International Journal of Ecology and Environmental Sciences
www.ecologyjournal.in
Online ISSN: 2664-7133; Print ISSN: 2664-7125
Received: 03-11-2018; Accepted: 06-12-2018; Published: 06-01-2019
Volume 1; Issue 1; 2019; Page No. 15-19

Optimization of fermentation condition for pectinase production from *Aspergillus flavus*, using African star cherry pectin as a carbon source

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Abstract
Pectinases are heterogenous group of enzymes which are used to hydrolyze pectic substances and thus have become of high interest in many industries such as paper, textile and food industries. This research was aimed at determining the optimal submerged fermentation conditions for producing pectinase by Aspergillus flavus using African star cherry pectin as a carbon source. The design of this work involves extraction of pectin from ground African star cherry peels, isolation of pectin-hydrolyzing Aspergillus flavus and the optimization of biochemical parameters such as effects of pH, time, temperature, nitrogen source and pectin concentration for production of pectinolytic enzyme under best conditions. African star cherry pectin extraction yield was calculated to be 8.5%. Aspergillus flavus isolate was screened for pectin depolymerizing ability using agar well diffusion assay and the result indicated a clearance zone of 16mM (1.6cm). Further studies on the effects of time, pH, temperature and nitrogen sources showed day 4, 5.5, 300C and Ammonium Sulphate optimal for pectinase production under submerged fermentation technique.

Keywords: *Aspergillus flavus,* African star cherry pectin, pectinase production, Optimization, 3, 5-dinitrosalicylic acid (DNS) reagent

Introduction

Pectin is a linear polysaccharide, like most other plant polysaccharides. It is both polydisperse and polymolecular and its composition varies with the source and the conditions applied during isolation (Raj *et al.,* 2012) [15]. The structure of pectin is very difficult to determine because pectin can change during isolation from plants, storage and processing of plant materials. Commercially pectin is extracted by treating the raw materials with hot dilute mineral acid at pH of about 2. The hot pectin is separated from the solid residue as efficiently as possible. This is not easy since the solids are by now soft and the liquid phase are viscous (Hoondal *et al*., 2002) [9]. The viscosity increases with increase in pectin concentration and molecular weight.

Pectinases are a varied group of related enzymes that hydrolyze the pectic substances, present mostly in plant materials. Pectic enzymes are widely spread in nature and are produced by bacteria, yeast, fungi and plants (Babu and Mayer, 2014) [1]. Many extracellular enzymes are produced by fungi which are capable of decomposing organic matter and one such enzyme is pectinolytic enzymes. Therefore, by means of breaking down pectic substances for nutrition, microbial pectinases play an important role in nature (Yadav *et al*., 2009) [20]. Pectinases are inducible, produced only when considered necessary and they add to the natural carbon cycle (Hoondal *et al*., 2002) [9].

Recently, there has been a large increase in industrial applications of enzymes owing to their important biotechnological prospect. Consequently, pectinase was put into marketable use for the first time in 1930 for the preparation of wines and fruit juices. But the chemical nature was obvious only in the 1960s and with this knowledge; scientists began to make greater use of this broad range of enzymes more proficiently (Pasha *et al*., 2013) [14]. Greater percentage of all industrial enzymes is produced in optimized submerged fermentation medium, using potent microorganisms that feed on the available substrate to release enzyme of interest. Submerged fermentation system has been extensively employed in production of highly priced materials and physiology aspects of synthesis enzymes (Darah *et al*., 2013) [5], especially in a condition where experimental design requires variation of biotechnological parameters for efficiency.

Materials and Methods

Collection of African star cherry fruit

Mature African star cherry fruits were purchased from Afor Ugbawka market in Nkanu East Local Government Area of Enugu State, Nigerian.

Collection of Microorganism

Some ripened African star cherry fruits were exposed in an open space and allowed to decay and got infected by fungi. Pectinase producing *Aspergillus flavus* was then isolated from the decayed pectin rich cherry, using a modified cherry pectin agar medium.

Extraction of pectin from cherry peels

Mature cherry fruits each were washed with water and peeled. The peels were cut into small bits and treated with hot 96% ethanol. The ethanol treated peels were washed with water and air-dried. The dried peels were ground with a milling machine until it became powder.

