Production of Bacterial Pectinase from Agro - industrial Wastes

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Abstract : Currently, the fundamental exploitation of agricultural and food wastes, which participate in pollution, is the controlled biological degradation of the wastes by microorganisms for the production of valuable compounds such as vitamins, enzymes etc. for medicinal and industrial uses. Pectin is a major component of primary cell wall of all land plants and encompasses a range of galacturonic acid rich polysaccharide. There are very few reports of Pectinase enzyme production by bacterial strains. In the present study, forty seven bacterial isolates from rotten vegetable and fruit samples were screened for Pectinase production. The strains showing maximum activities were identified as *Bacillus* sp. (VM1) and *Bacillus* sp.(R2).and used for further studies. Maximum quantities of Pectinase were produced when a 18 Hrs. old inoculum was used at 4.0 % (v/v) of production medium under shaking conditions for 72 Hrs. The optimal temperature and pH for bacterial growth and Pectinase production were found to be 40°C and 7.0, respectively. With both the isolates, maximum enzyme production resulted when 5% citrus peels were used as the carbon source. Peptone among organic nitrogen and Potassium nitrate among inorganic nitrogen source with Glucose as carbon source in presence of Magnesium sulphate as metal ion showed best results. The supplementation of media with 0.9% (w/v) D-Galacturonic acid led to a 20% & 14% increase in activity for *Bacillus* sp.(VM1) and *Bacillus* sp.(R2). Both the isolates were effective in fabric Bioscouring and decreasing fruit juice viscosity. Both the isolates due to their pectinase production at neutral pH would be potentially useful and suitable for the vegetable purees or other preparation which need almost neutral pH range.

Key words : *Bacillus* sp., Pectinase, Agro industrial wastes.

Introduction

Many plant pathogenic bacteria and fungi are known to produce pectinolytic enzymes useful for invading host tissues. Moreover, these enzymes are essential in the decay of dead plant material by non pathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere (Soares Marcia C.N. etal., 1999).Pectic substances are glycosidic macromolecules with high molecular weight. Pectinases are produced by a large number of organisms, such as bacteria, fungi, actinomycetes and yeast (Nadaroglu H. et al., 2010). At present almost all the pectinolytic enzymes used for industrial applications are produced by the fungi .There are a few reports of pectinases production by bacterial strains. (Jayani R. et al., 2010). Pectinases have been used in processes and industries where the elimination of pectin is essential; fruit juice processing, macerating of plants and vegetable tissue, waste water treatment, extracting vegetable oil, bleaching of paper, adding poultry feed , in textile and alcoholic beverages and food industries(Nadaroglu H. et al., 2010). The selection of a particular strain remains a tedious task and the choice gets tougher when commercially competent enzymes yields are to be achieved. Due to optimal pH of fungal pectinase (3.0 – 5.5) such preparations are not suited for production of vegetable purees or other preparations in which pH values are close to neutral. Furthermore, due to the relatively low temperature stability of the fungal enzyme preparations, maceration needs to be carried out at temperatures not exceeding 45°C, necessitating the incorporation of a pasteurization step to limit the growth of mesophillic microorganisms (Marcia M.C.N.Soares et al., 1999). Therefore bacterial strains producing commercial enzymes are always preferred over fungal strains because of ease of fermentation process (for production) and implementation of strain improvement techniques or any modern technique to increase the yield of production (Kumar A. and Sharma R., 2012).

The objective of the present research work was to enrich and isolate pectinase producing bacterial strains from decomposing and rottened agro industrial wastes followed by characterization and optimization of bioprocess parameters for the best producer of enzyme.

Materials and Methods

Isolation and identification of pectinase producing bacteria

The Agricultural and vegetable waste dump soil samples along with rotten fruits and vegetables in and around Ulhasnagar, District Thane, Maharashtra. were collected and serially diluted up to 10^7 dilutions. It was plated on Nutrient agar medium and incubated at room temperature for 24 hours. The isolated pure strains were screened for extra cellular pectinase production using Pectate agar medium : Peptone - 3g, Yeast extract -0.5g, kH_2PO_4 – 0.15g, $CaCl_2$ – 0.001g, Pectin – 0.5g, Na_2CO_3 – 0.5g at pH – 8. The substrate utilized zone around the colony was observed by flooding the plate and by agar cup method using 3.3% Cetyltrimethyl Ammonium Bromide (CTAB) solution overlaid on the medium and incubated for 10 min. (Elangovan Namasivayam et al., 2011)., the positive strain that produced

maximum pectinase enzyme was selected and identified by Bergey's manual of Determinative Bacteriology . The cell free supernatant was used for Pectinase assay and purification. The supernatant was partially purified by using Ammonium sulphate precipitation method followed by dialysis using cellophane membrane (Klug- Santner B.G. et al., 2006).

Pectinase Assay

Pectinase activity was assayed by the colorimetric method of Miller (1959). The standard graph was generated using standard glucose solution. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1µm glucose per min.

Protein determination

Protein of all enzymatic preparations was determined by the method of Lowry et al.(1951).

Parameters controlling the Pectinase activity

Effect of Incubation Time, Temperature, pH, Inoculum sizes and aeration conditions

The bacterial isolate was subjected to different culture conditions to derive the optimum conditions for pectinase production. Pectinase production was estimated at regular time intervals (24 , 48, 72, 96, and 120 h) . For selected temperatures (30 - 70 °C) and pH (4.0 to 8.0), the relative activities (as %ge) for both were expressed as the ratio of the Pectinase activity obtained at certain point of temperature / pH to the maximum activity obtained at the given temperature /pH range. Effect of varied Inoculum sizes of 0.1 OD at 615 nm (1 – 5 ml) and effect of aeration was studied by using different flasks volumes (100, 150, 250,500 and 1000ml) with fixed media volume(25 ml).

Effect of D-Galacturonic acid on Pectinase production

To study the effect of D-galacturonic acid on Pectinase production, it was added to the culture broth at a final concentration of 0.3 to 1.5 % w/v under aseptic conditions. The resulting extracellular Pectinase activity produced were measured by estimating the reduced groups produced (Jayani R. etal., 2010).

Effect of Carbon and Nitrogen sources

The carbon sources such as Maltose, Sucrose, Mannitol, Lactose, Glucose, Cellulose and Starch and Nitrogen sources such as Ammonium chloride, Potassium nitrate, Sodium nitrite, Ammonium citrate, Gelatin, Peptone and Casein were supplemented as individual components to the mineral salt media to check their effect on pectinase production, by keeping all predetermined factors constant.

