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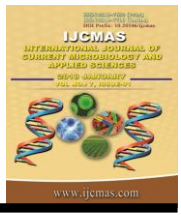

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Molecular Identification, Production and Optimization of Pectinase by using *Stenotrophomonas maltophilia* P9 Isolated from Algal Biomass of Himachal Pradesh, India

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A B S T R A C T

The demand for enzymes in the global market is projected to rise at a fast pace in recent years. There has been a great increase in industrial applications of pectinase owing to their significant biotechnological uses. Therefore the study was undertaken with aims of; screening pectinase producing bacteria from *Rhizoclonium* sp. algal biomass collected from different parts of Himachal Pradesh. The isolate P9 with high potential pectinase activity was identified molecularly by sequencing 16s rDNA region of the isolate and identified as *Stenotrophomonas maltophilia* P9 was selected for production. Various optimization steps by using classical one factor at a time approach (OFAT) were carried out to make the production of pectinase enzyme cost effective and commercially viable. The various parameters studied for pectinase production were different media, medium pH, temperature, inoculum size and incubation period. The maximum pectinase production was observed using Adessina *et al.*, (2013) media at initial pH of 5.0, at 30^{o}C with 10% inoculum size followed by incubation period of 72 h. In the present study, an attempt was made to isolate efficient pectinase producing bacteria from least explored source of enzymes i.e. algal biomass of Himachal Pradesh and can be further used for various biotechnological applications.

Introduction

Biotechnological answers for environmental sustainability are modern solutions that help in the growth of the nation and are a boon for the welfare of human beings for the present and forthcoming generations. Biotechnology operations for enzyme production is no longer an academic, it is potentially useful alternative proposition for the future (Mishra *et al.*, 2011). Enzymes are natural catalysts. They are produced by living organisms to increase the rate of an immense and diverse set of chemical reactions required for life. Furthermore, their ability to perform very specific chemical transformations has made them increasingly useful in industrial processes (Li *et al.*, 2012). Pectinase (EC 3.2.1.15) belongs to the class

hydrolase which are able to hydrolyse pectin more efficient than other Pectinases and their action are very specific, i.e., they acts only on pectin substrates. Pectin is a major constituent of cereals, vegetables, Pectin fibers are complex, high molecular weight heterogeneous and acidic structural polysaccharide. D-galacturonic acid is one of the major components of pectin (Mehta, 2013). Pectinolytic enzymes are classified according to their mode of action on the galacturonan part of the pectin molecule (Semenova *et al.,* 2016). Pectinolytic enzymes can be applied in various industrial sectors wherever the degradation of pectin is required for a particular process. Several microorganisms have been used to produce different types of pectinolytic enzymes (Jayani *et al.,* 2010). Among the various pectinase, bacterial extracellular pectinase are the most significant, compared with animals, plants, viruses and fungal extracellular pectinase.

Extracellular pectinase produced by *Bacillus* and *Cocci* species are of main interest from a biotechnology perspective, and are not only in scientific fields of protein chemistry and proteins engineering but also in applied fields such as foods, pharmaceutical and paper industries. Microbial pectinases account for 10% of the global food and industrial enzyme sales (Jayani *et al.,* 2005; Murad and Azzaz, 2011) and their market is increasing day by day. These are used extensively for fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibers, degumming of natural fibers, wastewater treatment, and as an analytical tool in the assessment of plant products (Alkorta *et al.,* 2008; Singh *et al.,* 2010). Owing to the enormous potential of pectinase in various sectors of industries whenever degradation of pectin is needed, it is important to undertake research in screening of microorganisms for pectinase and investigate optimal conditions for production of microbial pectinase.

In the light of aforementioned facts, current study was designed to utilize least explored and highly probable source of hydrolytic enzyme producing microorganisms, *Rhizoclonium* sp. algal biomass as a major source for production of pectinase that ultimately will decrease production cost on commercial scale. Among different isolates, *Stenotrophomonas maltophilia* P9 capable of producing pectinase was selected and subjected to optimization of different process parameters for producing appreciable levels of pectinase.