Pectin was extracted using the method described by McCready (1970) [11]. The ground cherry peel, 100g, was weighed into a 2000ml beaker containing 800ml of distilled water. Freshly

ground sodium hexametaphosphate (12g) was added and the initial pH was adjusted with 3N HCL to 2.2 ± 0.1. The mixture was heated in a water bath at 70^0C for 1hour and stirred with a propeller-type stirrer. The pH was checked at intervals of 15mins. The water lost was replaced at intervals except in the last 20mins of the extraction. The mixture was vacuum filtered through a muslin cloth and the residue was washed with 200ml of distilled water, and the washings were added to the filtrate. The filtrate was concentrated by evaporation on a hot plate to approximately 1/5th of the initial volume.

The concentrated cherry pectin was cooled to 50^oC and poured into a volume of ethanol in the ratio of 1:3.The ethanol contained 0.5M HCL. The mixture was stirred for 30mins and allowed to stand for 1 hour. The precipitate was vacuum-filtered and washed with ethanol-HCL solution. The extract was finally washed with acetone to remove traces of HCL and ethanol. The extract was dried in an oven at 40^oC for five days, then air-dried to constant weight and ground finely.

Isolation of pectinolytic fungi

Approximately 5g of the rotten cherry fruits were cut using sterile blade and homogenized in sterile medium of 1% cherry pectin; containing 0.14% of $(NH_4)_2 SO_4$, 0.2% of K_2HPO_4, 0.02% of $MgSO_4.7H_2O$, 0.1% of nutrient solution containing; 5g/L $FeSO_4.7H_2O$, 1.6mg/L $MnSO_4.H_2O$, 1.4mg/L $ZnSO_4.7H_2O$, 2.0mg/L $CoCl_2$ and pH 5.0, using a modification of Udewobele *et al* (2014). The pH of the medium was adjusted to pH 5.0 by using 1.0N HCl/1.0N NaOH. The mixture was incubated at room temperature (30^oC) for 24 hours. The liquid broth as prepared above was added to 3% agar-agar (the gelling agent) and the media autoclaved at 121^oC for 15min. The sterilized media poured aseptically into Petri dishes and allowed to form solid cherry pectin agar gel. The plates were then incubated 30^oC overnight to check for sterility. A loop each of homogenized extracts from the liquid media was streaked onto the solid cherry pectin agar media, under the flame of Bunsen burner. Streaks were made from each side of the plate, marking an initial point, with sterilization of the wire loop after each side has been completed. The plates were thereafter incubated at 30^oC till visible colonies of fungi were observed. *Aspergillus flavus* isolated from the morphological contrasting colonies was purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure *Aspergillus flavus* isolates was obtained. Pure fungal isolates was maintained on Potato Dextrose Agar (PDA) slopes or slants and stored at 4^oC as stock cultures. The PDA media were prepared according to the manufacture's description.

Agar well diffusion assay

The fungal isolate were qualitatively screened for pectinolytic activity, using agar well diffusion method. The cherry pectin agar plate was prepared and three wells were made with sterilized cork borer in the petri plate under aseptic conditions. The wells were filled with 5 days old culture filtrate and incubated at room temperature overnight. The substrate utilized zone around the colony was observed using iodine solution (Bijesh *et al*., 2015).

Quantitative screening of fungal isolates for pectinolytic activity

A modified cherry pectin liquid media was employed using 250ml Erlenmeyer flasks containing 100ml of sterile cultivation media optimized for pectinase production with 0.1% NH_4NO_3, 0.1% $NH_4 H_2PO_4$, 0.1% $MgS0_4.7H_2O$, 0.5% citrus pectin and pH adjusted to 5.0. The flasks were stoppered with aluminum foil and autoclaved at 121^oC 15psi for 15min. With the aid of a flamed, cooled cork borer (10mm) and transfer needle, one disc of fungal hyphae from edge of actively growing fruit agar culture was introduced into the liquid media. The flasks were covered with sterile foil and incubated at 30^oC for 4 days. After incubation, the cultures were harvested by filtration through Whatman No.1 filter paper. The filtrates were stored at 4^oC and used as the crude enzymes for screening quantitative pectinolytic activity.

Identification of Fungal Isolate

The culture was sent to Microbiology unit of Brain Phosphorelationship Laboratory, Ogui Enugu, Nigeria for species identification. The color, texture, nature of mycelia or spores and growth patterns were also observed. Photographs of the cultures were also taken.