Effect of metal ions

To determine the influence of metal ions viz. Ca^{+2}, EDTA, Cu^{+2}, Mg^{+2}, Ba^{+2} and Co^{+2} on Pectinase activity. The listed ions were added to the reaction mixture at concentration (1mM). Pectinase activity without added metal ions was taken as 100% activity (EI- Batal.A.I etal. 2013).

Effect of natural pectin

The growth medium was prepared and synthetic pectin was replaced by different natural pectin substrates such as Sugarcane baggase, Orange peels, Potato peels, Banana peels, Tea waste, Apple peels and Citrus peels . All other predetermined factors were kept constant .The cell free supernatant was analyzed for Pectinase activity (Marcia M.C.N. Soares et al., 1999).

Growth and enzyme production profile under optimized parameters

The growth and enzyme production profile of the microorganisms was studied by withdrawing the samples from the optimized media and parameters at regular intervals up to 96 Hrs. (Jayani R. et al., 2010).

Effect of mutagens

For the sake of strain improvement, the identified pectinolytic strains were treated with two mutagens viz. Ultraviolet irradiation as physical mutagen and hydrogen peroxide as chemical mutagen (Akbar S. et al., 2013). A loopful of parental strains were inoculated into the plate and was exposed to UV radiation for 10, 20,30,40,50 and 60 minutes time interval under beam of UV lamp (Germicidal lamp, UV tube, T-15c, 15W, 254 nm). The distance between lamp and the Petri plate was adjusted to 10 cm for each trail to obtain 95% death rate. On completion of predetermined time the plates were retained in dark for overnight to prevent photo reactivation of mutants.

Similarly, for chemical mutagen treatment, selective media was prepared and molten agar was mixed with different concentration of hydrogen peroxide ranging from 0.5-5% and inoculated with potential strain. The cfu count was recorded to plot a survival /kill curve. Further after physical / chemical treatments , the strains were tested quantitatively for enzyme production by shake flask culture fermentation.

Bioscouring of Cotton and Change in viscosity of fruit juice

Cotton fabric was treated with crude pectinase enzyme at R.T on shaker for 24 hrs. After treatment, fabric was dried, weighed and then weight loss was determined (Rajendran R. etal., 2011). Decrease in viscosity of apple juice was studied after treatment with extracted crude Pectinase enzyme by using viscometer (Prathyusha K. and Suneetha V.2011).

Results and Discussion

In the present study, forty seven bacterial isolates were isolated using agro industrial wastes from different vegetable markets and restaurants of Ulhasnagar, Dist.-Thane (MS). On screening of pectinolytic productivities of the forty seven bacterial isolates, eleven isolates were using pectin as sole carbon source. From these isolates, two potent isolates were selected on the basis of their Pectinase production and used for further studies.

Both the isolates were identified with reference to Bergey's manual of Determinative Bacteriology (2000) as *Bacillus* sp. and named as *Bacillus* sp. VM1 (isolated from discarded rotten vegetables and fruits from vegetable market) and *Bacillus* sp. R2 (isolated from Restaurant and juice centre discarded wastes).

Pectinase produced by isolates was purified using Ammonium sulphate precipitation, followed by dialysis. Results (data not shown) indicate a decrease in total proteins and total activity, where as specific activity increased. After purification, specific activity for pectinase from *Bacillus* sp. (VM1) was 5 micro mol/mg with increase in 4.24 purification fold while *Bacillus* sp.(R2) showed specific activity 3.96 micro mol/mg with 3.14 increase in purification fold compared to crude enzyme.

Maximal pectinase activity was observed between 72-96 Hrs.(Fig.-2), after which a decline in enzyme activity was observed . This might be due to denaturation and /or decomposition of pectinase as a result of interaction with other compounds in the fermented medium or due to sugar consumption.

The enzyme was active over a broad pH range (Fig.-3), displaying over 45 % of its activity in the pH range of 4.0 upto 9.0 with an optimum pH of 7.0. Concerning to the pectinase at pH 9.0, the relative activity decreased down upto 61% for *Bacillus* sp. (VM1) and 49 % for *Bacillus* sp. (R2). This could be attributed to histidine residues that have ionizable side chains, increasing the net negative charge on the molecule in the alkaline pH range and leading to repulsion between the strands, resulting in destabilization of the hydrogen bond structure of the enzyme. The optimum pH for PGase was higher than the majority of fungal PGase described, and they are acidic enzymes as reported by many workers. *Monileilla* sp. showed its maximum activity at pH 4.5 and at pH 4.5-5.0 for *Penicillum* sp. (EI-Batal A.I. et al., 2013).

The activity of the pectinase increased gradually at temperature ranged from 30°C up to 60°C (Fig.-4) .Moreover the optimum temperature was achieved at 40°C, meanwhile the relative activity was attained approximate 25% at 70°C, this clearly evidenced that the higher temperature resulted in a decrease in the enzyme activity. It was reported that extremely high temperature lead to deamination, hydrolysis of the peptide bonds, interchange, and destruction of disulphide bonds and oxidation of the amino acids side chains of the enzyme protein molecules ((EI-Batal A.I. et al., 2013).

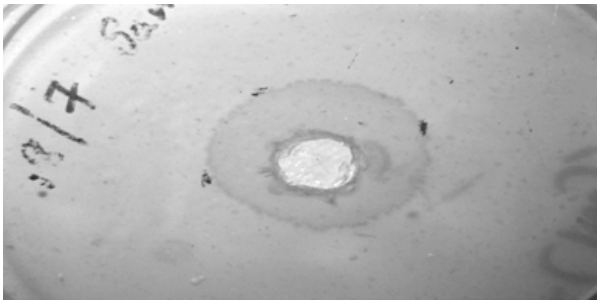


Fig.1- Pectinase activity by Agarcup method

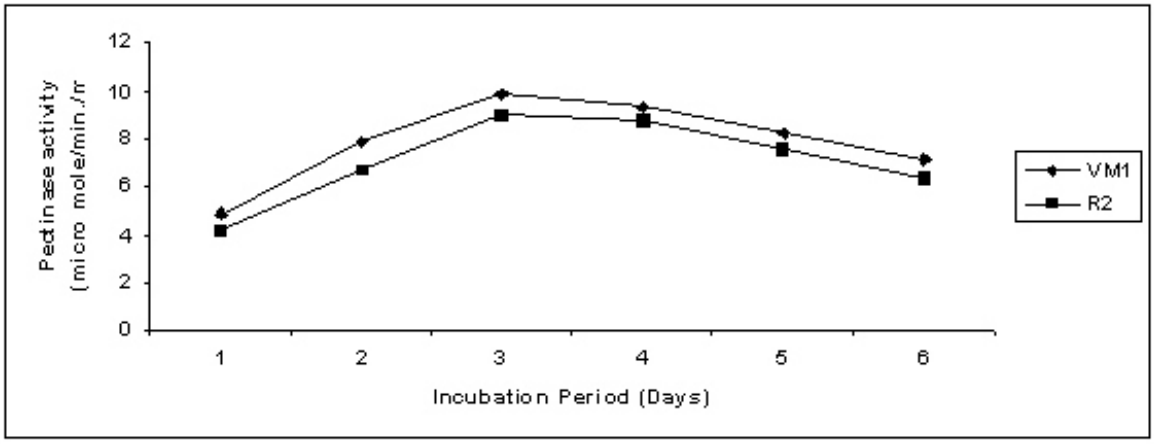


Fig.2 : Effect of Incubation period on the Pectinase activity of *Bacillus* isolates .

