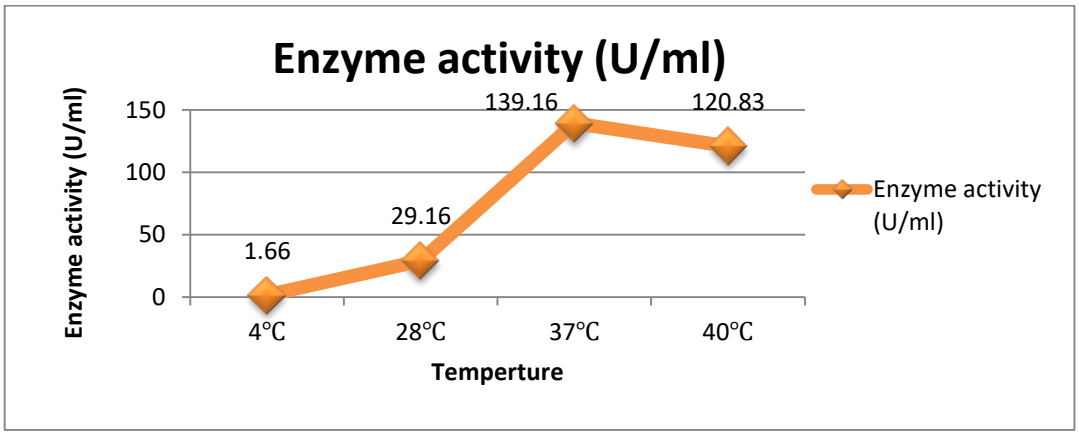

Figure 10: Optimization of Temperature

In 2014, Jabeen and Qazi have also studied the effect of various temperatures on the chitinase production and activity by using *Bacillus cereus* and reported the best activity at the temperature 30°C. In 2011, Karunya *et al*. have also performed the similar studies and reported the highest chitinase activity at temperature 35°C which was nearly similar with our studies. Shanmugaiah *et al*., in 2008, studied and reported the best chitinase activity at temperature 35°C using bacterium *B. laterosporous.*

Partial purification by Ammonium sulphate precipitation

Different % saturation of ammonium sulphate was studied to get the maximum activity of precipitation. Using all the optimized parameters, the production was done and then partially purified. The supernatant and the filtrate were used as crude enzyme and were further used for their enzymatic activity. 80 % ammonium sulphate was found to be optimum for precipitation with maximum enzymatic activity of 120.83 U/ml (Figure 11).

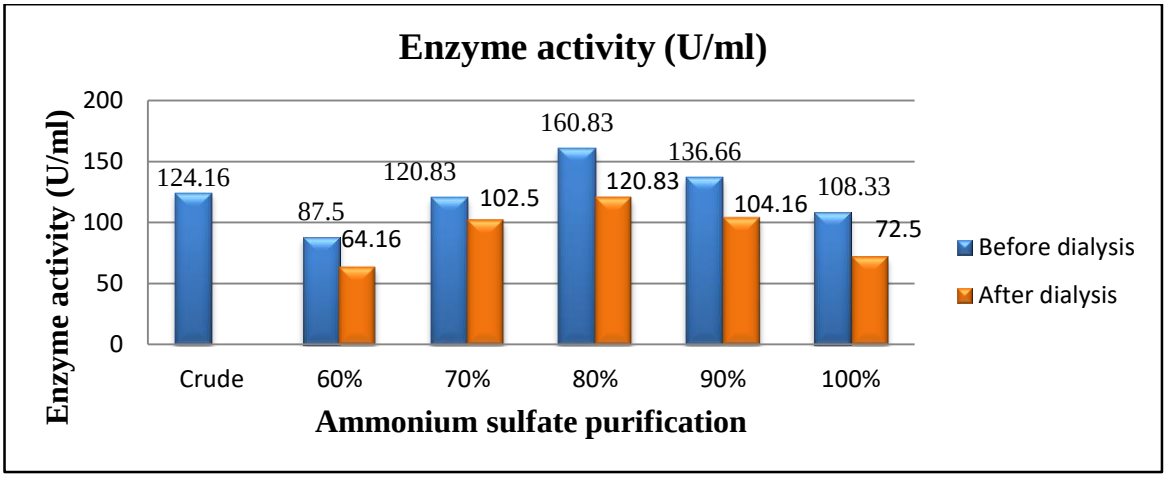

Figure 11: Ammonium sulphate purification

In the present study, chitinase activities using isolate AS7 were studied and found higher in Luria Bertaini broth with colloidal chitin substrate. The highest enzymatic activities were obtained at various optimized conditions that were pH 7, incubation at 37°C for 5 days of incubation.

CONCLUSION

Chitinase plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. In the present study, 16 chitinase producers were isolated from different soil samples of Valsad district and maximum isolates were obtained from Agricultural soil. All the isolates were characterized morphologically and biochemically where all the producers were found bacterial species. 4 isolates with higher chitinase producing ability were used for chitinase production by fermentation process. The fermentation of chitinase enzyme was carried out successfully in the present work. All the four isolates were tested for their chitinase producing ability and determined by DNSA method. The current study reveals that, the isolate AS7 showed highest enzymatic activity. In the present study, culture medium was optimized for the maximum production of chitinase enzyme by isolate AS7 wherein best production was obtained in Luria Bertaini broth with substrate colloidal chitin at optimum pH 7, temperature 37°C and incubation time of 5 days.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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