Materials and Methods

Collection of sample and screening of pectinase producing microorganism

Rhizoclonium sp. algal biomass collected from different sites of Himachal Pradesh in clean polythene bags and was brought to the laboratory. To algal biomass, 1% solution of pectin powder was added separately. The enriched samples had been serially diluted and plated on to nutrient agar medium supplemented with pectin substrate with initial pH of 7.0 and incubated at 37^{o}C for 2-3 days. The pure cultures were obtained and maintained at 4^{o}C on nutrient agar medium.

Qualitative assay of pectinase

An extensive screening of bacterial isolates capable of exhibiting appreciable levels of pectinolytic activity was done. The degradation of pectin was estimated by flooding plates with 0.1 % Congo red for 15 min and then washed with 1 N NaCl (Teather and Wood 1982).

Quantitative analysis

The bacterial isolates were screened for the production of extracellular enzymes pectinase activity. Each bacterial isolate was grown in

nutrient broth at 37^{o}C for 24 h. As soon as the substantial growth was observed. Then 5 ml of inoculum was added to 45 ml of nutrient broth supplemented with 1% of pectin powder. The inoculated flasks were then incubated at 37±2^{o}C for 24h.

Then, the cell-free supernatant was recovered by centrifugation (10,000 rpm, 10 min at 4^{o}C) and the clear supernatant was used to determine the pectinase enzyme activity.

Pectinase assay (Miller, 1959)

Pectinase activity was determined using 1% (w/v) pectin in 0.2M citrate buffer pH 6.0 as substrate (Miller, 1959). The released reducing sugars were quantified using a glucose standard curve as a reference. One International Unit (IU) of enzyme activity represents µ moles of glucose released/min/ml of enzyme.

Determination of total protein

Total protein content of cell free filtrate was determined by Lowry's method using bovine serum albumin as standard (Lowry *et al.,* 1951).

Molecular identification

Bacterial isolate P6 was grown overnight at 37^{o}C in nutrient broth. The cells were harvested and processed for DNA isolation by conventional method of Sambrook and Rusell (1989). Partial DNA fragment of the 16S rDNA was amplified by polymerase chain reaction (PCR) using gDNA as template.

The universal primers used for amplification were 8F: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'. The phylogeny and family of the bacterial strain was accessed using BLAST search.

Optimization of process parameters

Various process parameters influencing enzyme production during submerged fermentation were optimized. The strategy followed was to optimize each parameter, independent of the others, and subsequently optimal conditions were employed in all experiments.

In a sequential order, the various process parameters were optimized for maximal enzyme production as follows: fermentation medium, temperature, pH, inoculum size and incubation period by using conventional approach i.e. one factor at a time (OFAT).

Optimization of different media

Effect of different media i.e. Media I (Adesina *et al.,* 2013), Media II (Joshi *et al.,* 2013), Media III (Kashyapet *et al.,* 2000), Pectin media (Kumar *et al.,* 2012), Nutrient broth pectin (Joshi *et al.,* 2013) were studied for pectinase production.

Effect of different pH

pH of the growth media was adjusted from 5.0 to 9.0 to find pH suitable for fermentation and ultimately for maximum enzyme production. The inoculated broth was incubated for fermentation at 37°C for 72 h.

Optimization of different temperature

In order to attain high enzyme titer, the optimum fermentation temperature was determined by incubating broth media at different temperature ranging from 20°C to 45°C (pH 7.0).

Effect of different inoculum size

Optimum inoculum concentration for enzyme production was studied by varying the

inoculums concentration (7.5% to 17.5%) in enzyme production medium and incubated at 30°C for 72 h.

Time course for pectinase production

The optimized growth media was inoculated and incubated at 30°C for different time intervals ranging from 24 to 144 h and enzyme activity was measured. Maximum enzyme production was observed in growth medium after 72 h of incubation.

Statistical analysis

All the experiments were conducted in triplicates and results are presented as mean. Further the significant differences among the mean values were evaluated by one way ANOVA and using online software tool OPSTAT.

Results and Discussion

Screening of pectinolytic bacteria

Based upon Qualitative test, 3 bacteria were found to be positive for pectinase production and produced zone of hydrolysis. To screen out maximum pectinase producer, bacterial isolates were subjected to quantitative assay. In which isolate P9, produced maximum amount of pectinase as 3.59 IU/ml (Table 1).

Genotypic identification

Molecular characterization of the selected pectinase producing strain P9 was done at genomic level by using 16S rRNA gene technique Genomic DNA.

