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SCREENING OF STARCH AND PECTIN DEGRADING ORGANISMS FROM WASTE AND EVALUATION OF THEIR ACTIVITY ON NATURAL SUBSTRATE

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ABSTRACT

This study investigate the micro organism spoiling fruits during transportation, storage or waiting to be processed and rendering themselves unedible and disposal of such spoiled fruits is also a problem leading to environmental pollution. With the view of value addition of the so-called waste, the present study is emphasized on isolation of some useful microorganism from such spoiled fruit. Organism was screened specifically for starch and pectin degradation. Amyl lyticorganism screened belonged to *Bacillus* and *Aspergillus* and Pectin lytic organism belonged to *Aspergillus*, *Mucor* and *Yeast*. Comparative activity of this microorganism against certain natural substrate containing starch and pectin content was performed. Amyloyltic activity was determined against rice, corn, and tapioca. All three isolates showed maximum activity on rice (14.5µg//ml/min, 13.16µg/ml/min, 11.25µg/ml/min) and minimum activity on tapioca (6µg/ml/min, 8.4µg/ml/min, 5.5µg/ml/min). Three pectinolytic activities were determined against apple, guava and carrot. Here pectinase of *Aspergillus* showed maximum activity on apple (6.0µg/ml/min), Polygalacturonase of *Mucor* and *Yeast* showed maximum activity on guava (1.4µg/ml/min and 3.4µg/ml/min), Pectin lyase of *Aspergillus* and *Yeast* showed Minimum activity on carrot (0.0019µg/ml/min, 0.0002µg/ml/min) and also pectinase of *Mucor* showed minimum activity on carrot (0.0012µg/ml/min). Pectinlyase of *Mucor* and *Yeast* showed no activity on carrot and apple.

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INTRODUCTION

In nature diversity of microorganism exhibits wide range of activity, association and interaction with each other and with their environment. The study of the relation between microorganism and food degradation by enzymes was established since from the knowledge of alcohol and organic acid production from food. These enzymes play a major role in degradation of organic compounds and decomposition of organic substrate. This microbial activities yield with beneficial microbial products like wine, beer, cheese and curd etc. Starch is the major form of stored carbohydrates in plants. Which accounts for 70% of un dried plant material. It occurs in the form of water insoluble granules. Starches are produced commercially from the seeds of plants, such as corn, wheat, sorghum or rice, from the tubers and roots of plants such as cassava, potato and pith of sago palm. It is composed of a mixture of two substances Amylose and Amylopectin. Amylose is a linear polysaccharide made of D-Glucose with α-1, 4 linkages. They consist of 200-20,000 glucose units, which form a helix as result of the bond angles between the glucose units. Natural starches contain 10-20% amylase. Amylopectin is a highly branched polysaccharide made of D-Glucose with α-1, 4 linkages differs from amylase in being highly branched. Short side chains of about 30 glucose units are attached with α-1, 6 linkages approximately every twenty to thirty glucose unit along the chain. They may contain up to two million glucose units. Natural starches contain 60-90% amylopectin. In recent years number of new enzymes associated with degradation of starch have been detected and studied (Boyer and Ingle, 1972; Buonocore *et al*., 1976; Griffin and Fogarty, 1973; Fogarty and Griffin, 1975). The amylase is starch-degrading enzymes classified depending on how they act on the starch molecules. They include:

1. α-amylase which randomly hydrolyzes the α-1, 4 glycoside linkage of amylose and amylopectin but not the 1,6 glycosidic linkage of amylopectin.
2. β-amylase which splits only the second α-1, 4 glucoside linkage from the non-reducing chain ends, detaching one molecule of maltose at a time from the chain and finally leaving limit dextrin's.

