Vol III Issue VII August 2013
Impact Factor : 0.2105 ISSN No : 2230-7850

Monthly Multidisciplinary
Research Journal

Indian Streams Research Journal

IMPACT FACTOR : 0.2105

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RNI MAHMUL/2011/38595 **ISSN No.2230-7850**

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Indian Streams Research Journal

ISSN 2230-7850
Volume-3, Issue-7, August-2013

Pectinase Production By Aspergillus Species In Submerged Fermentation By Using Agricultural Wastes As A Substrate

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Abstract: The present investigation was undertaken to produce pectinase enzyme from waste residues of different agricultural wastes by using Aspergillus species in submerged fermentation. The maximum amount of pectinase (90units/ml) was obtained from citrus peels followed by Watermelon (80units/ml), Pineapple (70units/ml), Sorghum straws (60units/ml). The minimum amount of pectinase was obtained from Corn cobs (50units/ml). Optimization of different parameters such as substrate concentration, pH, temperature and incubation time for pectinase production was studied. The maximum amount of pectinase was produced by the Aspergillus spp. at 6% Citrus peels substrate concentration, pH 6, temperature 350C and incubation time 24 hrs by submerged fermentation .

Keyword: Agricultural wastes, Aspergillus species. Submerged fermentation, pectinase production

INTRODUCTION:

Many bacteria, fungi, yeasts and actinomycetes are known to produce pectinolytic enzyme called pectinases that breakdown pectin substrate. Among these microorganisms Fungi are known to be efficient producers of different enzymes. Aspergillus niger is commonly cultured for the industrial production of many substances. Many useful enzymes are produced by fermentation by using Aspergillus niger. For example, glucoamylase and pectinases have been used in several conventional industrial processes such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater containing pectinic materials etc.

Currently, the various types organic wastes such as agricultural and food wastes which are potential polluting agents are controlled by biological degradation of these wastes by using microorganisms for the production of valuable compounds such as proteins, polysaccharides, oligosaccharides, vitamins, hormones, enzymes and other raw materials for medicinal and industrial uses (El-Sheekh et al., 2009). Biowastes are highly perishable materials and their disposal is often a serious problem in processing industries. Use of such biowastes as substrate for production of various enzymes by microbial fermentation has gained importance recently. Various fungi are especially used for biodegradation of biowastes quite profitably (Anand and Maini, 1997).

Pectin is important component of middle lamella and primary cell wall of higher plants, fruits, and vegetables. Pectic substances are widely distributed in fruits and vegetables; hence they form important natural substrates for pectinases (Elangovan et al., 2011). Generally, agro-industrial wastes are employed for the pectinase production. Various substrates that are being used are sugarcane bagasse, wheat bran, rice bran, wheat straw, rice straw, sawdust, corn cobs, coconut coir pith, banana waste, tea waste, sugar beet pulp, apple pomace, orange peel etc (Pilar et al., 1999).

Pectin is hydrolyzed by pectinases produced extracellularly by micro flora present in our natural environment. With the help of these pectinase enzyme, micro-organisms can convert wastes such as citrus into sugars which can be used for food and value added products. These micro-organisms can also be exploited for production of pectinases which are industrially important. The advantage of using micro-organisms for the production of enzymes is that these are not influenced by climatic and seasonal factors, and can be subjected to genetic and environmental manipulations to increase the yield (Vibha and Neelam, 2010).

Among the different enzymes, pectinases are important in the food processing industry and it is estimated that the pectinase market in the world for industrial processes is about 1000 million pounds per annum (Singh, 2000). In industries, acidic pectinases are used during extraction and clarification of fruit juices whereas alkaliphilic pectinases are used immensely in the degumming of fibres, treatment of effluents discharged from fruit processing units (Nitinkumar et al., 2010). Pectinases have been used in processes and industries where the elimination of pectin is essential; fruit juice processing, coffee and tea processing, macerating of

plants and vegetable tissue, degumming of plant fibers, treatment waste water, extracting vegetable oil, bleaching of paper, adding poultry feed and in the textile, alcoholic beverages and food industries.

