

Pectinolytic activities of pectinase produced by some bacterial isolates cultured from deteriorating fruits

Torimiro, N.,^{1*} Adediwura, V. A.,¹ Ojo, S. T.,¹ Oluwadare, A. O.¹ and Okonji, R. E.²

¹Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria

²Department of Biochemistry and Molecular Biology, Obafemi Awolowo University, Ile-Ife, Nigeria

Copyright resides with the authors in terms of the Creative Commons License 4.0.

(See <http://creativecommons.org/licenses/by/4.0/>).

Condition of use: The user may copy, distribute, transmit and adapt the work, but must recognize the authors and the Nigerian Journal of Biotechnology.

Abstract

Microbially derived pectinases have been shown to have due advantages over those produced from other sources because of the ease in manipulating the microorganisms to increase yield. The study was undertaken to determine the pectinolytic activities of pectinase produced by some bacterial isolates cultured from deteriorating fruits under submerged fermentation. Deteriorating oranges and grapefruits were collected and surfaced sterilized. Thereafter, 1 g of each sample was homogenized and transferred into 10ml sterile distilled water. The suspensions were agitated, streaked onto nutrient agar and incubated at 37 °C for 24 h. The isolates were screened for pectinolytic activities and identified using standard protocols. The specific activities of the enzyme and the physicochemical factors were analysed. The isolates identified were *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Klebsiella aerogenes* isolates. The specific activities of pectinase produced by the isolates were between 26.40- 52.27 U/ml. The isolates reacted differently to the different substrates used. The optimum temperature for the activity of pectinase observed from the isolates was 50 °C and the pH varied between pH 4 and 8. Sodium azide at 1 mM and EDTA at 10 mM were observed to inhibit pectinase activity in all the isolates. The isolates showed promising potential for pectinase production under optimal physicochemical conditions.

Keywords: Bacteria, Pectinase, deteriorating fruits, submerged fermentation, physicochemical parameters

*Corresponding Author- ntorimiro@gmail.com

Introduction

Enzymes are appreciably utilized in various industrial processes. It has been reported that microorganisms possess the abilities to synthesize various enzymes required for various uses (Pandey et al., 1999). Enzymes obtained from different microorganisms are known to be superior and they perform reactions in bio-processes in an economical and environmentally friendly way as opposed to the chemical catalyst (Poonam, 2013). Microorganisms have been shown to possess the ability to synthesize different types of pectinolytic enzymes with a different mechanism

of action and biochemical properties (Gummadi & Panda, 2003; Favela-Torres et. al., 2005). Pectinases are a complex group of enzymes that act specifically on pectic substances and influence intracellular adhesivity and tissue rigidity (Tatiana da Costa & Flavo, 2005).

Microorganisms have a number of advantages in the production of pectinase through the application of selection methods, increase of biosynthesis via the conditions of cultivation, in-depth interaction on various substrates, a wide spectrum of enzyme complex and can genetically be modified (Vibha & Neelam, 2010). Bacteria have been exploited in

pectinase production and the capacity of selected species to produce and secrete large quantities of extracellular enzymes has been reported (Namasivayam et. al., 2011; Geetha et. al., 2012; Torimiro & Okonji, 2013). Enzymes are easily denatured and their catalytic activity may be significantly reduced by a variety of physical or chemical agents. However, if these conditions are maintained at the optimum, increasing enzymes activity and longer stability may be achieved (Amutha & Jaya, 2011).

Pectic substances which form important natural substrates of pectinase are widely distributed in fruits and vegetables. Namasivayam et. al., 2011 reported that 10-30% pectic substances are found in peels of oranges and various fruits. In a study carried out by Geetha et. al. (2012), pectinase producing organisms isolated from fruit peel wastes were found to produce significant quantities of pectinolytic enzymes. Microorganisms that produce a high yield of pectinase under optimal physicochemical conditions are desirable to reduce production costs (Vibha & Neelam, 2010; Torimiro & Okonji, 2013). This present study aims to determine the pectinolytic activities of pectinase produced under various physiochemical conditions by bacterial isolates cultured from deteriorating fruits.