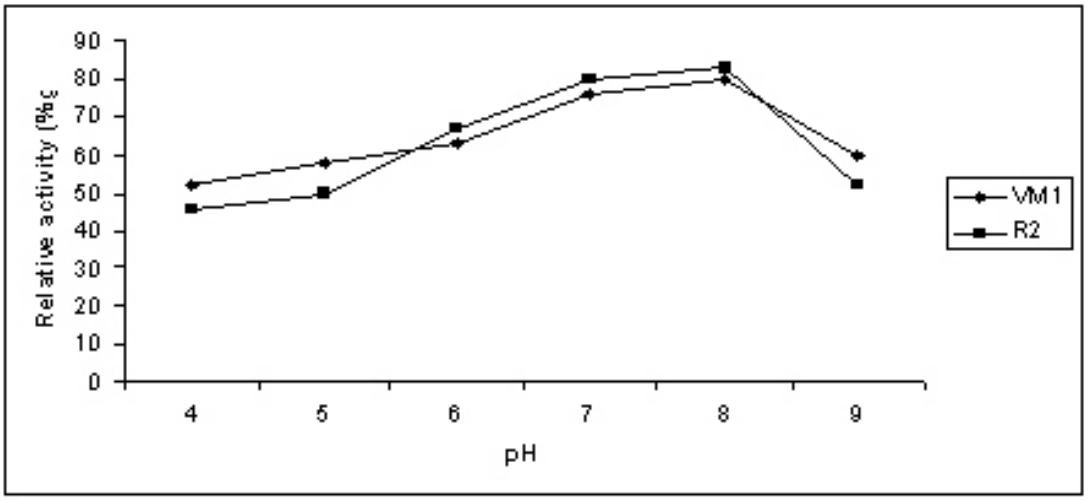


Fig. 3 : Effect of pH on the Pectinase activity of *Bacillus* isolates .

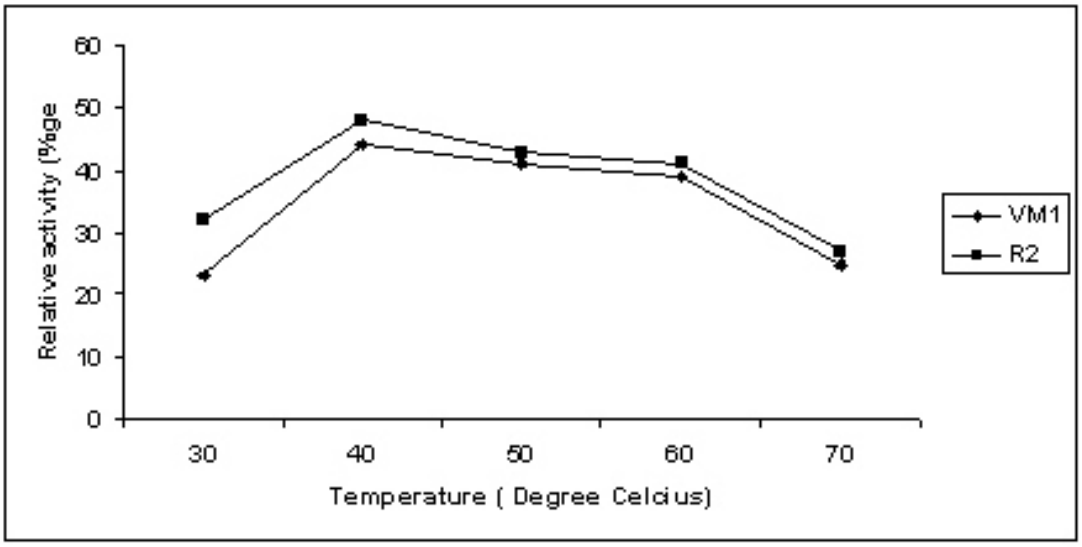


Fig. 4 : Effect of temperatures on the Pectinase activity of *Bacillus* isolates .

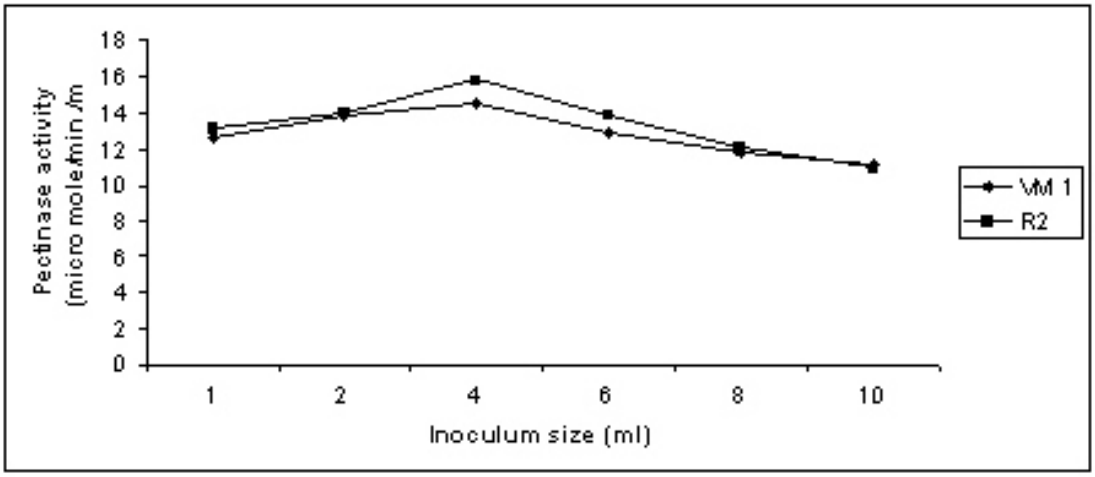


Fig.5 : Effect of inoculum size on the Pectinase activity of *Bacillus* isolates .