The ability to synthesize pectinolytic enzymes is very common in groups of microorganisms. Different types of micro-organisms have been exploited for the production of enzymes but fungi are preferred on an industrial scale. This is because about 90% of the enzymes produced mostly secreted in the culture medium (Blandino et al., 2001). Aspergillus niger is the most commonly used fungal species for industrial production of pectinolytic enzymes. (Vibha and Nilam, 2010).

Present study was undertaken to isolate and identify pectinase producing fungi from soil sample

and pectinase production through submerged fermentation techniques by using Aspergillus species. Optimization of the fermentation conditions for maximum production of pectinase was also studied.

MATERIALAND METHODS:

Collection of agricultural wastes:

Fruit peels (Pineapple, Lemon, and Watermelon) were collected from different fruit juice centers in Solapur city. Agricultural wastes (Corn cobs, and Sorghum straws) were collected from fields nearby Solapur city. Fruit peels were first washed thoroughly with sterile distilled water to remove all adhering substances. Small pieces of peels were made using knife and these were then shade dried in trays. The dried fruit peels and agricultural wastes were then made to powder using a mechanical grinder.

Estimation of pectin from substrate sources:

Pectin was extracted from selected substrates and saponified. It was precipitated as calcium pectate by the addition of calcium chloride to an acid solution. The precipitate was thoroughly washed to eliminate chloride ions, and then it was dried and weighed. The estimation of pectin was done by using gravimetric method. (Ranganna, 1979)

Isolation and Identification of pectinase producing fungi:

Soil sample was collected from the vegetable -fruit market of Solapur city. It was serially diluted and 0.1 ml of each dilution was inoculated on pectin containing agar medium plates. After 48 hrs incubation at 30^0C, colony showing zone of clearance was selected for further study. On the basis of microscopic observations, morphological, colony characteristics the fungus was identified as Aspergillus species.

Determination of the pectinolytic activity of isolated Aspergillus species:

The pectinolytic activity of Aspergillus spp. was studied by spot inoculation on pectin containing agar media. The inoculated plates were incubated at 300C for 48 hrs. After incubation the plates were flooded with 1% (w/v) aqueous solution of hexadecyl trimethyl ammonium bromide solution.(Deshmukh, 1997).The zone of clearance around the colonies in an opaque white background indicated the pectinase production ability of Aspergillus species. The stock culture was maintained on potato dextrose agar (Sarvamangala et al., 2006) slant stored at 40C.

Qualitative Test for pectinase production from substrates used:

Aspergillus species was allowed to grow on a pectin source from substrate containing agar plate to which, 1% (w/v) aqueous solution of hexadecyl trimethyl ammonium bromide was added to observe the zone of clearance which was indication of pectinase production (Nitinkumar et al.,2010).This test was carried to observe pectinase production from each substrate separately..

Preparation of inoculum:

Aspergillus media broth [composition: Glucose 20$(NH_4)2NO_3$1.0g KH_2PO_4 0.4g, $MgSO_4.7H_2O$0.5g, $FeCl_3$0.016g, $ZnSO_4.7H_2O$, 0.005g, Distilled water 100ml, pH5.0] (Deshmukh,1997) was prepared, sterilized and spores of Aspergillus species were added from Potato dextrose agar slants and incubated at 30^0C for 96 hrs. After incubation the spore suspension (1×107spores /ml) inoculated with 0.01% Tween 80 solution was used as a source of inoculum.