Materials & Methods

Isolation and screening of bacterial isolates for pectinolytic activities

Deteriorating oranges and grapefruits were collected and surface sterilized with 70% ethanol. A pooled one gram of the samples was each homogenized and transferred into 10ml sterile distilled water and agitated. A 0.1ml of the aliquot was inoculated unto nutrient agar plates, seeded using the spread plate technique and incubated at 35°C for 24 h. Pure colonies of the bacterial isolates were obtained and were screened for their ability to produce pectinase using the modified Pectin Agar plate method (Al-Aljani et. al., 2012). The isolates were streaked on the Pectin Agar plate and incubated at 35°C for 48 h. Thereafter, the plates were flooded with Lugol's iodine and the appearance of a clear halo zone around the colonies was an indication of pectinase production.

Identification of the bacterial isolates

The isolates with the ability to produce pectinase were streaked unto nutrient agar and incubated at 35°C for 18-24 h. The bacterial isolates were subjected to morphological and various biochemical tests as described by Olutiola et. al. (1991) and the identity of the isolates was determined by referencing Bergey's Manual of Determinative Bacteriology (Holt et. al., 1994).

The production of the pectinolytic enzyme by the bacterial Isolates

The bacterial isolates were standardized to an optical density (OD) of 0.3 at 1% (v/v) and subsequently inoculated into 100 ml defined enzyme production growth medium containing; 0.5g citrus pectin, 0.1g Yeast extract, 0.715g NH₄Cl, 0.45g Na₂HPO₄.12H₂O, 0.63g KH₂PO₄, 0.075g KCl, and 0.025g MgSO₄ (Kumar & Sharma, 2012). The culturing was done under submerged fermentation in triplicates. The cultures were incubated in a rotary shaker incubator at 37°C for 36 h at 120 rpm and centrifuged at 4000rpm for 10 min to separate the supernatant from the cell biomass. The cell-free supernatants were used to assess the pectinase activity of the isolates (Kumar & Sharma, 2012).

Determination of the Total protein concentration

The protein concentration was routinely determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard, where the absorbance of protein was extrapolated from the standard curve. The reaction mixture contained 0.2 ml of the enzyme and 1.0 ml Bradford reagent. The absorbance was taken at 595nm under a visible spectrophotometer.

Pectinase Assay

The crude enzyme produced by the test isolates were assayed using the modified method as described (Miller, 1959). Five milliliters (5 ml) of cell-free supernatant was incubated with 1% pectin in 0.1 M Citrate buffer at pH 4.8 and the reaction mixture was incubated at 50 °C for 10 min. After adding 1.0 ml of DNSA reagent (3, 5- Dinitrosalicylic acid), the mixture was boiled for 5 min and was stopped by adding

1 ml of Rochelle's salt (Sodium potassium tartrate- Sigma USA). The absorbance was read at 540 nm to estimate the reducing sugars released. One unit of pectinase activity was defined as the amount of enzyme that liberated reducing sugar equivalent to 1 μ mol glucose per minute under the specified assay conditions (Karthik et. al., 2011).

Determination of kinetic parameters of pectinase

The kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying the concentration of pectin between 4 mM and 52 mM in 0.1M citrate buffer, pH 4.8. The kinetic parameters were then determined from the double reciprocal plot (Lineweaver & Burk, 1934).

Effect of substrate concentration on pectinase activities

The effect of different substrates (Apple pomace, Orange peel, and banana peel) was determined. The substrates were obtained by drying the peels of various fruits in an oven maintained at 50°C between 6 and 8 hours till they were completely dried. The dried peels were ground to a powder which was then used to prepare 1% solution of each of the substrate in 0.1M citrate buffer, pH 4.8. In a typical pectinase assay, 5 ml of cell-free supernatant was incubated with 1% of the fruit peels (substrates) in 0.1 M citrate buffer at pH 4.8 and then incubated at 50°C for 10 min. After adding 1.0 ml of DNSA reagent, the mixture was boiled for 5 min and the absorbance was read at 540 nm. The assay with the pectin was taken as the control.

Effect of temperature on pectinase activity

The effect of temperature on the activity of the enzyme was determined by carrying out the assay at temperatures between 30 and 70°C.