The three-day old pure culture was used in preparing microscopic slides. A little bit of the mycelia was dropped on the slide and a drop of lacto-phenol blue was added to it. A cover slip was placed over it and examination performed under the light microscope at X400 magnification. Identification was carried out by relating features and the micrographs to "Atlas of mycology" by Barnett and Hunter (1972). Species identification was by examining both macroscopic and microscopic features of a three day old pure culture. Colour, texture, nature of mycelia and/or spores produced, growth pattern in addition to microscopic features such as separation, spore shapes and so on were examined and confirmed *Aspergillus flavus*.

Fermentation Culture

Submerged fermentation (SmF) technique was employed, using the method described by Ezugwu *et al* (2014), with the following modifications; five 250ml Erlenmeyer flasks containing 100ml of sterile cultivation media made up of 0.1% NH_4NO_3, 0.1% $NH_4H_2PO_4$, 0.1% $MgS0_4.7H_2O$ and 0.5% cherry pectin, adjusted to pH 5.0 by using 1.0N HCl/1.0N NaOH. The flasks were stoppered with aluminium foil and autoclaved at 121^oC 15psi for 15min. Three day old cherry pectin agar culture of *Aspergillus flavus* was used to inoculate the flasks. In every sterile flask, one disc of fungal hyphae from edge of actively growing cherry pectin agar culture was added respectively using a flamed and cooled cork borer of diameter 10mm and then plugged properly. The culture was incubated for 5days at 30^oC. At 24h interval, a flask was selected and the mycelia biomass separated by filtration through Whatman No.1 filter paper. The filtrates were analyzed daily for pectinase activity and extracellular protein concentration till the 5th day of fermentation.

Pectinase Assay

Pectinase activity was evaluated by assaying for polgalacturonase (Pg) activity of the enzyme. This was achieved by measuring the release of reducing groups from cherry pectins using a modification of the 3,5 dinitrosalicylic acid (DNS) reagent assay method described by Miller (1959) [12] as contained in Wang *et al*.(1997) [19] with little modifications.

The reaction mixture containing 0.8ml of 0.2% cherry pectin in 0.05M sodium acetate buffer of pH 5.0 and 0.2ml of enzyme solution were incubated for 20min. 1ml of DNS reagent was added and the reaction was stopped by boiling the mixture in a boiling water bath for 10mins. The mixture volume was made up to 4ml with 1ml of Rochelle salt solution and 1ml of distilled water. The reaction mixture was allowed to cool and then absorbance read at 575nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of one micromole of galacturonic acid per minute.

Protein determination

Protein content of the enzyme was determined by the method of Lowry *et al.* (1951) [10], using Bovine Serum Albumin as standard. For protein standard curve, the reaction mixture contained 0.0-1.0 ml of protein stock solution (2 mg/ml BSA) in test tubes arranged in triplicates. The volume was made up to 1ml with distilled water. But for the test mixture, 0.1 ml of sample enzyme was mixed with 0.9ml of distilled water. In either case, 5 ml of solution E was added and allowed to stand at room temperature for about 10min. 0.5 ml of solution C (dilute Folin-Ciocalteau phenol reagent) was added with rapid mixing. After standing at room temperature for 30min, absorbance was read at 750 nm using spectrophotometer. Absorbance values were converted to protein concentration by extrapolation from the standard curve.

Optimization of fermentation condition

Effect of nitrogen sources on pectinase production

The effects of different inorganic nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulphate, sodium nitrate) were determined. The various nitrogen sources were used separately at an equivalent concentration of 0.1% fermentation media. Crude pectinases extracted on day 4 were used to assay for pectinase activity and protein concentration using the methods described by Miller (1959) [12] as contained in Wang *et al* (1997) [19] with little modifications and Lowry *et al.* (1951) [10] respectively.

Effect of pectin concentration on pectinase production

Effect of substrate (pectin) concentration on the production of pectinase was determined by carrying out 4 days submerged fermentation with 0.2, 0.4, 0.6, and 1.0% of cherry pectin at optimum (other factors being constant)**.** Pectinase activity and protein concentration were determined using the methods described by Miller (1959) [12] as contained in Wang *et al* (1997) [19] with little modifications and Lowry *et al.* (1951) [10] respectively.

Effect of pH on pectinase production

The production of pectinase from the fungus at different pHs (3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) at 30°C under optimal condition (other factors being constant) was carried out to determine the optimum pH for pectinase production. Pectinase activity and protein concentration were determined using same methods as for effects of pH.