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3. Amyloglucosidases: hydrolyzes both 1,6 and 1,4-glucoside linkages to produce glucose without intermediate dextrin's and maltose.
4. Pullulanase and other disbranching enzymes: Hydrolyze only α-1, 6 linkages.
5. α-Glucosidases: Hydrolyse preferentially α-1, 4 linkages in short chain oligosaccharides produced after hydrolysis of amylose and amylopectin by other amylases.
6. *Bacillus macerans*amylase (cyclodextrin producing enzymes): Hydrolyze starch to a series of nonreducing cyclic D-glucosyl polymers (cyclodextirins or sachardinger dextrin's (source: Fogarty and Kelly, 1979)

Pectin substances are complex high molecular mass glycosidic macromolecules found in higher plants. They containa α-1, 4-polygalacturonic acid backbones which can be randomly acetylated and methylated. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. They are responsible for the structural integrity layer formed between the walls of adjacent young cells. They are responsible for the structural integrity and cohesion of plant tissue (Rombouts FM, Pilnik W. Pectic enzymes. In: Rose AH, Ed. Microbial Enzymes and Bioconversions. Academic Press, London. 1980; 5:227-72. Alkorta I, Gabirsu C, Lhama MJ, Serra JL. Industrial applications of pectic enzymes: a review. Proc Biochem 1998; 33: 21-8)

Three major pectic polysaccharide groups are recognized, all containing D-galacturonic acid to a greater or a lesser extent.

1. Homogalcturonan (HG): is a linear polymer made of α-(1,4) Galacturonic acid, which can be o-acetylated at C-2, and C-3positions and their carboxyl groups are methyl esterified. It can be called smooth regions of pectin. The molecule is classified according to its etherification level. Pectin has at least 75% of the carboxyl groups methylated; pectinic acid or polygalacturonic acid has no methyl esterified carboxyl groups.
2. Rhamnogalacturonan I: This contains alternating α- (1,4) galacturonosyl and α- (1,2) rhamnosyl residues, with primary oligo α- (1,3) arabinose and oligo β- (1,4) galactose branching. The galacturonic residues can be acetylated and both residues can carry side chains of neutral sugars as galactose, arabinose and xylose.
3. Rhamnogalacturonan II: RGII is a homogalacturonan chain with complex side chains attached to the galacturonic residues (Willats *et al.* 2006. Vincken and coworkers have proposed a pectin molecule structure model in which HG and RGII are long side chains of RGI backbone. Both RG chains are also called hairy regions of pectin molecule.

Pectinase are group of enzymes that attack pectin and depolymerize it by hydrolysis and trans elimination as well as by de-esterification reactions, which hydrolyses the ester bond between carboxyl and methyl groups of pectin (Ceci and Loranzo, 1998). Pectinases account for 10% of the global industrial enzymes produced (Stutzenberger, 1992). They are classified according to their mode of secretion as extracellular and intracellular pectinase. An extracellular enzyme is excreted outside the cell into the medium in which that cell is living. They usually convert large substrate molecules (i.e. food for the cell or organism) into smaller molecules that can then be more easily transported into the cell, whereas an intracellular enzyme operates within the confines of the cell membrane. Both intracellular extracellular pectinases are classified on the mode of their attack on the galacturonan part of pectin molecule. They include:

1. Pectin methylesteranses (PE) (EC 3.1.11.1): It is a specific enzyme that gradually remove units of methanol and pectic acid from the terminal pectin chains (Pilnik and Voragen, 1993)
2. Pectin depolymerize: These are endopectinases attacking the links at 1-4 of pectin chains i.e. they attack in the middle of the chain and not from the terminals (Pastore, 2001)
3. Endopolygalaturonase (Polygalacturonase; EC 3.2.2.1): They split glycosidic linkages next to free carboxyl groups by hydrolysis.
4. Pectate lyase or acid Endo-pectin transelininase: (EC 4.2.2.3) splits glycosidic linkage next to free carboxyl groups by β- elimination.
5. Endopectin transeliminase (EC 4.2.2.3) split glycoside bonds of highly methylated pectin.
6. Pectin lyase: These enzymes act on highly methylated pectin's.

Exoenzymes: These are enzymes, which release galactutonic acid from the terminal pectin chain. Only exo Polygalacturonases and exo PALs are known. Rhizopus tritici, Gleosporium kake, Ciniothrium diplodella and *Aspergillus niger* have been reported to produce exo-Polygalacturonases as well as endo Polygalacturonases (Kawano *et al,* 1999).