Effect of pH on pectinase activity

The effect of pH on pectinase activity was studied by assaying the enzyme using the following buffers of different pH values: 0.1 M citrate buffer (pH 3.0,4.0 and 5.0), 0.1 M phosphate buffer (pH 6.0 and 7.0), 0.1M Tris buffer (pH 8.0) and 0.1 M borate buffer (pH 9.0). A solution of 1% pectin was first prepared in the different buffers and this was used in a typical pectinase assay: 5 ml of cell-free supernatant was incubated with 1% of the pectin in 0.1 M of the different buffer and the reaction mixture was incubated at 50°C for 10 min. After adding 1.0 ml

of DNSA reagent, the mixture was boiled for 5 min and the absorbance was read at 540 nm. The assay with 0.1 M citrate buffer pH 4.7 was taken as control.

Effect of metal on pectinase activity

The effect of different metal ions (Na^+ , K^+ , Mn^{2+} , Mg^{2+} and Ba^{2+}) at a concentration of 1 mM and 10 mM on the pectinase activity of each crude enzyme was determined. Chloride salt solutions of each metal were added in 0.5 ml of 1% pectin in 0.1M citrate buffer and incubated at 50°C for 10 min, the mixture was then boiled for 5 min after the addition of 1.0 ml DNSA. The absorbance was read at 540 nm.

Effect of inhibitors pectinase enzyme activity

The effect of two inhibitors (Sodium azide and Ethylenediaminetetraacetic acid (EDTA) at a concentration of 1 mM and 10 mM on the pectinase activity of each crude enzyme was determined. The different concentrations of each of the compounds were added in 0.5 ml of 1% pectin in 0.1M citrate buffer and incubated at 50°C for 10 min, the mixture was then boiled for 5 min after the addition of 1.0 ml DNSA. The absorbance was read at 540 nm.

Results

A total of five bacterial isolates with pectinolytic activity belonging to four genera were identified. These include *Bacillus subtilis*-1, *S. aureus* and *Bacillus subtilis*-2 cultured from grape fruits while *Klebsiella aerogenes* and *Bacillus cereus* were cultured from deteriorating orange.

The range of the specific activity observed in all the isolates is between 26.40 - 52.27 U/mg. The specific activity of *B. cereus* is high at 52.27 U/mg while *S. aureus* had a low k_m of 6.82 mM as shown (Table 1). High pectinase activity by the isolates was observed especially when orange peels were used as substrate (Fig 1). The optimum temperature for pectinase activity for all the isolates was 50°C and there was a gradual decrease of activity after the optimum temperature (Fig 2). The pH range is between 4.0 -8.0 (Fig. 3). The metal ions $MnCl_2 \cdot 4H_2O$ and $MgCl_2 \cdot 7H_2O$ at 10 mM concentration were observed to have the highest inhibitory effect on enzyme produced by all the test isolates (Table 2). Figure 4 shows that Sodium Azide at 1 mM and EDTA at 10 mM respectively, inhibited the enzyme activity.

Table 1: Pectinase activity of the Bacterial Isolates with kinetic parameters

| Enzyme source | Specific Activity (U/mg) | K _m (mM) | V _{max} (Activity) |
|---------------|--------------------------|---------------------|-----------------------------|
| B. subtilis | 28.69 | 84.77 | 29.85 |
| B. cereus | 52.27 | 81.39 | 38.75 |
| S. aureus | 32.80 | 6.82 | 22.72 |
| K. aerogenes | 26.40 | 39.17 | 23.04 |
| B. subtilis 2 | 45.50 | 22.14 | 17.85 |