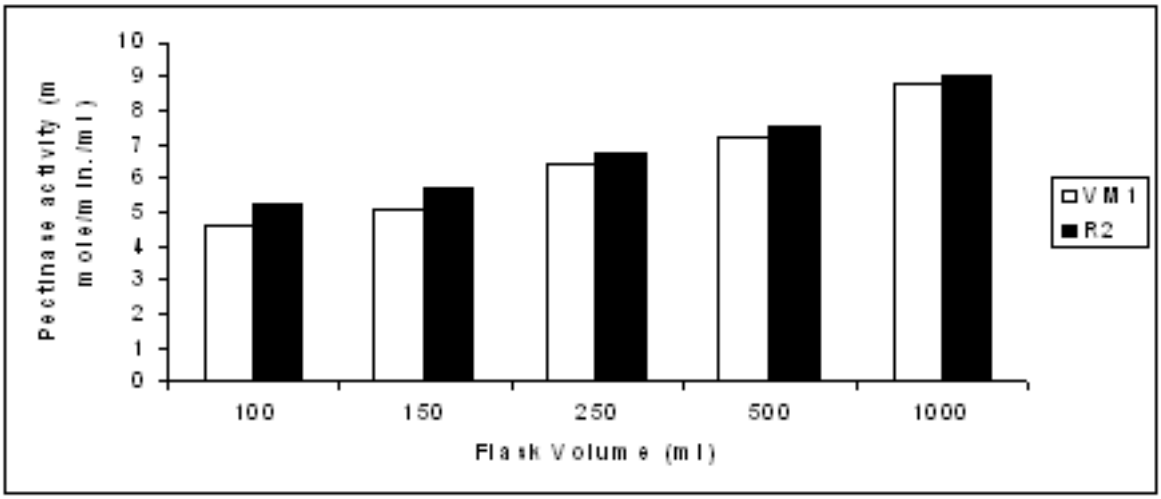


Fig. 6 : Effect of different flask volumes (aeration)on the Pectinase activity of *Bacillus* isolates.

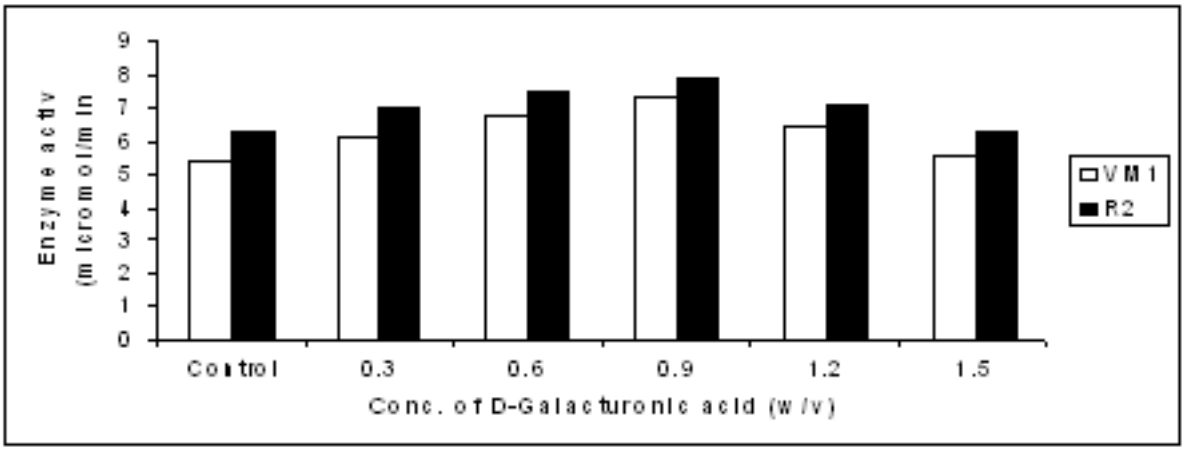


Fig. 7 : Effect of D-galacturonic acid on Pectinase production by *Bacillus* isolates.

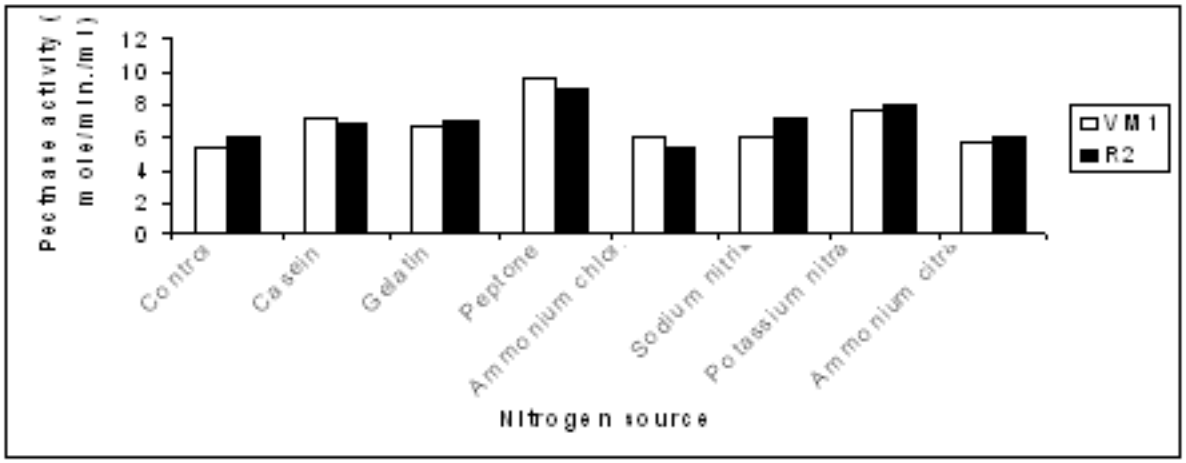


Fig. 8 : Effect of Nitrogen source on the Pectinase activity shown by *Bacillus* isolates.