Effect of temperature on pectinase production

The optimum temperature for pectinase production was determined by varying the submerged fermentation temperature at 30 – 60°C interval of 10°C for 2 days, pH 5.0 and under optimal condition (other factors being constant). Pectinase activity and protein concentration were determined using same methods as above.

Results and discussion

In this research work, pectin was extracted from African star cherry with an extraction yield of 8.5% at pH of 2.2,temperature of 70°C and extraction time of 60mins using ethanol-Hcl method described by McCready (1970) [11]. Fungal strain was collected from decayed cherry fruit and then cultured in cherry pectin based agar medium. Based on macroscopic and microscopic characteristics of the fungal isolate, *Aspergillus flavus* was confirmed (Figure 1). The screening of fungal isolate for pectinolytic activity was of two methods; first, Agar well diffusion assay which gave a clearance zone of 16 mm (1.6cm). Reddy and sreeramulu (2012) [16] isolated 44 fungal strains from agricultural soils of Chittor district in which *Aspergillus flavus* showed maximum zone of clearance of 15.0mm for pectinolytic activity. The result of this work is comparable with that of the above mentioned author. The second method which involved examination of the fungal isolate for quantitative pectinolytic activity gave a specific activity of 2.13 U/mg on the maximum day (4th day) of enzyme production.

Fig 1: Pure culture of *Aspergillus flavus*

During the fermentation period of 5days, daily evaluation of pectinase activity and extracellular protein secretion by the organism were carried out and results used to generate daily specific activity. Highest specific activity of 2.13 U/mg was obtained on the 4th day, making the 4th day the maximum day of pectinase production (Figure 2). The result of this project is agreement with that of Doughari and Onyebarachi (2019) [7] who observed optimum production of polygalacturonase at incubation period of 96 h, pH 4.5 and at 35°C from *Aspergillus flavus* grown on orange peel.

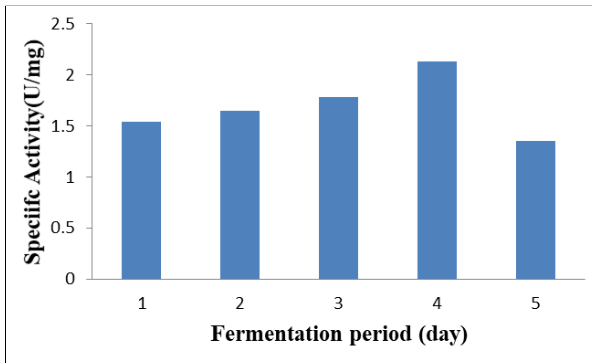

Fig 2: Effect time of pectinase production

The effect of pH on pectinase production was carried out within the pH ranges of 3.5-6.0 (at interval of 0.5) and the results revealed pH 5.5 best for pectinase production from *Aspergillus flavus* with a specific activity of 1.98U/mg (Figure 3). Panda *et al* (2012) [13] reported an optimum pH of 6.0 for pectinase production by *Aspergillus flavus*. The pH of 5.5 reported in this work is in agreement with that of Thangaratham and Manimegalai (2014) who observed high pectinase production at initial pH of 5.5 with temperature of 35ºC. The effect of temperature ranges from 30 – 60ºC at interval of 10ºC for 2 days revealed temperature of 30ºC ideal for pectinase production with specific activity of 2.07 U/mg (Figure 4). Either increase or decrease beyond the optimum value show decline in enzyme production for both temperature and pH effects except at optimal conditions where highest enzyme concentration is extracted. This result is in agreement with the optimum temperature of 30°C though at pH 6.0 reported by Panda *et al* (2012) [13] during their studies on pectinolytic and cellulolytic activity of soil fungal isolates from similipal bioreserve forest.