Industrial production of dextrose powder and dextrose crystals from starch using L-amylase and glucoamylae began in 1959. Since then, amylase is being used for various purposes. Conversion of starch into sugar, syrups and dextrin's forms the major part of starch processing industry. They are commercially used in many industries like in Starch Conversion (for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielsen, J.E.; Borchert, T.V. (2000)), detergent industry, food industry, textile industry, brewing industry, paper industry, manufacture of maltose, high fructose containing syrup, oligosaccharides mixture, maltotetraose syrup, production of Alo-mixture, manufacturing of high molecular weight branched dextrin's, removal of starch sizer from textile (desizing), treatment of starch processing waste water (SPW), direct fermentation of starch to ethanol.

Pectinase production occupies about 10% of the overall manufacturing of enzyme preparations. Apple pomace has been reported to be an attractive raw material for production of pectinase by *Aspergillums foetidus* in solid-state culture Hours *et al* (1988). Pectinase production by *Aspergillus* strain has observed to be higher in SSF than in SmF Soils *et al* (1996). They are used in many field like Fruit juice extraction, textile processing and bio scouring of cotton fibers, degumming of plant bast fibers, retting of plant fibers, waste water treatment, coffee and tea fermentation, paper and pulp industry, purification of plant viruses, animal feed, improvement of chromaticity and stability of red wines.

MATERIALS AND METHODS

Sample Procurement

Sample of Spoiled apple fruits were collected from different fruit shops / vendors in Yelahanka, in month of September 2007, Bangalore. These materials were transferred to lab, maintained in optimum 28^0 C – 32^0 C. Fig-1 shows the spoiled apple.

Fig. 1. Spoiled apple sample

Isolation of Amylase and Pectinase producing micro organism
Micro organism was isolated from rotten and fungal infected part of Apple by serial dilution method (Experiments in microbiology, plant pathology an biotechnology by K.R.Aneja). For isolation, suspension of the sample will be prepared using 10gms of sample in the 100ml water blank kept in a rotary shaker at 80rpm 1hr for uniform distribution of organism. Sample was Serial diluted upto 10^7 and plated on starch agar media (Bacto pepton-5.0g, beef extract-3g, starch soluble-20g, agar-15g, dis.water-1000ml, pH-6.8-7.2) pectin agar media (NaNo3-2.0g, Kcl-0.5g, MgSo4 7H20-0.5g, K2HPO4 -1g, FeSO47H20-0.01g, Pectin-10g, Agar-20g, Dis. Water-1000ml).

Screening of isolates for efficient Amylase and pectinase producer:

Isolates will be tested qualitatively by growing the culture on modified starch agar medium with incubation period of 48hr at 37°C for bacterial and 72-96hr at 25°C for fungi. After colonies reached around 3mm plates were flooded with grams iodine solution (iodine-1.0gm, potassium iodide-2.0gm, and distilled water-300.0ml) to detect clearance zones. For screening pectinolytic organism, isolates were plated on Czapekagar media (Czapek cocncentrate -10.0ml, K2HPO4-1.0g, yeast extract-4.0g, sucrose-30.0g, agar-15.0g, dis. Water-1000ml) having 1% pure pectin as sole carbon source and Congo red @ 150mg / Ltrs to visualize the formed clear zones. Simultaneously, control plate was set having Congo red without pectin or any other carbon source. Plates were then incubated 30°C for 12 days selection was done on basis of formation of clear zone. Amylase and pectinase producing isolate will be selected and maintain on nutrient agar/potato dextrose agar media for further studies.

Morphological Examination of Screened Isolate:

Based on the colonies observed on the experimental plates, bacteria, fungi and yeast were stained. For bacteria Gram staining, for Fungi lacto phenol cotton blue staining and for Yeast simple staining with grams iodine was performed.