Further these amplified 16S rRNA sequences of the bacterial strains was blasted using online tool (mBLAST NCBI). The isolate P9 showed 96% homology with *Stenotrophomonas maltophilia.* The 16S rRNA gene sequences of the isolate has been deposited to National Centre for Biotechnology Information (NCBI) gene bank using Bankit program and has been registered in the databases vide accession number *Stenotrophomonas maltophilia* P9 |MF443881|. The taxonomical identification was done by the phylogenetic tree construction and the comparison of bacterial strain sequences with other homologous bacterial sequences.

Optimization of process parameters

The pectinolytic bacteria *Stenotrophomonas maltophilia* P9 was taken for optimization studies through submerged fermentation by varying the media, temperature, pH, inoculum size and incubation period, since the production of pectinase enzyme is influenced by diverse physico-chemical and biological factors.

Effect of media

Stenotrophomonas maltophilia P9 showed maximum growth in Adessina *et al.,* (2013) medium containing Pectin, 0.3 g, Yeast extract, 1.0 g, Sucrose, 10 g, KNO_3, 0.6, KH_2PO_4, 1.0 g, $MgSO_4$, 0.25g, $CaCl_2.2H_2O$, 0.1, K_2HPO_4, 0.5g, KCL, 0.5g, dH_2O- 1000 ml, pH- 7.0. Cell free broth obtained from this medium showed highest enzyme yield of 3.95 IU with a specific activity of 3.16 IU/mg (Fig. 2).

Other media however, showed lower yields of enzymes which could be attributed to solution of complex ions which might have induced inhibitory effect on enzyme production. Various nitrogen sources, minerals and phosphates like KH_2PO_4, K_2HPO_4 has been found best for pectinase production (Vyas *et al.,* 2005; Narayana *et al.,* 2014; Anuradha *et al.,* 2010). Marcia *et al.,* (2013) have reported a pectinolytic activity of 82.152 U/ml for strains of *Bacillus* spp. in Hankin's medium.

Table.1 Production of pectinase from screened bacteria isolated from *Rhizoclonium* sp. algal biomass

Isolates	Pectinase activity(IU)*	Protein(mg/ml)**	Specific activity***
P9	**3.59**	**1.76**	**2.04**
P17	2.33	1.21	1.93
P19	1.61	1.05	1.53
$C.D._{0.05}$	0.87	0.49	N/S
S.E.(m)	0.25	0.14	0.24

*Enzyme activity (IU): µmoles of reducing sugars released/min/ml of enzyme
**Protein concentration: mg/ml
***Specific activity: enzyme activity/mg of protein

Table.2 Effect of different pH on pectinase production from *Stenotrophomonas maltophilia* P9

pH	Pectinase activity (IU)*	Protein(mg/ml)**	Specific activity***
5.0	**3.99**	**2.32**	**1.70**
6.0	1.82	1.00	1.12
7.0	1.67	1.56	1.07
8.0	1.61	0.59	1.03
9.0	1.03	0.99	1.04
$CD_{0.05}$	0.64	0.39	0.34
S.E.(m)	0.20	0.12	0.11

*Enzyme activity (IU): µmoles of reducing sugars released/min/ml of enzyme
**Protein concentration: mg/ml
***Specific activity: enzyme activity/mg of protein

Table.3 Effect of different inoculum size on pectinase production from *Stenotrophomonas maltophilia* P9

Inoculum size (%)	Pectinase activity (IU)*	Protein (mg/ml)**	Specific activity***
7.5	2.52	1.70	1.48
10.0	**5.92**	**3.13**	**1.79**
12.5	4.04	2.54	1.59
15.0	1.92	1.01	1.91
17.5	1.54	0.94	1.64
$C.D._{0.05}$	0.70	0.40	N/S
S.E.(m)	0.22	0.13	0.14

*Enzyme activity (IU): µmoles of reducing sugars released/min/ml of enzyme
**Protein concentration: mg/ml
***Specific activity: enzyme activity/mg of protein

Fig.1 Neighbor-joining tree showing phylogenetic relationship of *Stenotrophomonas maltophilia* P9 based on a distance matrix analysis of 16S rRNA sequences