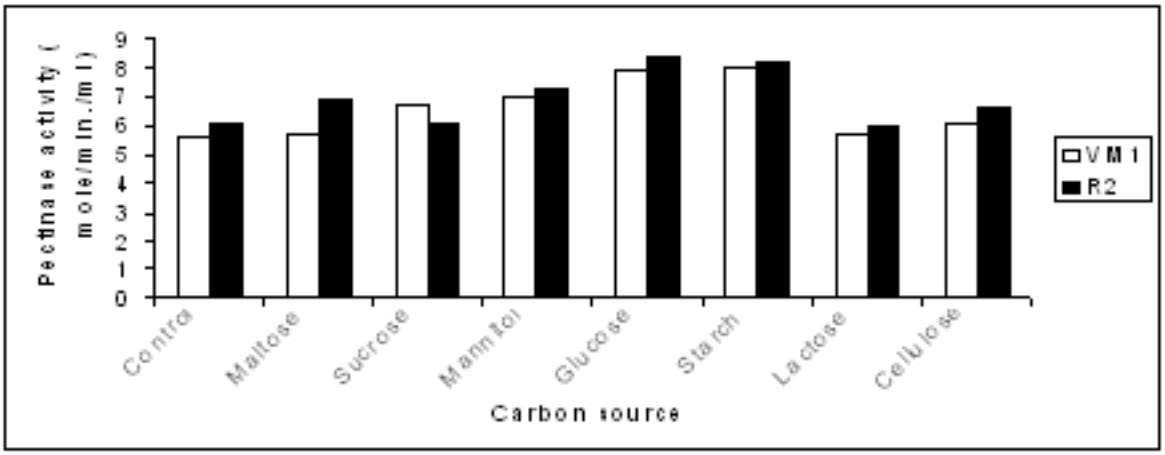


Fig. 9 : Effect of Carbon source on the Pectinase activity shown by *Bacillus* isolates.

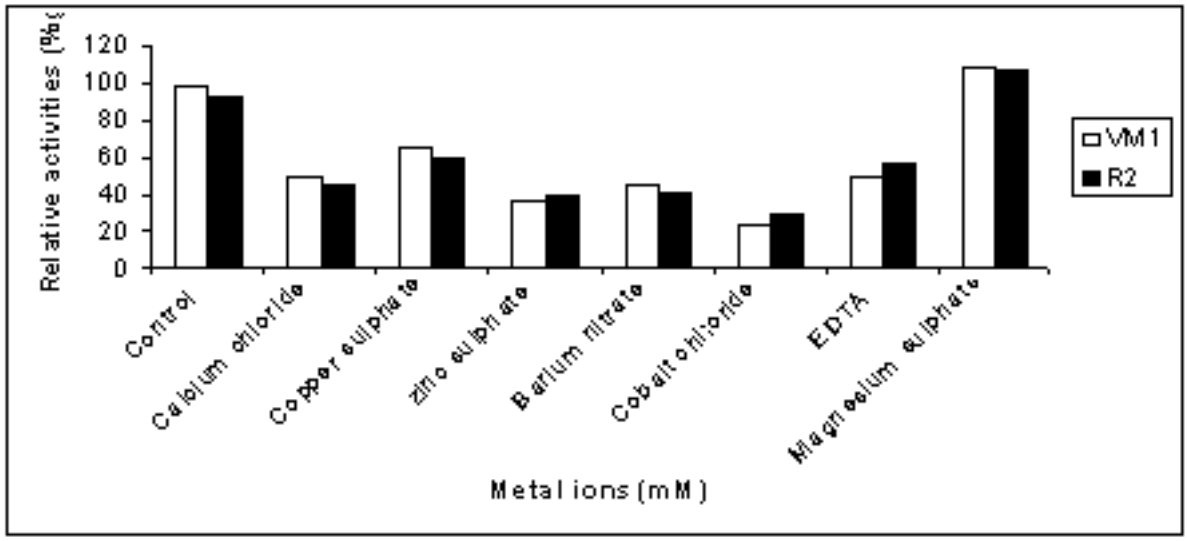


Fig.10 : Effect of metal ions on the Pectinase activity of *Bacillus* isolates.

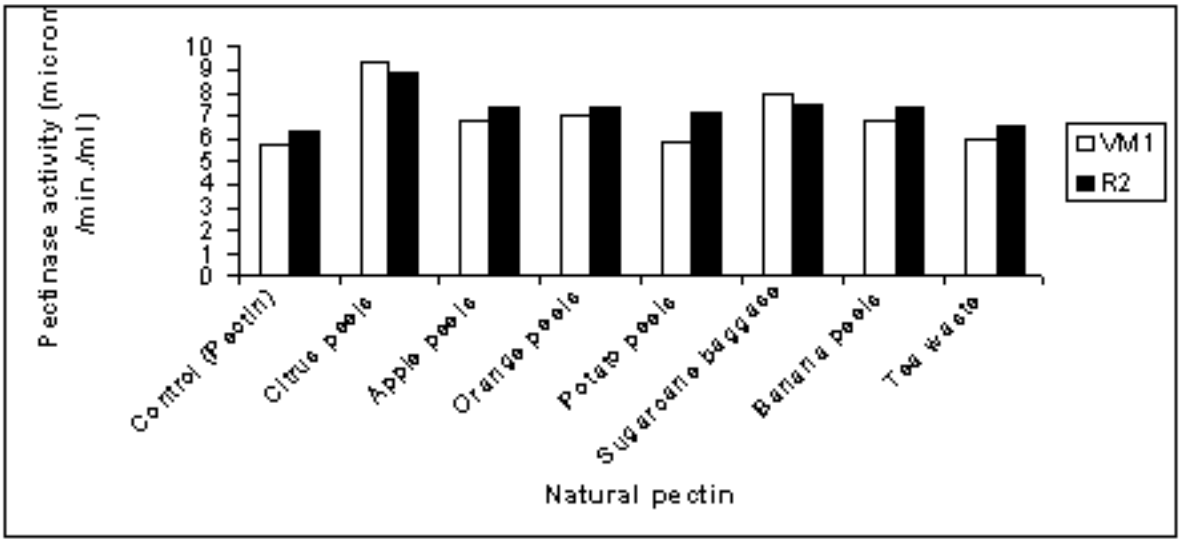


Fig.11 : Effect of natural pectin sources on the Pectinase activity of *Bacillus* isolates.

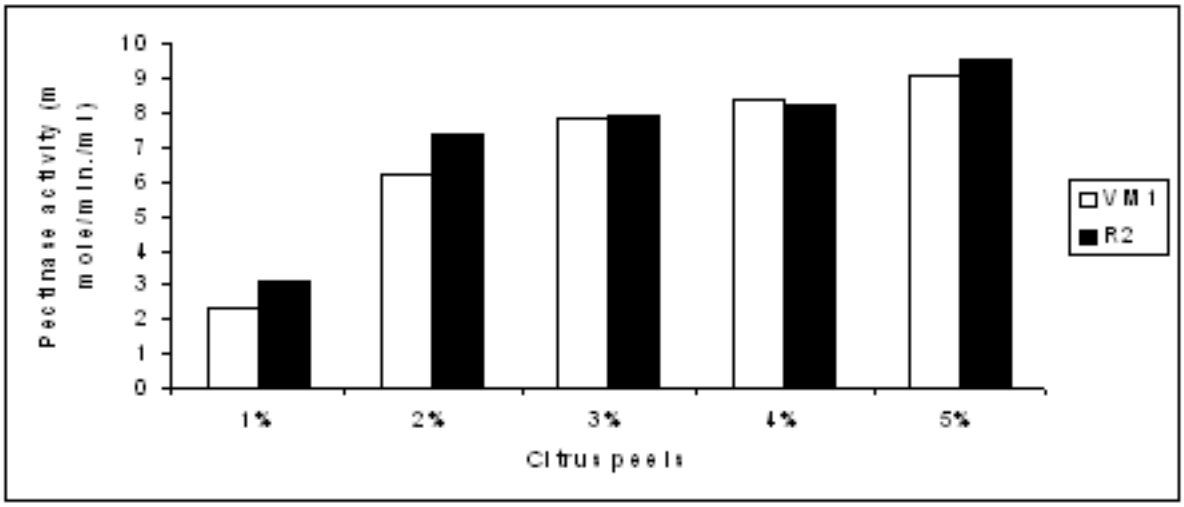


Fig. 12 : Effect of Citrus peels concentration on the Pectinase activity of *Bacillus* isolates.