Enzyme Assay for Amylase:

Strains presenting large clearing zone were used for enzyme producing assay against substrates like rice, corn, tapioca (for amylolytic organism) and apple, guava carrot (for pectinolytic organism). Suspension of substrate was prepared, sterilized and inoculated with culture containing 10^6 cells/ml and incubated for 37^0c with intermittent shaking. After 6th and 12th day of incubation biomass was separated by centrifugation at 1000rpm for 20min and supernatant used to evaluate enzyme activity.

Amylase activity was determined by a modified method of Miller, (1959) Briefly, 0.5ml of 1% starch solution mixed with 1.0 ml of 0.02M phosphate buffer, pH 6.9 as a blank and 0.5ml of 1% starch solution, 0.2ml of 0.02M phosphate buffer and 0.8ml of enzyme extract (cell free supernatant) as a reaction mixture was prepared. The reaction was allowed to take place for 3min at room temperature. After incubation 2 ml DNS reagents was added and again incubate in the boiling water bath for 15min. Then these were cooled and 10ml of distilled water was added to the contents of each tube. The resulting color due to reaction of DNS and reducing sugar was measured at 540 nm wavelengths. One enzyme unit is equivalent to release of 1.0 μM maltose per unit time per unit volume.

Enzyme Assay for Pectinase:

Assay was conducted according to WBC Home Manual. 6ml of 0.5% polygalacturonic acid mixed with 1ml of 0.1M Phosphate buffer, pH 5 as a blank and reaction mixture contained 6ml of 0.5% polygalacturonic acid and 1 ml enzyme extract. Tubes were incubated in water bath at room temperature for 60min with shaking. After incubation cool the test tube. Draw 100µl from each test tube and pipette into another test tubes placed in ice water. Add 2ml color reagent A Sodium carbonate-40.0g in 600ml water, glycine-16.0g, copper sulphate pentahydrate-0.450g, make up to 1L with water) and 2ml color reagent B (Neocuprine-HCl-1-2g make up to lL with water and store at 4^0c in a brown bottle).Keep it in boiling water bath for 13min. cool the test tubes and add 2ml-distilled water. The absorbance was measured at 450nm.

Enzyme Assay for Polygalacturonase:

Reaction mixture contained 2ml of 0.1M sodium acetate, 0.25ml of 0.15M Sodium chloride, 0.5ml of 1% polygalacturonic acid and 0.5ml enzyme solution. Incubate in water bath at 37^0c for 15min with shaking. To the 0.1ml of above solution, 2ml distilled water and 1ml of alkaline copper tartarate was added and boiled in water bath for 10min. cool the test tubes and add 1ml arcenomolybdate reagent. Add 10ml distilled water and check the absorbance at 620nm (Baldwin and pressy, 1989).

Enzyme Assay for pectin lyase

Assay was made by using reaction mixture (0.2ml of 0.1M Sodium acetate, pH5.5, 2.5ml of0.12M Sodium chloride, 0.5ml of 1% pectin, pH5.5 and 0.05ml of enzyme solution). Incubate at 37□c for 15min in water bath with shaking. After

incubation add 5ml distilled water and absorbance was observed at 235nm. (Albersheim and Killas (1962))

RESULTS

11 isolates was screened for amylolytic organism on selective starch Agar media having pure starch as the only carbon source. A clear inhibition zone was visualized in 9 isolates of which eight were of *Bacillus* species (BBPs1, BBPs2, BBPs3, BBPs5, BBPs6, BBPs7, BBPs9, BBPs10), Under microscope appearing as purple color with rod shape and remaining one seemed to be *Aspergillus* species (BBPs11) showing respective colony characteristic. (Fig-2a, 2b, 2c)