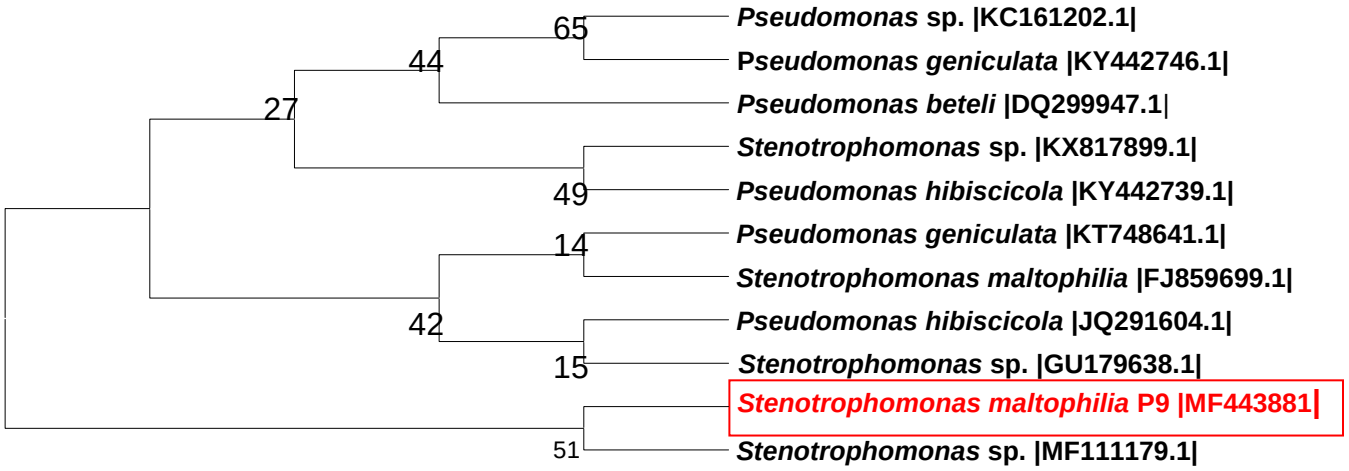


Fig.2 Effect of different media on extracellular Pectinase production from *Stenotrophomonas maltophilia* P9

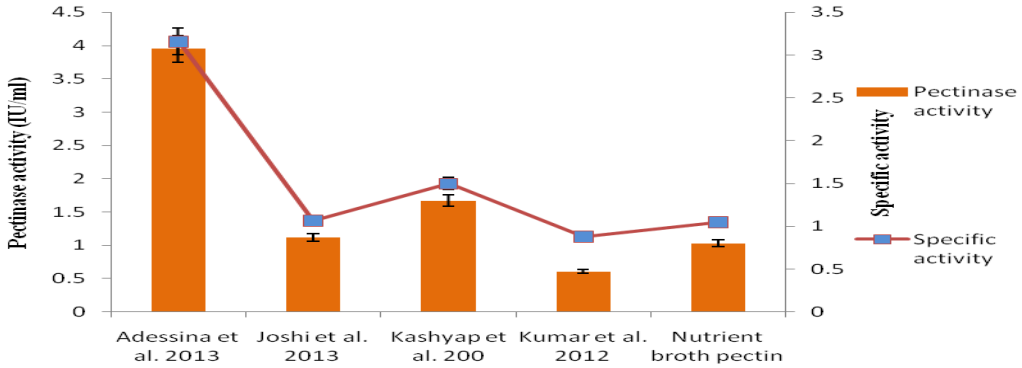


Fig.3 Effect of different temperature on pectinase production from *Stenotrophomonas maltophilia* P9

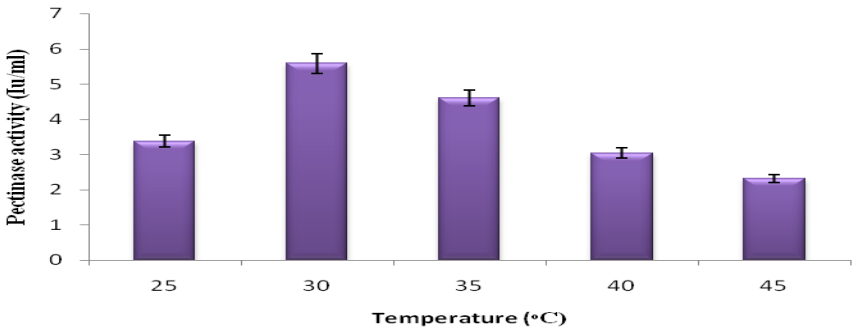


Fig.4 Effect of different incubation time on pectinase production from *Stenotrophomonas maltophilia* P9