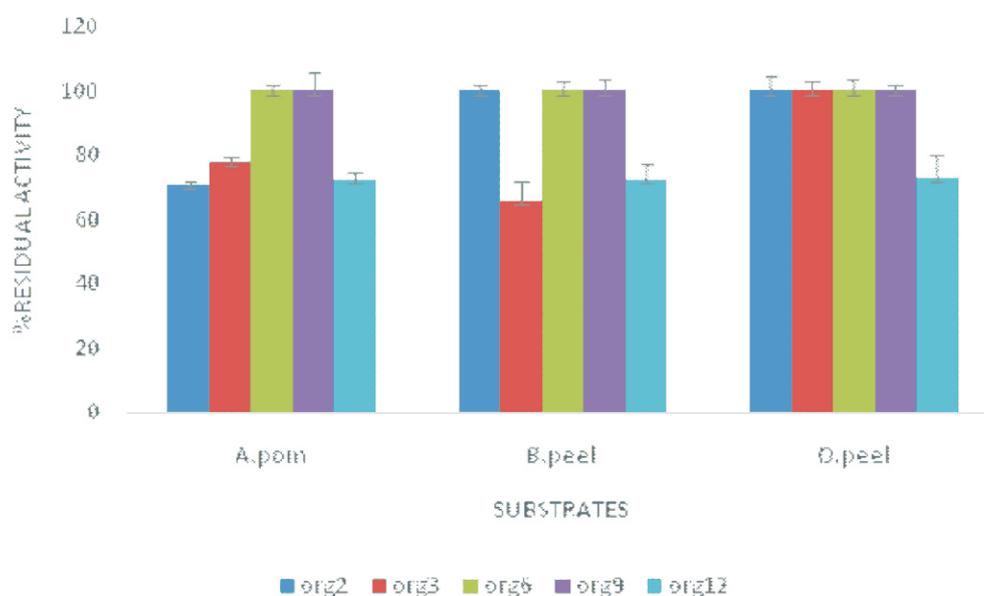


Figure 1: Effect of the substrate (apple pomace, banana peel, and orange peel) on the pectinase activity. Org2= *B. subtilis*-1; Org3 = *B. cereus*; Org 6 = *S. aureus*; Org 9 = *Klebsiella aerogenes*.; Org 12 = *B. subtilis*-

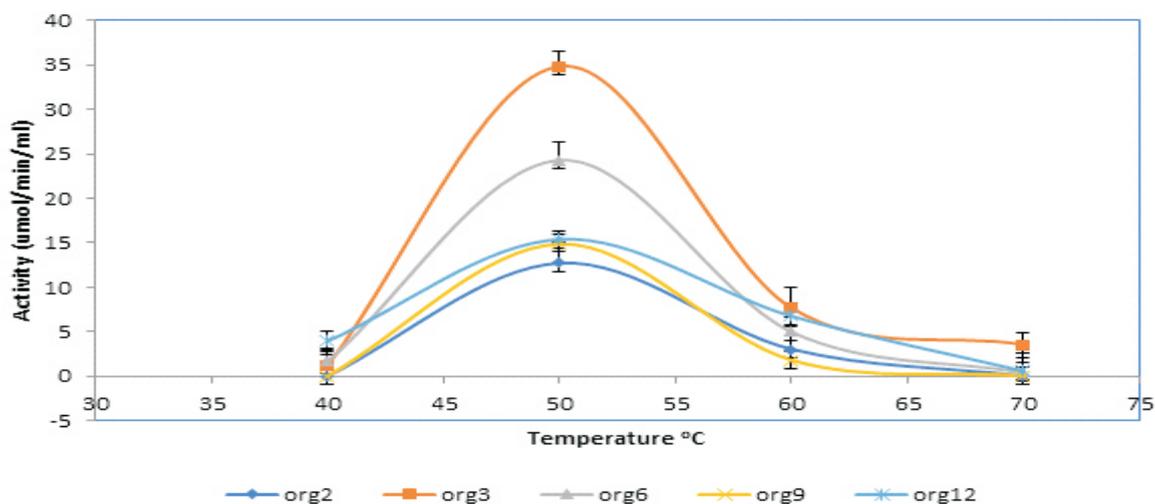


Figure 2: Effect of temperature on the activities of the enzymes. Org2= *B. subtilis*-1; Org3 = *B. cereus*; Org 6 = *S. aureus*; Org 9 = *Klebsiella aerogenes*.; Org 12 = *B. subtilis*-2