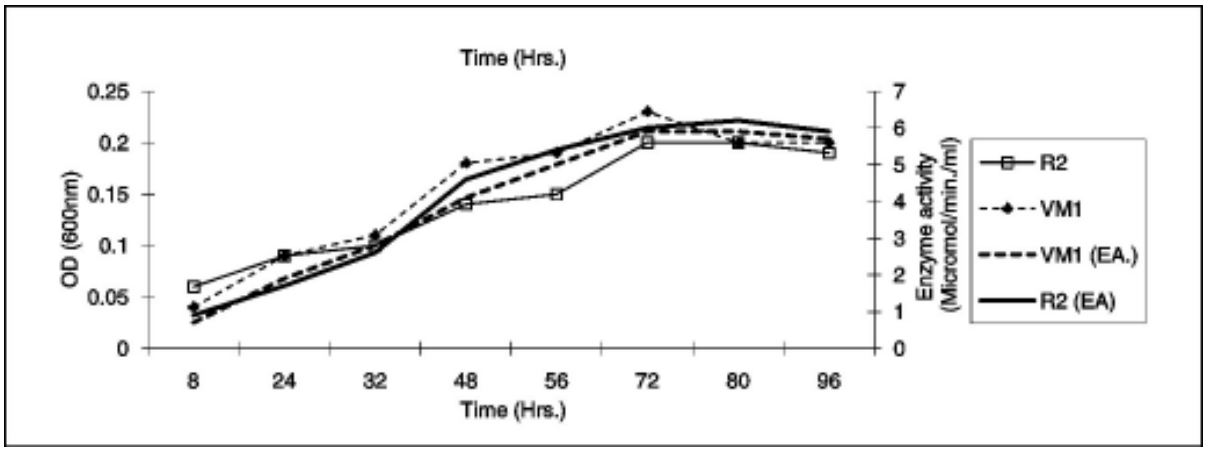


Fig. 13 – Growth and enzyme production profile of *Bacillus* isolates under optimized parameters and media.