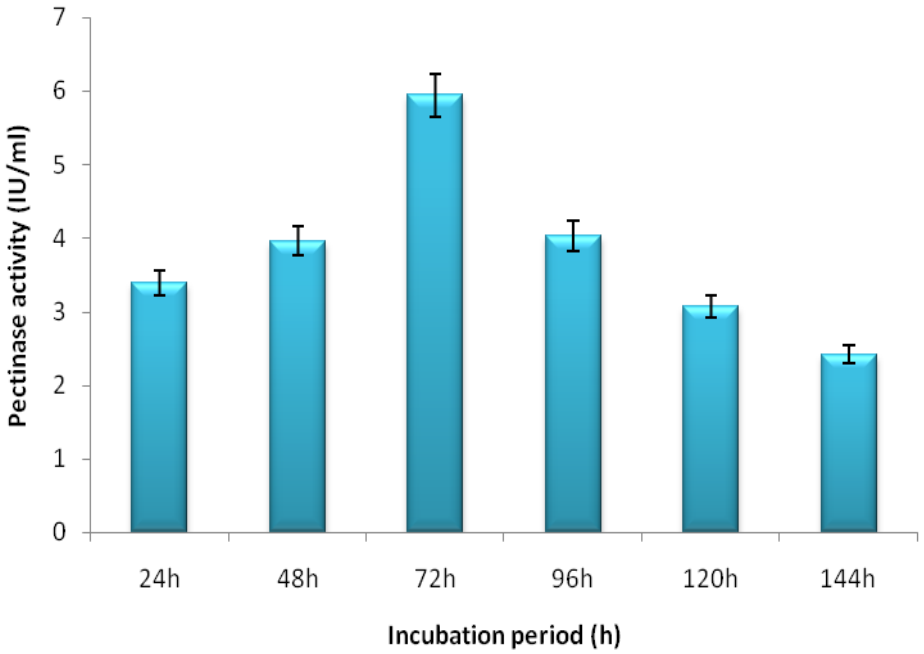
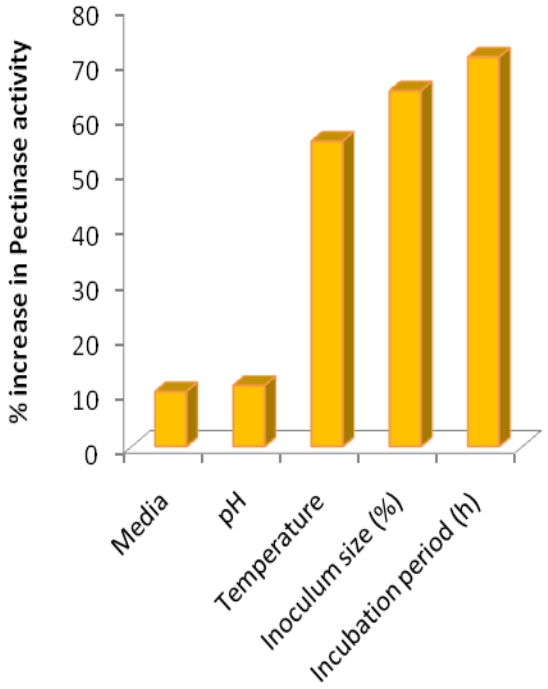


Fig.5 An overview of percent increase in pectinase activity from *Stenotrophomonas maltophilia* P9 after optimization of different parameters



Effect of pH

Table 2 represents the effect of pH on the enzyme production potential from *Stenotrophomonas maltophilia* P9. The highest pectinase activity of 3.99 IU was observed at acidic pH 5.0 with specific activity 1.70 IU/mg. At higher pH of 7.0 and 8.0, a respective pectinase activity of 1.67 IU and 1.61 IU has been observed.

This implies that the pH of the medium influences the growth of microorganisms and hence the enzyme production. Each microorganism possesses a specific pH range for its growth and activity. Maximum pectinase production 35.84 U/ml from *Bacillus* sp. has been reported at pH 5.0 by Laha *et al.,* (2014). Highest pectinase activity (1.6 IU/ml) was observed at pH 6.0 from *Bacillus* sp. isolated from vegetable waste soil (Kaur *et al.,* 2016).