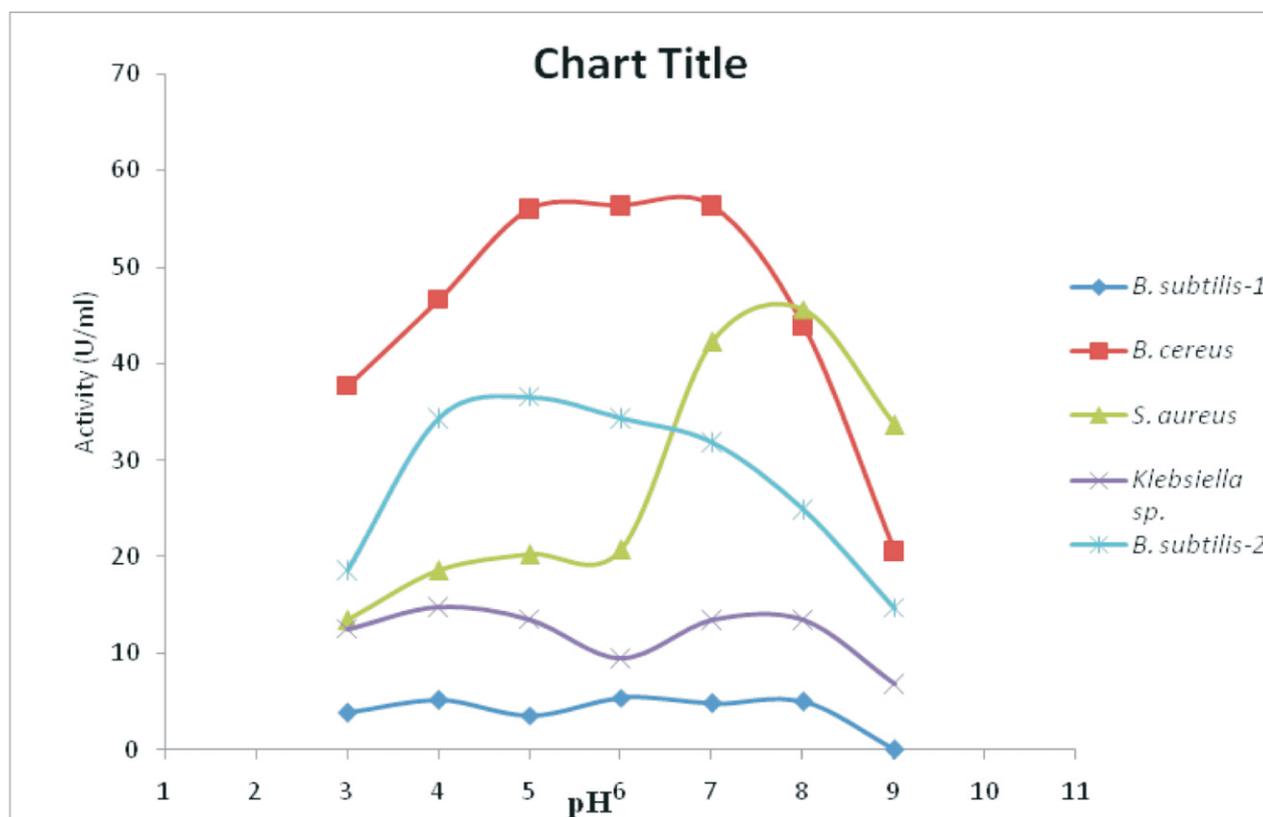


Figure 3: Effect of pH on the pectinase activities

Table 2: Effect of metal ions on pectinase activity

| METAL IONS. | <i>B.subtilis 1</i> | | <i>B.cereus</i> | | <i>S.aureus</i> | | <i>Kaerogenes</i> | | <i>B.subtilis -2</i> | |
|--------------------------------------|---------------------|-------|-----------------|-------|-----------------|-------|-------------------|-------|----------------------|-------|
| | 1mM | 10mM | 1mM | 10mM | 1mM | 10mM | 1mM | 10mM | 1mM | 10mM |
| KCl | 22.47 | 15.34 | 83.28 | 55.13 | 67.38 | 47.02 | 70.53 | 45.00 | 53.00 | 33.30 |
| NaCl | 42.32 | 29.50 | 42.75 | 40.32 | 44.44 | 37.13 | 33.95 | 12.63 | 63.56 | 19.60 |
| MgCl ₂ .7H ₂ O | 19.18 | 0 | 83.34 | 27.46 | 86.45 | 28.62 | 74.47 | 24.74 | 33.02 | 13.99 |
| BaCl ₂ | 27.95 | 17.53 | 72.70 | 40.32 | 79.50 | 51.47 | 68.68 | 30.53 | 45.29 | 28.16 |
| MnCl ₂ .4H ₂ O | 37.62 | 0 | 91.62 | 17.57 | 100 | 3.87 | 100 | 0 | 60.66 | 4.47 |