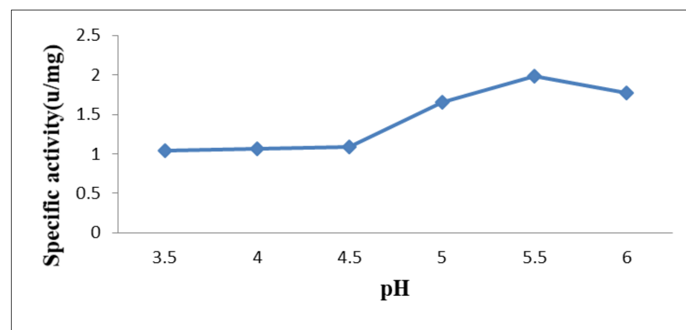

Fig 3: Effect of pH on pectinase production

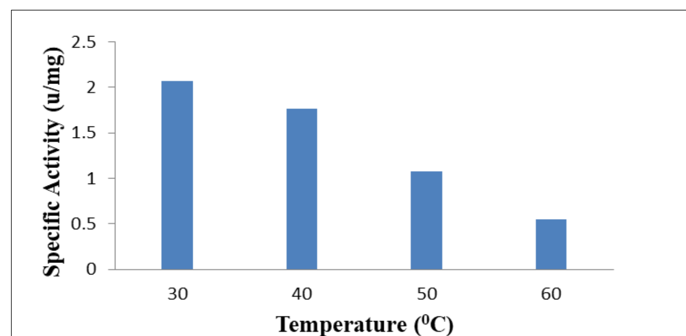

Fig 4: Effect of temperature on pectinase production

The Effect of inorganic nitrogen sources on pectinase production using ammonium chloride, ammonium sulphate, ammonium nitrate and sodium nitrate indicated ammonium sulphate best nitrogen source for pectinase production with specific activity of 2.17 U/mg and ammonium chloride the least nitrogen source with specific activity of 1.67 U/mg (Figure 5). Doughari and Onyebarachi (2019) [7] reported optimum production of polygalacturonase using ammonium sulphate as source of inorganic nitrogen and orange peel as a carbon source, at incubation period of 96 h, pH 4.5 with temperature of 35°C from *Aspergillus flavus*. Furthermore, four days submerged fermentation with 0.2, 0.4, 0.6 and 1.0% of African star cherry pectin revealed 1.0% pectin best for pectinase production with a specific activity of 1.97U/mg (Figure 7). Dhital *et al* (2013) [6] produced highest pectinase concentration with 1.5% substrate (pectin) concentration in their research on optimization of cultural conditions for the production of pectinase from selected fungal strain. Also Banakar and Thippeswamy (2012) [2] reported 1% pectin concentration at an optimal condition of temperature 28 ± 1 ºC, pH 7.0 for 5 days of incubation for fungal pectinase and exo-polygalacturonases production under submerged fermentation.

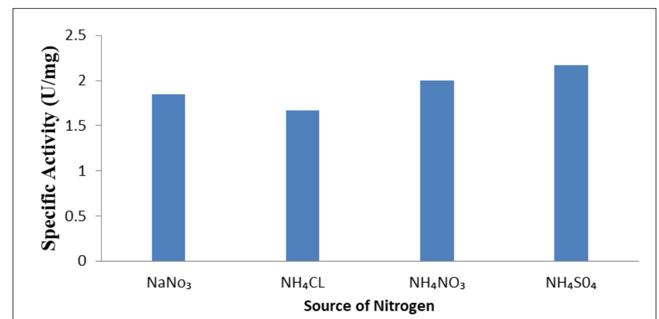

Fig 5: Effect of inorganic nitrogen source on pectinase production

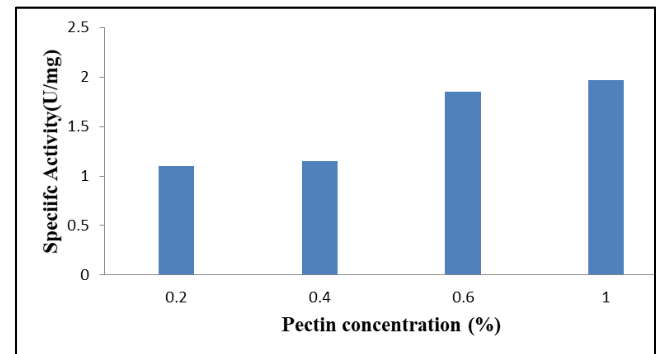

Fig 6: Effect of temperature on pectinase production

Conclusion

The results obtained in the study revealed that *Aspergillus flavus* is efficient for the production of pectinase using African star cherry pectin as a carbon source in a submerged fermentation system. African star cherry fruit is cheap, highly economical and rich in pectic substances which can be used in the production of pectinase. Powdered African star cherry pectin is highly nutritive and a potent inducer that supported the growth of *Aspergillus flavus* and led to the production of highly active pectinase, under optimal conditions reported in this work. The pectinase obtained

from the fungus could find applications in food and wine industries.