Effect of temperature

Maximum pectinase titres were produced at 30^{o}C i.e. (5.59 IU) whereas; least pectinase production was found at 45^{o}C (2.31 IU) from *Stenotrophomonas maltophilia* P9. Incubation temperature is the most important physical factor which affects enzyme production dramatically and their stability. Maximum enzyme activity at optimum temperature may be due to the faster metabolic activity and increase in protein content and extracellular enzyme production in culture supernatant. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity (Willey *et al.,* 2008) (Fig. 3).

Kumar *et al.,* (2012) studied the production and optimization of pectinase from *Bacillus* sp. MFW7 and high pectinase production (1.6 IU/ml) was observed at temperature 35^{o}C. Nithisha *et al.,* (2016) studied the optimization of pectinase from *Bacillus* sp. and reported maximum enzyme activity was shown as 40 units/ml at optimum temperature 35^{o}C.

Effect of inoculum size

The data in Table 3 revealed that optimum inoculum size found to be 10.0% (V/V) for pectinase production revealing 5.92 IU. This inoculum size has statistically significant differences over other inoculum sizes. The least pectinase units were recorded at 17.5% (1.54 IU) as in Table 3. Thus, it is evident from the data that the enzyme titers were significantly affected by different inoculum sizes.

Highest pectinase titres of 5.92 IU from *Stenotrophomonas maltophilia* P9 was observed at 10% inoculum size having statistically significant differences over the range of variable inoculum sizes. There was a steady increase in the enzyme production from 7.5 to 10% however there was a steep decrease in the enzyme activity beyond this inoculum size because of an overload of the cells in the production medium resulting in nutrient starvation. A lower inoculum size 5% of has been reported optimum for pectinase production from *Bacillus* sp. by Hazziz *et al.,* (2013). Venkata *et al.,* (2016) reported an enzyme activity of 136 U/ml from *B. circulans* at an inoculum size of 5%.

Effect of different incubation period

To determine the effect of incubation period on pectinase production from *Stenotrophomonas maltophilia* P9, enzyme activity was measured at regular intervals from 24 h to a period of 144 h (Fig. 4). Highest pectinase activity was measured at 72h (6.94 IU) from followed by gradual

decline on either side. Least enzyme production was observed at 144 h (2.42 IU) of fermentation time. Statistically enzyme production was found significantly higher at 72 h than others timings.

Afterwards incubation beyond 72 h resulted in a decreased enzyme activity that could be due to depletion of nutrients available causing a stressed microbial physiology eventually resulting in an inactivation of enzyme (Flores *et al.*, 1997). Maximum yield of pectinase (40 U/ml) was obtained after 72 h incubation from *Bacillus* sp. isolated from soil by Nithisha *et al.,* (2016). Venkata and Diwakar (2013) found that 48 hours was the optimum duration for maximum pectinase enzyme activity (166U/ml) from *Bacillus circulans* isolated from dump yards of vegetable wastes. Above this period the pectinase enzyme activity started to decrease. In case of pectinase production enzyme activity increased from 3.95 IU to 6.94 IU and 75.69 percent increase was noticed in pectinase production after optimizing different process parameters as shown in Figure 5. Classical approach for one Variable at a time (OVAT) used in the present enzymes optimization study has resulted in an increase in the production of different enzymes proving the direct utility of this technique in increasing enzyme titers.

In the current study a pectinase producing bacteria *Stenotrophomoans maltophilia* P9 was isolated from *Rhizoclonium* sp. algal biomass collected from different sites of Himachal Pradesh. Laboratory scale optimization of different conditions for enhanced eznyme production by carried out by classical one factor at a time approach (OFAT). The maximum productivity of pectinase achieved by optimized process parameters such as temperature 30^{o}C, pH 5, incubation time 72 h, inoculum size 10% was 6.94 IU. It can be concluded from the above studies, *Stenotrophomonas maltophilia* P9 based on its capability to produce pectinase could be considered as potential candidate for industrial applications and this genus is first time reported for pectinase production. Moreover the study also reveals the values as well as the microbial wealth of pectinase producing bacteria which can be a boon for the development of biotechnological processes. However, these parameters have to be tested in mass cultures in automated incubators so as to confirm the optimum conditions for enzyme production for industrial application.

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