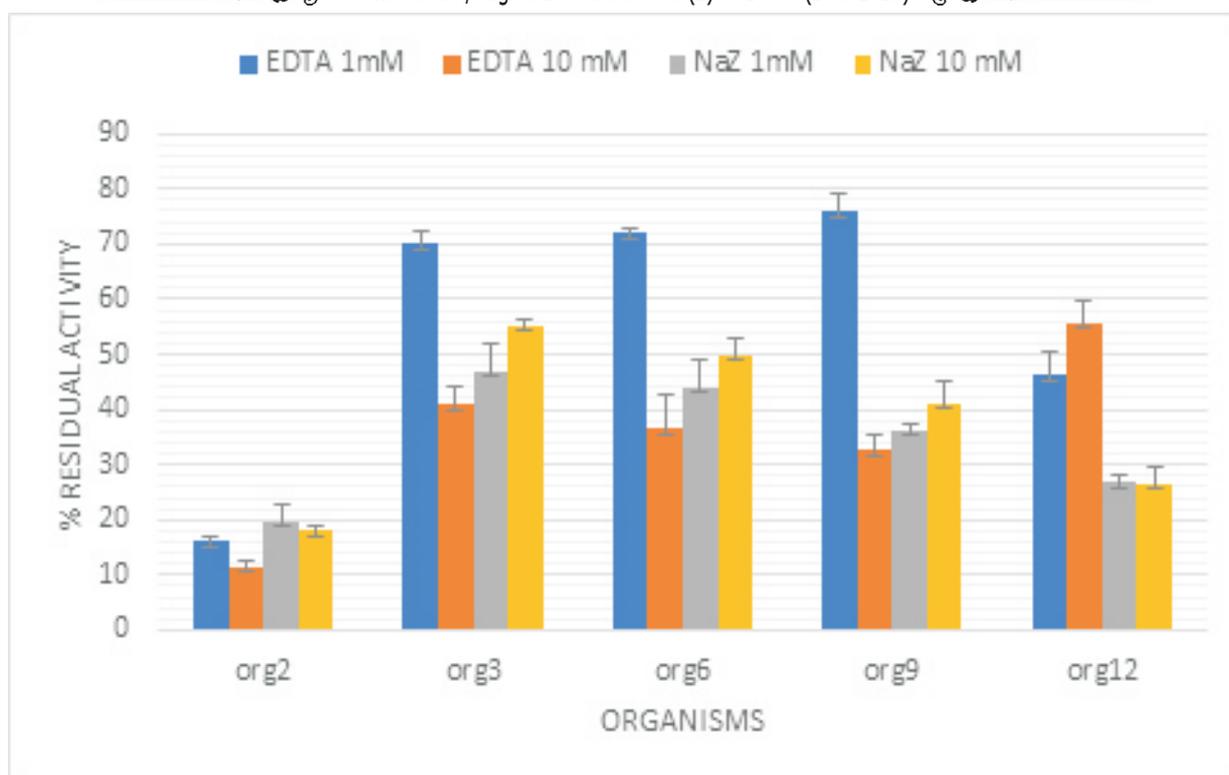


Figure 4: Effect of sodium azide and EDTA on pectinase activity. Org2= *B. subtilis*-1; Org3 = *B. cereus*; Org 6 = *S. aureus*; Org 9 = *Klebsiella aerogenes*.; Org 12 = *B. subtilis*-2