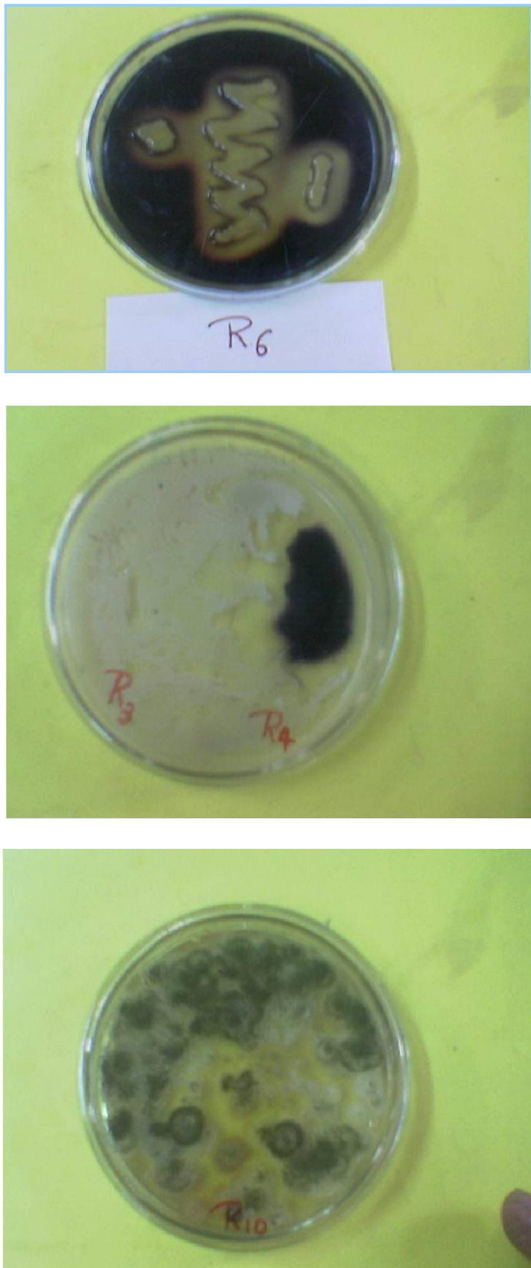

Fig-2a. Plate showing clear zone of inhibition in Amylolytic Isolate #BBPs3 &no zone of inhibition with BBPs4. Fig-2b. zone of inhibition in isolate#BBPs11. Fig-2c .zone of inhibition in isolate#BBPs10

5 isolates was screened for pectinolytic activity on selective czapek media having pure pectin as only carbon source, of which 3 isolate showed positive result. (Fig-3a, 3b, 3c)

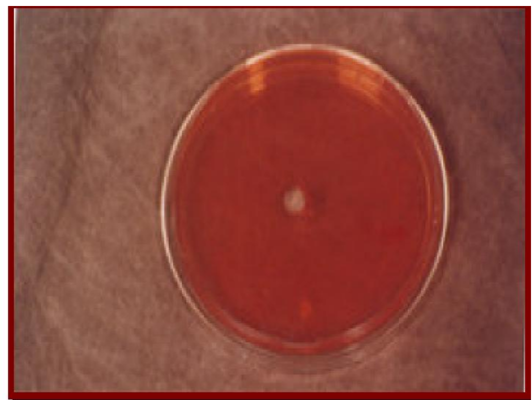

Fig. 3a, 3b, 3c. Plates showing clear zone of inhibition in Pectinolytic Isolate#BBPp1, #BBPp2, #BBPp3

Isolate BBPp1belonged *gillus*genus with their mycelia greenish, conidiophores with bulge vesicle and spores bearing head were large and globular, tightly packed *Aspergillus.*

Isolate BBPp2 clearly showed presence of mycelia without septa. Sporangiospore were arising from node and sporangia present at the end of sporangiophores, hence detected as *Mucor* genus. Isolate BBPp3 belonged to yeast showing cream color moist colonies in culture and Microscopically cells appeared oval with budding cells. Amylase activity of two *Bacillus* and *Aspergillus* Isolates was determined against substrate rice, corn, and tapioca. Wherein maximum activity was seen in rice and minimum activity in tapioca from one of the *Bacillus* genus. (Fig-4)