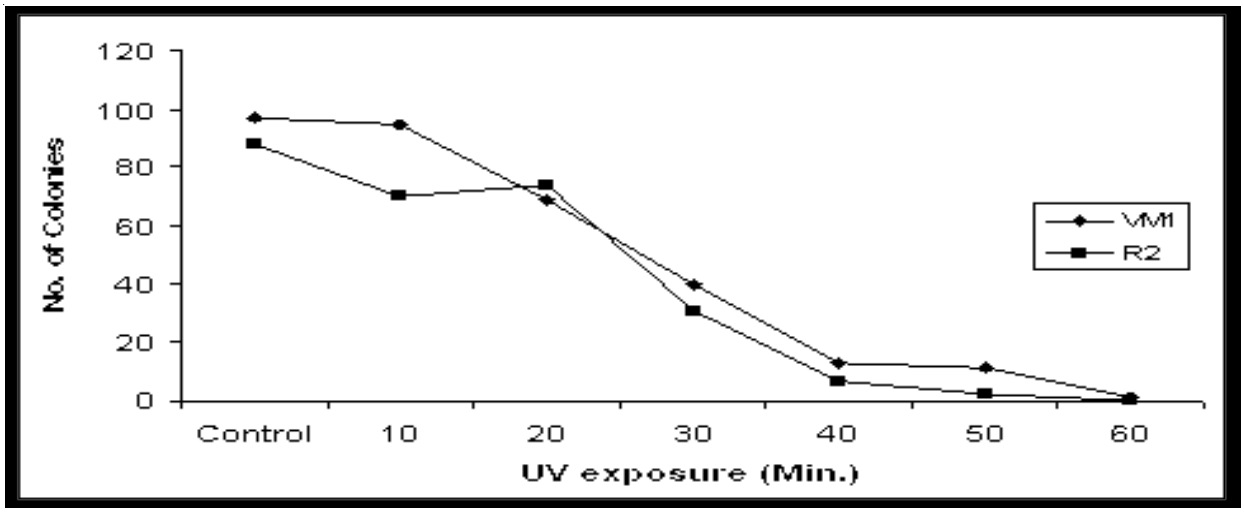


Fig. 14 - Survival curve of UV mutant *Bacillus* isolates

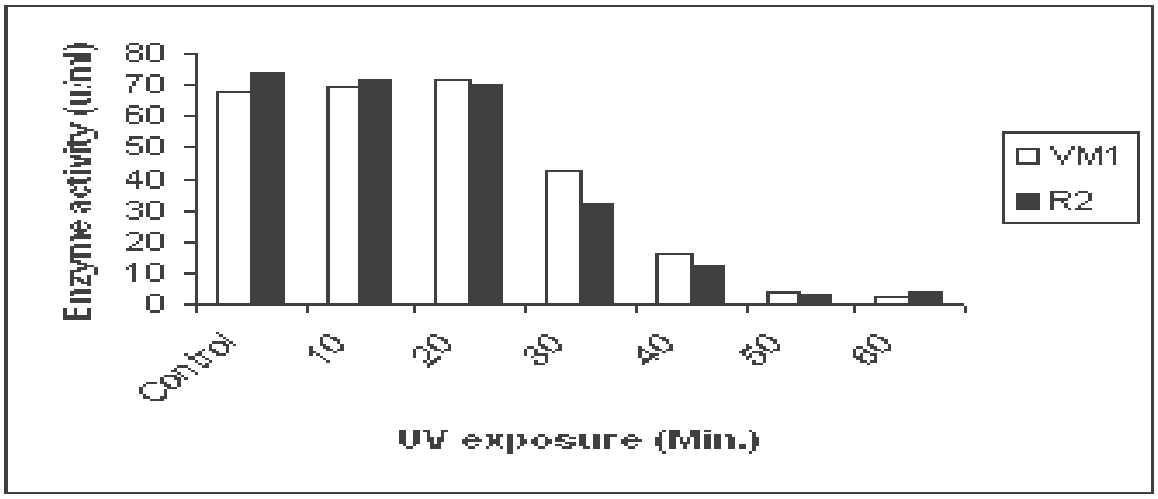


Fig.15– Pectinase activity of UV mutant isolates

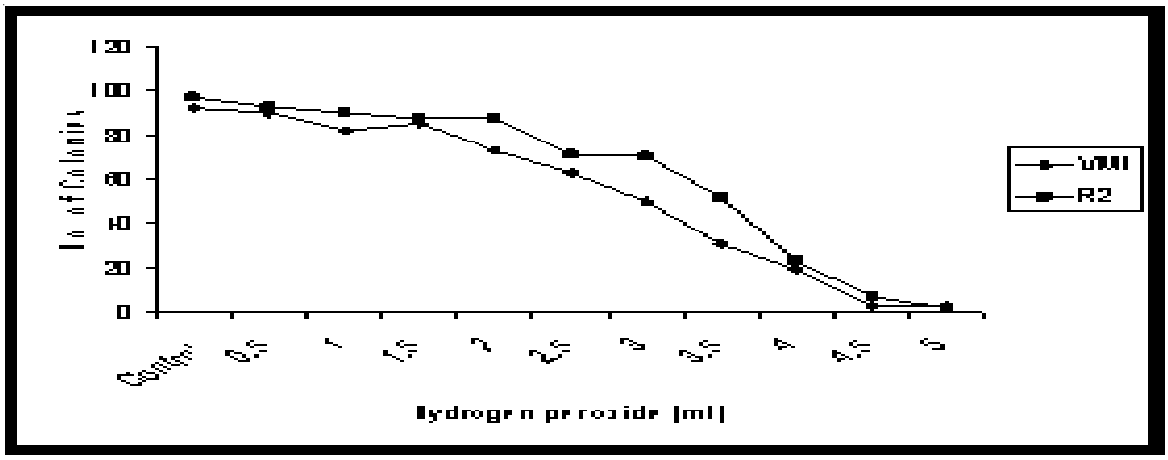


Fig.16 - Survival curve of H_2O_2 mutant isolates

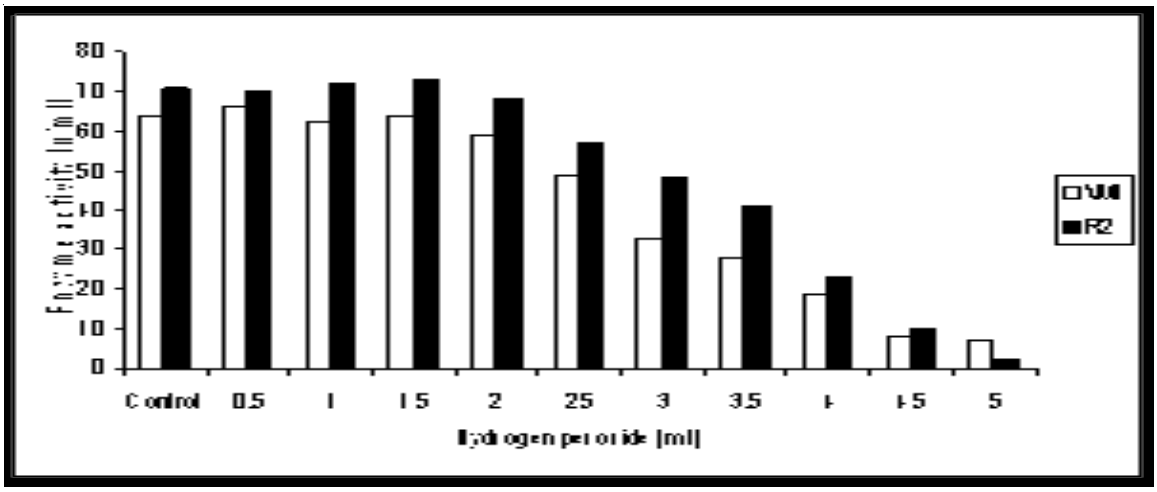
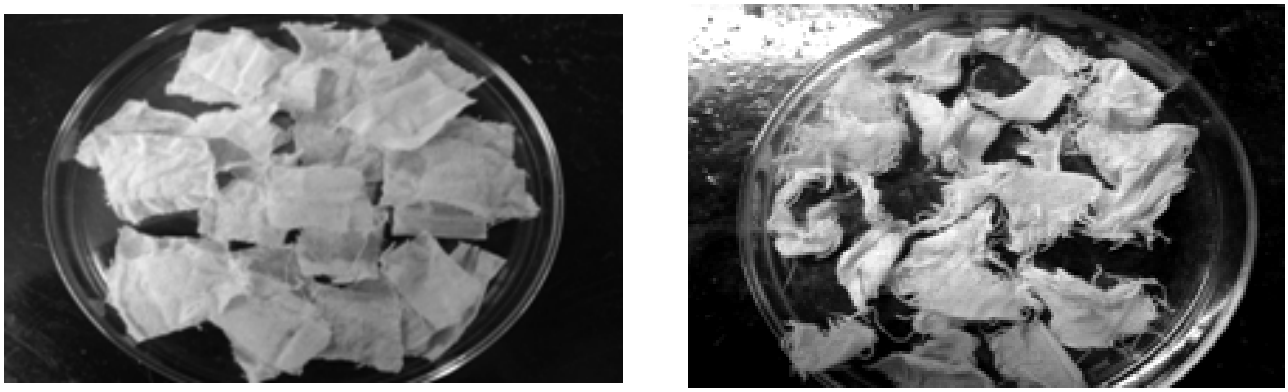


Fig.17 – Pectinase activity of H_2O_2 mutant isolates.