Discussion

Microbially derived pectinases have been shown to have advantages over those produced from other sources because of the ease in manipulating them to increase yield. The study was undertaken to determine the pectinolytic activities of pectinase produced by some bacterial isolates cultured from deteriorating fruits under submerged fermentation. Bacterial isolates which included *B. subtilis*, *B. cereus*, *S. aureus*, and *K. aerogenes* were identified to possess pectinolytic enzyme potentials. *Bacillus sp.* were observed to show high specific activity for pectinase especially *B. cereus*. This observation is in agreement with findings reported that selected *Bacillus sp.* can produce large quantities of pectinase (Schallemey et. al., 2001; Namasiyavan et. al., 2011). The study revealed that *S. aureus* possesses the capabilities to produce pectinolytic enzyme and has a low Km indicating a high affinity for pectin. Studies have shown that *S. aureus* and other *Staphylococcus sp.* isolated from vegetable dump yards have been reported to possess pectinolytic potentials (Raju & Divakar, 2013; Rokade et. al., 2015).

It has been reported that orange

bagasse gives the optimum yield of pectinase (Giese et. al., 2008; Madu et. al., 2014). In this study, it was observed that the isolates showed high specificity for the substrate used but most of the isolates produced pectinase at the optimum with orange peel as compared to other substrates an indication that orange peels could be a good alternative substrate in pectinase production. The optimum temperature of 50°C was recorded for all the isolates in this study and this in agreement with the report of Patil et. al. (2012) who used carrot waste in their experiment and reported maximum activity of 43 U/ml at 50°C for pectinase produced by *Bacillus sp.* However, a steady decline in pectinase activity by all the isolates was observed after the optimum temperature was attained which is an indication there will be reduction in pectinase yield at temperature above 50 °C.

A high pectinolytic enzyme activity was observed for *B. cereus* and *S. aureus* between pH 7-8 in this study. Most bacteria have been observed to produce maximally at pH 7 and above (Silley, 1986; Torimiro & Okonji 2013). The effect of metal ions on the activities of the isolate showed relative inhibition of the enzyme. At 10mM concentration of $MgCl_2$.

7H₂O and MnCl₂· 4H₂O a total loss of pectinase activity of *B. subtilis*1 and a reduced pectinase activity of *B. cereus*, *S. aureus*, *K. aerogenes* and *B. subtilis*2 was observed while MnCl₂· 4H₂O caused a total loss of pectinase activity in *K. aerogenes*. However, it was observed that pectinase activity is enhanced at a low concentration (1mM) for all the isolates. A report has shown that Ca²⁺, Mg²⁺, and Zn²⁺ activate pectinase produced by *Penicillium italicum* (Alana et. al., 1990). It was observed that an increase in the concentration of Ethylenediaminetetraacetic acid (EDTA) increases its inhibitory effect while an increase in the concentration decreases the inhibitory effect of sodium azide. This is in agreement with the report of Banu et. al. (2010), who reported that EDTA exhibited maximum inhibition of 40% on pectinase activity of *Penicillium chrysogenum*.

In conclusion, the various bacterial isolates showed appreciable production of pectinases at varying physicochemical parameters. However, *B. cereus* followed by *S. aureus* showed promising potential for pectinase activity under optimal physicochemical conditions, an indication they can be exploited to increase yield during the production of the enzyme.