References

1. Babu Y, Bayer M. Plant polygalacturonases involved in cell elongation and separation—the same but different? Plants. 2014; 3(4):613-623.
2. Banakar SP, Thippeswamy B. Isolation, production and partial purification of fungal extracellular pectinolytic enzymes from the forest soils of Bhadra Wildlife Sanctuary, Western Ghats of Southern India. Journal of Biochemical Technology. 2012; 3(5):S138-143.
3. Barnett HL, Hunter BB. Illustrated general of imperfect fungi. 3rd edn. Burgess, Minneapolis Minnesota, USA, 1972, 20-130.
4. Bijesh K, Steni KT, Denoj S. Co-production of pectinase and biosurfactant by the newly isolated strain Bacillus subtilis BKDS1. British Microbiology Research Journal. 2015; 10(2):1-12.
5. Darah I, Haritharan W, Lim SH. Involvement of Physicochemical Parameters on Pectinase Production by Aspergillus niger HFD5A-1. Journal of Pure And Applied Microbiology. 2013; 7(4):2541-2549.
6. Dhital R, Panta OP, Rki TBK. Optimization of cultural conditions for the production of pectinase from selected fungal strain. Journal of Food Science and Technology Nepal. 2013; 8:65-70.
7. Doughari JH, Onyebarachi GC. Production, purification and characterization of polygalacturonase from aspergillus flavus grown on orange peel. Applied Microbiology: Open Access. 2019; 4(3):1-7.
8. Ezugwu AL, Ezike TC, Ibeawuchi AN, Nsude CA, Udenwobele DI, Eze SOO, *et al*. Comparative studies on pectinases obtained from Aspergillus fumigatus and Aspergillus niger in submerged fermentation system using pectin extracted from mango, orange and pineapple peels as carbon sources. Nigeria Journal of Biotechnology. 2014; 28:26-34.
9. Hoondal GS, Tiwari RP, Tewari R, Dahiya N, Beg QK. Microbial alkaline pectinases and their industrial applications. A Review; Biotechnology. 2002; 59(4-5):409-418.
10. Lowry OH, Rosenbrough PJ, Fass AL, Randall RJ. Protein measurement with Folin-phenol reagent. Journal of Biochemistry. 1951; 193:265-275.
11. McCready RM. Methods on food analysis. Academic Press, New York and London, 2nd edition, 1970, 365.
12. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Analytical Chemistry. 1959; 31:426-428.
13. Panda SS, Sahoo K, Das R, Dhal NK. Pectinolytic and cellulolytic activity of soil fungal isolates from similipal bioreserve forest, World Environment. 2012; 2(2):1-3.
14. Pasha KM, Anuradha P, Subbarao D. Applications of pectinases in industrial sector. International Journal of Pure and Applied Sciences and Technology. 2013; 16(1):89-95.
15. Raj AAS, Rubila S, Jayabalan R, Ranganathan TV. A Review on pectin: chemistry due to General properties of pectin and its pharmaceutical uses. Open Access Scientific Reports. 2012; 1(12):1-4.
16. Reddy PL, Sreeramulu A. Isolation, identification and screening of Pectinolytic fungi from differentsoil samples of chittoor district. International Journal of Life Sciences Biotechnology and Pharma Research. 2012; 1(3):186-193.
17. Thangaratham T, Manimegalai G. Optimization and production of pectinase using agro waste by solid state and submerged fermentation. Internatinal of Journal of Current Microbiology and Applied Science. 2014; 3(9):357-365.
18. Udenwobele DI, Nsude CA, Ezugwu AL, Eze SOO, Anyawu CU, Uzoegwu PN, Chilaka FC. Extraction, partial purification and characterization of pectinases isolated from Aspergillus species cultured in mango peels. African journal of biotechnology. 2014; 13(24):2445-2454.
19. Wang G, Michailides TJ, Bostock RM. Improved detection of polygalacturonase activity due to Mucorpiriformis with a modified dinitrosalicylic acid reagent. Phytopathology. 1997; 87:161-163.
20. Yadav S, Yadav PK, Yadav D, Yadav KDS. Pectin lyase: A review. Process Biochemistry. 2009; 44(1):1.