Submerged fermentation:

In submerged fermentation, 250 ml Erlenmeyer flask containing 100 ml of fermentation medium (Deshmukh, 1997) [Substrate as pectin source 2.0g,$(NH_4)_2SO_4$ 1.4g, K_2HPO_4 2.0g ,$MgSO_4.7H_2O$ Nutrient solution 1.0ml (Composition of nutrient solution (g/l): $FeSO_4.7H_2O$, $MnSO_4.7H_2O$ 1.6g, $ZnSO_4.7H_2O$ 1.4g ,$CoCl_2$ 2.0g, pH 5) Distilled water 1000ml] was inoculated with 5ml of inoculum. The flasks were incubated on rotary shaker (150rpm) for 96 hrs at 30^0C.

Optimization of the fermentation conditions:

Process parameters optimized included pH, temperature, and substrate concentration and incubation period.

Optimization of pH:

250 ml flasks containing 100ml of fermentation medium with different pH ranging from 4 to 7 were inoculated with 5ml of inoculum to each flask and incubated on rotary shaker (150rpm) at 30^0C.The enzyme production was analyzed by pectinase assay done after every 24 hrs of fermentation.

Optimization of Temperature:

250 ml flasks containing 100ml of fermentation medium with constant pH 6 were inoculated with 5ml of inoculum to each flask and incubated on rotary shaker (150rpm) at between25^0C, 30^0C, 35^0C, and 40^0C.The enzyme production was analyzed by pectinase assay done after every 24 hrs of fermentation.

Optimization of substrate concentration:

For substrate concentration optimization, the pH

and Temperature for each substrate was kept constant and substrate concentrations used were 4g, 6g, 8g and 10g in 100ml of fermentation medium taken separately in 250 ml flasks. The enzyme production was analyzed by pectinase assay done after every 24 hrs of fermentation.

Optimization of Incubation time:

250 ml flasks containing 100ml of fermentation medium with constant pH and substrate concentration were inoculated with 5ml of inoculum to each flask and incubated on rotary shaker (150rpm) at 30^0C. The enzyme production was analyzed by pectinase assay done after 24 hrs, 48 hrs, 72 hrs and 96 hrs of fermentation.

Enzyme extraction from Fermented broth:

After every 24 hrs of incubation, 10ml fermented broth was separated by filtration using Whatmann filter paper and centrifuged at 6000rpm for 20min at 4^0C. The supernatant obtained was used as source of crude enzyme for determination of enzyme activity. (Mrudula and Anitharaj, 2011).

Enzymatic assay of pectinase:

The enzyme units /ml of filtered fermented broth were determined by enzymatic assay of pectinase using titration method (Kertesz, 1955), (Yogesh, 2009).

RESULTS AND DISCUSSION:

Two different fungus cultures were selectively isolated by enrichment technique. These cultures were processed for their pectinolytic activity on pectin containing agar medium. On the basis of prominence of clearance zone on pectin agar medium, one fungal culture was selected for further study. Based on its colonial, cultural characteristics and microscopic observations, the fungus culture was identified to genus level as strain of Aspergillus species.

Estimation of pectin present in various substrates indicated that the maximum amount of pectin was present in the lemon peel followed by watermelon, pineapple, Sorghum straws and corn cobs respectively. i.e. 2.5%, 2.0%, 1.5%, 1.0% and1.0% (Table 1).

The pectinolytic activity was determined by spot inoculating previously isolated culture of Aspergillus spp. on pectin containing agar medium. After 48 hrs incubation period, zone of clearance around the colony against opaque white background and pectinase production was detected (Figure 6).

The pectinase production was carried out by submerged fermentation using different agro wastes such as Lemon peel, Watermelon rind, Pineapple peel, Corn cobs, and Sorghum straws as a sole carbon source. The partial optimization of submerged fermentation process was carried out with respect to incubation period, pH, temperature and substrate concentration.