References

- Al-Ajlani, M. M., Ahmad, Z. and Hasnain, S.(2012). Pectinase activity of wild-type *Bacillus subtilis* isolated from Pakistan. *Biotech. Res.* 1(2): 7-12.
- Alana, A., Alkorta, I., Dominguez, J. B., Liama M. J. and Serra J. L. (1990). Pectin lyase activity in a *Penicillium italicum* strata. *Appl. Environ. Microbiol.* 56: 3755-759.
- Amutha K. and Jaya K. (2011). Effect of pH, temperature and metal ions amylase activity from *Bacillus subtilis* KCX 006. *Inter. J. Pharm. Biosci.* 2:1-7.
- Banu, R. A., Devi, K. M., Gnanaprabhal, G. R., Pradeep, B. V. and Palaniswam, V. (2010). Production and characterization of pectinase enzyme from *Penicillin chrysogenum*. *Ind. J. Sci. Tech.* 3(4): 377-81.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye binding. *Analy. Biochem.* 72: 248-54.
- Favela-Torres, E., Aguilar C. N., Contreras-Esquivel, J.C. and Viniestra-Gonzalez, G. (2005). Pectinases: In *Enzyme Technology*. A. Pandey, C. Webb, C. R. Soccol, C. Larroche, Eds, Aviotech Publishers Inc., pp 265-287.
- Geetha, M., Saranraj P., Mahalakshmi S. and Reetha D. (2012). Screening of pectinase producing bacteria and fungi for its pectinolytic activity using fruit wastes. *Inter. J. Biochem. Biotech. Sci.* 1: 30-42.
- Giese, E.C., Dekker, R. F. H. and Barbosa, A. M. (2008). Orange Bagasse as substrate for the production of pectinase and laccase by *Botryosphaeria rhodiana* mamb-05 in submerged and solid-state fermentation. *Biores.* 3 (2): 335-45.
- Gummadi, S. N. and Panda, T. (2003). Purification and biochemical properties of microbial pectinases: A review. *Process Biochem.* 38: 987-96
- Holt, J. S., Krieg, N. R., Sneath, D. H. A., Stanley, J. T. and William, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*, 9th Ed. Baltimore. The Williams and Wilkins company.
- Karthik, L. J., Gaurav K. and Bhuskara-Rao, K. V. (2011). Screening of pectinase producing microorganism from agricultural waste dump soil. *Asian J. Biochem. Pharma. Res.* 1(2): 329-37.
- Kumar, A. and Sharma, R. (2012). Production of alkaline pectinase by bacteria (cocci species) isolated from decomposing fruits materials. *J. of Phytology.* 4(1): 1-5
- Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* 56: 658-66.

- Madu, J. O., Torimiro, N., Okonji, R. E., James, I.E. and Agboola, F. K. (2014). Physicochemical factors influencing pectinolytic enzyme produced by *Bacillus licheniformis* under submerged fermentation. *Nat. and Sci.* 12(8): 110-16.
- Miller, G.L. (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugars. *Analy. Chem.* 31: 426-28.
- Namasivayam, E., Ravinder, J. D., Mariappan, K., Jiji, A. and Kumar, M. (2011). Production of extracellular pectinase by *Bacillus cereus* isolated from market solid waste. *J. Bioanaly. Biomed.* 3: 70-75.
- Olutiola, P. O., Famurewa, O. and Sonntag, H. G. (1991). *An Introduction to General Microbiology: A Practical Approach*, 1st Ed. Heidelberg, Germany.
- Pandey, A., Selvakumar, P., Soccol, C. R. and Nigam, P. (1999). Solid-state fermentation for the production of industrial enzymes. *Curr. Sci.* 77; 149-62.
- Patil, R. C., Tushar P. M. and Shamim, A. S. (2012). Extraction of pectinase from pectinolytic bacteria isolated from carrot waste. *Inter. J. Pharm and Bio Sci.* 3(1): 261-65.
- Poonam, S. N. (2013). Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules* 23; 3(3): 597-611.
- Raju, E. V. N. and Divakar G. (2013). Production of Pectinase by using *Bacillus circulans* isolated from dump yards of vegetable waste. *Inter. J. Pharma. Sci. Res.* 4 (7): 2615-622.
- Rokade, D. D., Vaidya, S. L., Rehman- Naziya, M. A. and Dixit, P. P. (2015). Screening of pectinase producing bacterial, isolated from Osamanabad of fruit market soil. *Inter. J. Interdiscipl. and Multidiscipl. Studies* 2(6): 141-45.
- Schallemeij, M., Ajay, S. and Owen, P. (2001). *Microbial identification System References*. MIDI Inc.
- Silley, P. (1986). The production and properties of a crude pectin lyase from *Lachnospira multiparus*. *Lett. Appl. Microbiol.* 2:29-31.
- Tatiana da Costa, R. P. and Flavo, F. (2005). Extraction and assay of pectic enzymes from Peruvian carrot (*Arracacia xanthorrhiza* Brancroft). *Food Chem.* 89: 85-92.
- Torimiro, N. and Okonji, R. E. (2013). A comparative study of pectinolytic enzyme production by *Bacillus species*. *Afr. J. Biotech.* 12(46): 6498-503.
- Vibha, B. and Neelam, G. (2010). Exploitation of microorganisms for isolation and screening of pectinase from the environment. 8th International Conference, Globelics. Malaysia, November 1-3, pp. 2-16.