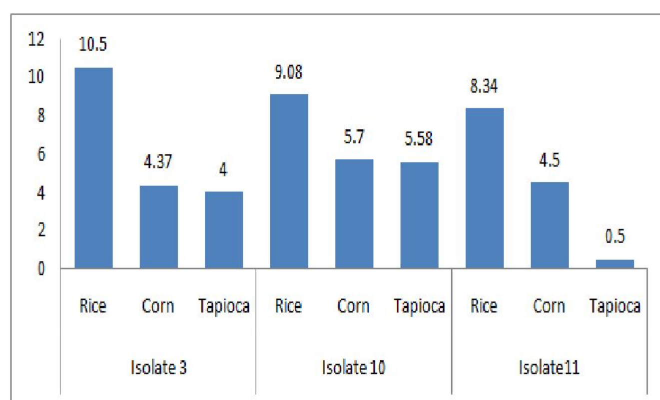

Fig. 4. Effect of Amylase activity (µg/ml/min) of the Isolates on the substrates on the substrates at the 12th day of incubation

Three organisms were screened as pectinolytic and were stained and detected as the genus *Aspergillus, Mucor,* and *Yeast.* In each of the screened pectinolytic organism three of the enzymes (Pectinase, Polygalacturonas, Pectinlyase) were tested against the substrate apple, guava and carrot. Here Pectinase of *Aspergillus* showed maximum activity on apple. Polygalacturonase of *Mucor* and *Yeast* showed maximum activity on guava. Pectinase of *Aspergillus* and *Yeast* showed least activity on carrot and pectinlyase of *Mucor* showed least activity on apple. No activity of Pectinlyase of *Mucor* and *Yeast* was seen in carrotFig-5a, 5b, and 5c)

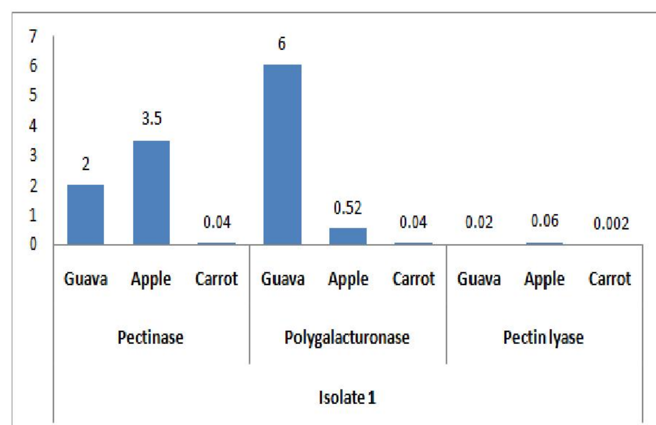

Fig. 5a. Effect of enzyme activity (µg/ml/min) showed by isolate #BBPp1 on following substrate

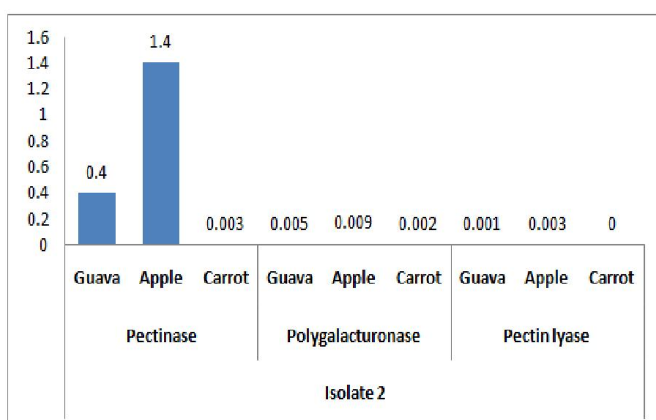

Fig. 5b. Effect of enzyme activity (µg/ml/min) showed by isolate #BBPp2 on following substrates

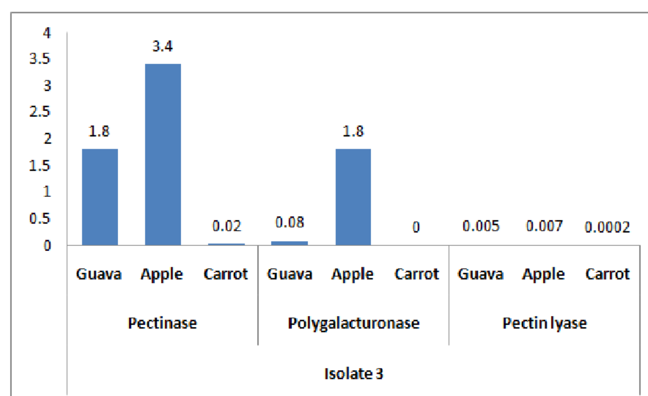

Fig. 5c. Effect of enzyme activity (µg/ml/min) showed by isolate #BBPp3 on following substrates