Among five substrate screened lemon peel and watermelon was found to be most significant for pectinase production by Aspergillus spp. followed by pineapple peel, Jawar straw, and corn cobs respectively. Maximum pectinase production was observed at 24 hours of incubation period. (Table 5)

The effect of pH was studied by varying the pH from 4 to7 for production of pectinase enzyme. The enzyme production was analyzed for every 24 hrs of fermentation. The maximum activity of pectinase was observed at pH-6.0 and was found to be 50 units per ml of filtered culture broth. (Table 3)

Incubation time and pH of fermentation medium was kept constant and temperature was varied between 25-400C for 96 hrs. It was found that at incubation temperature 35 0C maximum enzyme activity. i.e. 70 units per ml of filtered culture broth were detected (Table 4)

The production of pectinase was studied by optimizing the different substrate concentrations ranging from 4-10% at optimum pH 6.0, temperature 35^0C and incubation time 24 hrs. The maximum pectinase enzyme activity was observed for citrus peel (90 units per ml), watermelon (80 units per ml), and pineapple (70 units per ml) at 6% substrate concentration. Similarly 8% substrate concentration showed maximum enzyme activity for Sorghum straws (60 units per ml) followed by corn cobs (50 units per ml). (Table 5) The optimization studies showed the The maximum amount of pectinase was produced by the Aspergillus spp. at 6% Citrus peels substrate concentration, pH 6, temperature 35^0C and incubation time 24 hrs by submerged fermentation .

Processing of agricultural wastes from agro industries needs the commercial production of pectinase enzyme. Although synthesis of pectinolytic enzyme is very common in group of microorganisms but fungus is preferred on an industrial scale. Aspergillus niger is the most commonly used fungal species for industrial production of pectinolytic enzymes. (Vibha and Neelam, 2010).

In concern with use of various agricultural wastes and agro industrial wastes especially fruit processing industries, wine industries generates gallons of wastes during preparation of different juices (Vibha et al., 2010). It's dumping in nature causes pollution problems. Such problems can be solved by exploiting these agro wastes for pectinase production by using potential microorganisms by fermentation process.

In present study citrus peel and watermelon rind were found to be best substrate for pectinase production by Aspergillus spp. using submerged fermentation. Similar study can be extended to optimization of process by using different carbon sources, nitrogen sources and leaching conditions for getting maximum yield of pectinase. As these substrates are very cheap, abundantly available and could be easily stored after sun drying can be exploited for pectinase production on large scale. The use of these substrates for pectinase production will not only reduce the production costs of the enzyme but also helps to decrease the pollution load of agro industrial wastes.

Table 1 Estimation of pectin from substrate sources :

Substrate	Initial weight of filter paper	Final weight of filter paper	% of calcium pectate
Lemon	1.120	1.170	2.5
Watermelon	1.060	1.090	2.0
Pineapple	1.070	1.100	1.5
Corn cobs	0.990	1.010	1.0
Jawar straw	1.030	1.050	1.0

Fig1 :Estimation of pectin from substrate source:

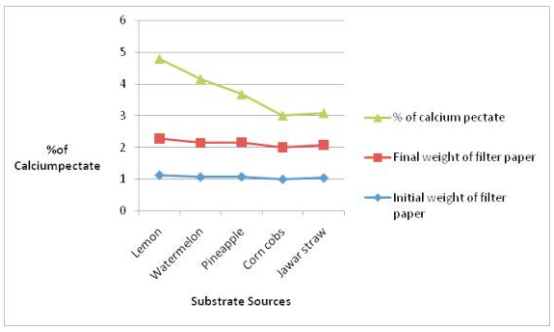

Table 2: Effect of incubation period on pectinase enzyme production:

Incubation period (hrs)	24	48	72	96
Substrate	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)
Lemon	40	30	20	10
Watermelon	30	30	20	10
Pineapple	20	10	00	00
Corn cobs	10	10	00	00
Jawar straw	20	20	10	00

Figure 2: Effect of incubation period on pectinase enzyme production:

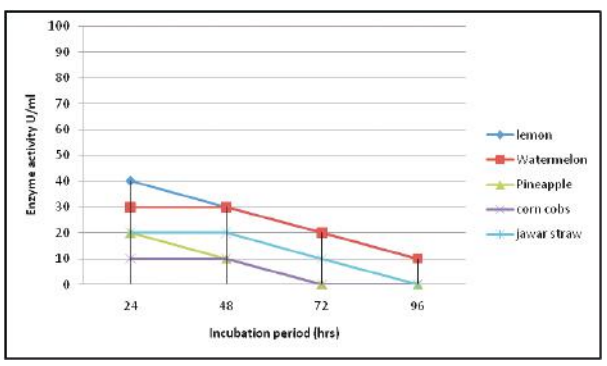

Table 3: Effect of pH on pectinase enzyme production

pH	4	5	6	7
Substrate	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)
Lemon	30	40	50	30
Watermelon	20	30	40	30
Pineapple	20	20	30	20
Corn cobs	10	10	20	10
Jawar straw	10	20	20	10

Figure 3: Effect of pH on pectinase enzyme production

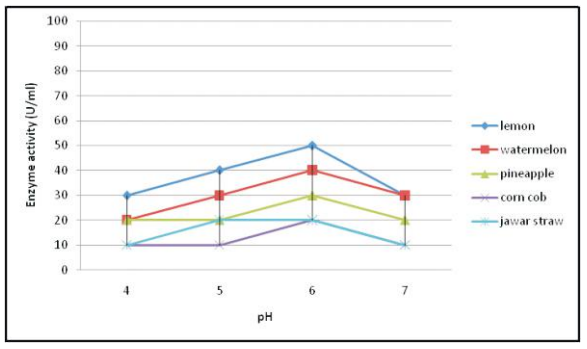

Table 4: Effect of Temperature on pectinase enzyme production

Temperature	25	30	35	40
Substrate	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)
Lemon	40	50	70	60
Watermelon	30	50	60	50
Pineapple	20	40	60	40
Corn cobs	00	20	50	30
Jawar straw	10	20	40	30

Figure 4: Effect of Temperature on pectinase enzyme production

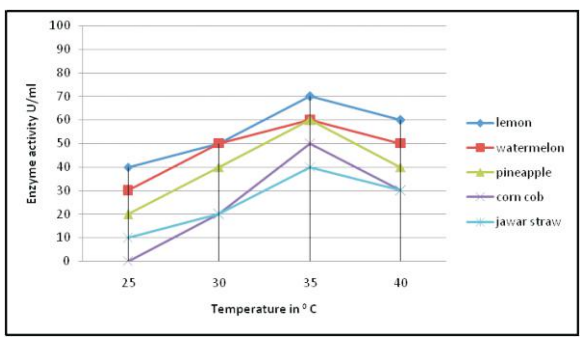

Table 5: Effect of Substrate concentration on pectinase enzyme production

Substrate concentration(gm)	4	6	8	10
Substrate	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)	Enzyme activity (U/ml)
Lemon	70	90	80	60
Watermelon	60	80	80	70
Pineapple	60	70	60	40
Corn cobs	30	40	50	40
Jawar straw	40	50	60	50

Figure 5: Effect of Substrate concentration on pectinase enzyme production

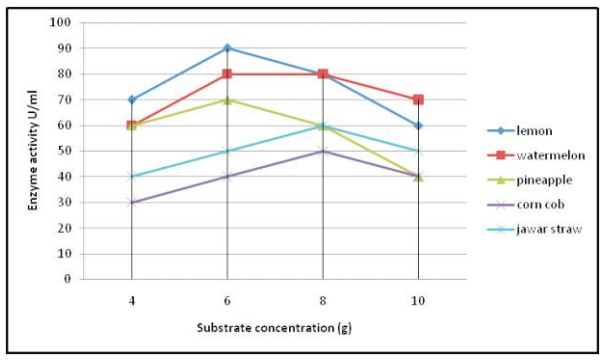

Figure 6: Zone of clearance shown by Aspergillus spp. on pectinolytic media.

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ACKNOWLEDGEMENT:

The authors would like to thank to Principal, Walchand College of Arts and Science, Solapur for providing the Laboratory facilities.