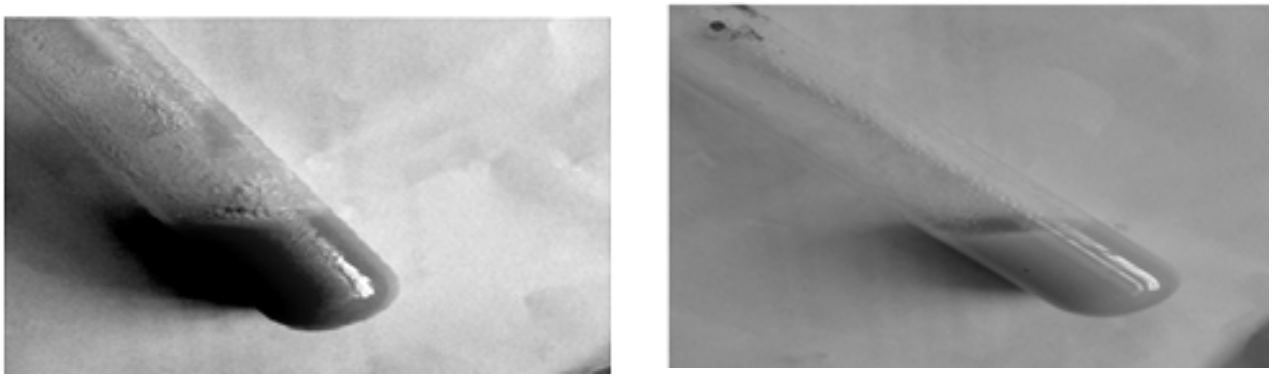
Table 1 : Applications of Pectinase

No.	Treatment	*Bacillus* Isolate	Before treatment	After treatment	%ge decrease
1	Bioscouring of cotton fabric	VM1 R2	1.09 1.10	0.97 0.95	11.01 13.64
2	Decrease in viscosity of Apple juice	VM1 R2	290 Sec. 287 Sec.	67 Sec. 59 Sec.	79.93 82.61



Before treatment **After treatment**

Fig. 18 : Bioscouring of Cotton fabric



Before treatment **After treatment**

Fig 19: Decrease in viscosity of apple juice

The optimal inoculum size needed to produce the highest yield of pectinase production was 4.0 ml (10^8 cells/ml) for both the isolates (Fig.-5). Beyond this Inoculum density, enzyme activity gradually decrease, may be due to the limitation of substrate concentration in the flask.

The effect of aeration conditions, (Fig.-6) revealed that, 1000 ml flask volume was more favorable for pectinase production with activities viz. 8.8 micro mol/min./ml for *Bacillus* sp. (VM1) and 9.1 micromole/min./ml for *Bacillus* sp. (R2).This may be due to 1000 ml flask volume supply enough aeration needed for respiration and metabolic activities. Some workers had reported that improvement in product yield is expected in the fermenter as compared to that in flasks, because of better control of process parameters in the former (Bayoumi R.A. et al., 2008).

An increase in activity of about 20 % for *Bacillus* sp. (VM1) and 14 % for *Bacillus* sp. (R2) was observed when the production medium was supplemented with 0.9% w/v D-Galacturonic acid (Fig.-7). Decreasing or increasing the concentration of D-galacturonic acid had an antagonistic effect on the production of the enzyme. The stimulatory effect of the addition of D-Galacturonic acid in the production medium on pectinase production by *Sclerotina sclerotiorum* and *Aspergillus niger* has been reported previously (Jayani R. et al., 2010).

Among different nitrogen sources (Fig.-8),Peptone, Gelatin, Casein and Potassium nitrate were the best Pectinase inducers for both the *Bacillus* sp. This might be due peptone and gelatin are natural nitrogen sources. Where as in the case of remaining nitrogen sources Pectinase production was affected might be due to poor growth of the *Bacillus* isolates on these nitrogen sources.

Among carbon sources (Fig.-9), Glucose proved the best supporting carbon source for both the isolates in competition with Starch and Mannitol followed by with moderate response showed by Cellulose, Sucrose and Maltose. While lactose had no any supportive role in pectinase production.

During study for effect of metal ions (Fig.10) on the enzyme activity, among different ions, Magnesium sulphate showed increase in enzyme activity viz. 10% for *Bacillus* sp. (VM1) and 14% for *Bacillus* sp. (R2) while other ions were responsible for decrease in enzyme activity. It was reported that the formation of a chelate complex between the substrate and the metal ions could form a more stable metal- enzyme- substrate complex and stabilizing the catalytically active protein confirmation.

On use of natural pectin source (Fig.11 & 12) , citrus peels had shown maximum activity for both the isolates. On comparison for pectinase production under solid state fermentation (SSF) and submerged fermentation (SmF), The SSF (data not shown) showed higher pectinase for both the isolates viz. for *Bacillus* sp. VM1 (9.7 micro mol/min./ml) and for *Bacillus* sp. R2 (9.3 micro mol/min./ml).The SSF is usually simpler and can use wastes of agro industrial substrates for enzyme production (Abhasi H. etal., 2011). The minimal amount of water in SSF allows the production of metabolites in a more concentrate form making the downstream processing less time consuming and less expensive. Higher production of pectinase in solid state fermentation process may be due to reason that solid substrate not only supplies the nutrient to the microbial cultures growing in it, but also serves as anchorage for the cells allowing them to utilize the substrate effectively (Bayoumi R.A. et al., 2008).

When the growth and enzyme production profile of *Bacillus* isolates was studied, a rapid increase in biomass during first 72 Hrs. of fermentation was observed, after which the growth became almost constant probably due to exhaustion of nutrients in the culture medium. The enzyme production started increasing after 32 Hrs. and reached its maximum after a 72 Hrs. remain stagnant for longer .The results under optimized conditions were compatible with enzyme production under non optimized conditions(Fig.13).

The adverse effects of physical & chemical mutagen were seen as decrease in cfu/ml and enzyme activity after 20 min. of UV exposure and at 2.0 ml concentration of Hydrogen peroxide in media (Fig.14 to Fig.17). Therefore strain improvement can be tried by using other standard conditions/agents in order to achieve strain improvements for enzyme production.

On applications of crude enzyme for Bioscouring of cotton fabric and decrease in viscosity of apple juice, pectinase from both the isolates showed good response in the given time with enhanced activity for pectinase from *Bacillus* sp.(R2) comparatively (Table-1 and Fig.18 -19).

Conclusion

The present study made a successful primary attempt to enrich and isolate the potential bacterial strain from the natural reservoir (rotten agro-industrial wastes) producing industrially important pectinase enzyme. The isolated both bacterial strains were identified as *Bacillus* sp. The production and optimization studies revealed that isolates requires 40^{o}C, pH 7.0, 72 Hrs. of incubation time , 4.0 ml of 1X10^{8} cells/ml culture density, Glucose(Carbon source), Peptone (Nitrogen source), 0.9 % (w/v) of D-Galacturonic acid (w/v), and Large volume flask for higher pectinase enzyme production. Citrus peels and Magnesium sulphate acts as a good agro waste substrate and supplement respectively. Weighing all its potent pectinase applications, more emphasis is to be laid not only on screening the novel pectinolytic bacteria, but also on production of high yielding strains. Utilization of fruit processed industrial byproducts and wastes as substrate acts to recycle the waste and to decrease the production cost making it economical. Hence furious work in this area is found to be an adept opportune both to the researches and to be industry.

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