DISCUSSION

Enzyme production is a growing field of biotechnology and the world market for enzyme is estimated at $3.3 billion by 2010 only. This market is expected to reach $4.4 billion by 2015(Global Market, BCC Research, January1, 2011, pub ID: WA6060223) the majority of the industrial enzymes are of microbial origin. Fruit and vegetables are utilized by all of us but when they get spoiled they become uneadible, which has to be disposed. Even this waste can be useful since microorganism in them are commercially utilized in many industries like food, textiles etc. So it is far necessary to isolate and screen organism from waste. Apple pomace has been reported to be an attractive raw material for production of pectinases by *Aspergillus foetidus* in solid-state cultures (Hours *et al*, 1988). Rotten and fungal infected kinnow were used to isolated some fungal species with pectinolytic activity. Seventy three fungi and bacteria were isolated from twenty different wastes, e.g. food-industrial wastes, daily home wastes, expired foodstuff wastes, and some agricultural wastes.Starch saccharifiying enzyme has been synthesized in solid culture (Selvakumar *et al*). Amylase and pectinase has been produced by *Aspergillus* species in SSF and observed is production is high in SSF than in SmF. (Alva *et al* (2007), soils *et al* (1996)). Amylase obtained from *Thermomyces lanuginosus* fermented with wheat bran showed its maximum activity of 534U/g for 120hrs at 50°C. (Adinarayana *et al* (2005)).

Similarly, glucoamylase of *Aspergillus* strain (As. J-GI 12) obtained for seeds was subjected to mixed fermentation with 2.5gm of wheat bran at 25°C for 6 days. Here rice bran and gingelly oil cake showed maximum and minimum activity of 16.42U/g and 2.03U/g (Alva *et al* (2007)). Fungal pectinase (Rhizopus genus) obtained from kinnow peel powder at 30°C for 6 days. Here pectinase, PG, PL showed activity of 436.27nanomoles, 52.39 and 758.83 but PL of *Aspergillus* species showed no activity. In the present study 15 organisms were isolated from spoiled apple and screened for amylolytic and pectinolytic activity having starch and pectin as a sole source of carbon in them. Of which few showed to present respective enzyme activity. Eight bacteria and one fungi showed amylase presence in them and *Mucor, Yeast*, and a Fungi showed pectinase presence in them.Work was conducted on both amylase and pectinase organism obtained from spoiled apple. The isolates were fermented with

different substrates at room temperature (37°C) for a week and activity was determined during 6th day and 12th day of incubation. It was analyzed that for amylase, Isolate BBPs3 (*Bacillus genus*) showed maximum activity of 14.5µmoles/ml/min on rice and BBPs10 (*Bacillus)* showed minimum activity of 5.58µmoles/ml/min on tapioca. In isolate BBPp1 (*Aspergillus),* pectinase showed maximum activity of 6.05µmoles/ml/min on apple. PL of BBPp2, BBPp3 (*Mucor)* showed no activity on carrot and apple. Amylase and pectinase are present in many fungal and bacterial species. Efficient enzymes can be obtained by fermenting such organisms for a respective period using natural byproducts like wheat bran, rice bran, spoiled apple, kinnow waste etc., as a substrate. It showed that these enzymes were successful in utilizing starch and pectin present in the different substrate and was capable of showing their maximum activity. From the above observation it can be analyzed that amylase and pectinase is an essential enzymes, commercially used for many purpose. Apart from biochemical and genetically engineering method using simple substrate like natural byproducts, spoiled fruits and vegetables can also produce these enzymes, which can be commercialized.

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