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Pectinase Production from *Aspergillus niger* RBF96 by Solid State Fermentation using Citrus Peel

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Microbial pectinases have penetrated the industrial market and have become one of the most promising enzymes recently due to their economical value. In this study, pectinolytic fungi were isolated from rotten ber fruit and screened for pectinolytic activity. The highest potency index (3.25) was observed in the isolate RBF96 compared to the reference strain *Aspergillus niger* MTCC 1344 (2.99). Based on the potency index and activities of polygalacturonase, pectin lyase and pectin esterase, RBF96 was found to be the most potent isolate and was identified as *Aspergillus niger,* based on morphological as well as molecular characterization. The process parameters for maximum enzyme production under SSF using citrus peel were optimized. The optimum moisture was found to be 65 per cent. The optimum pH for production of polygalacturonase, pectin lyase and pectin esterase were 4.0, 6.0 and 5.0 respectively. The optimum temperature was 35° C with maximum enzyme activity produced at six days of incubation period.

Key words: Rotten ber fruit, Pectinase Potency index, Citrus peel, Solid state fermentation,*Aspergillus niger* RBF96.

Pectinase are group of enzymes that attack pectin and depolymerise it by hydrolysis and transelimination as well as by deesterification reactions, which hydrolyse the ester bond between carboxyl and methyl groups of pectin[1]. Depending on their specificity and the type of reaction they catalyze, pectinases are classified into different types. Polygalacturonases catalyze the hydrolytic cleavage of the polygalacturonic acid chain through the introduction of water across the oxygen bridge. They are the most extensively studied among the family of pectinolytic enzymes[2]. Pectin lyases perform non-hydrolytic breakdown of pectates or pectinates. Pectin esterase catalyzes the de-esterification of methyl ester linkages of the galacturonan backbone of pectic substances to release acidic pectins and methanol[3]. A combined action of pectinesterase and polygalacturonases is required for degrading pectic substances. Pectin lyase is the only enzyme capable of depolymerizing the pectin molecules without the prior action of other enzymes[4].

Pectinolytic enzymes are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage. They also aid in maintaining ecological balance by causing decomposition and recycling of waste plant materials. These macerating enzymes are playing a key role in food biotechnology and their demand will likely to increase for extraction of juice from array of fruits and vegetables.

Pectinolytic microorganisms are widely distributed in soil, spoiled fruits, vegetables, decayed leaves and wood etc. Fungi like *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium expansum*, which are generally regarded as safe (GRAS) by United States Food and Drugs

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Administration (USFDA) are employed for pectinase production required in food industry. It has been reported that microbial pectinases account for 25 per cent of the global sales of food enzymes. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes[5].

Selection of an efficient strain, isolated from the natural environment however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. Once an efficient strain is selected, its fermentation condition needs to be optimized. Pectinases can be produced by both submerged and solid state fermentation (SSF). Submerged fermentation (SmF) is the cultivation of microorganisms in liquid broth. It requires high volumes of water, continuous agitation and generates lot of effluents. SSF has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms[6]. Agro-industrial residues are generally considered as the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. From data (2008) of United States Department of Agriculture (USDA) the worldwide industrial citrus peel wastes may be estimated is more than $15x10^6$t. Considerable quantity of citrus pulp in the fresh state is lost as a result of the difficulty of rapidly disposing a large quantity of the wastes in a fairly short period of time, and then a huge amount of it is still discarded to nature, causing several environmental problems[8]. A number of such substrates have been employed for the cultivation of microorganisms to produce pectinase enzyme. There is an increasing tendency among the people to use chemical free foods. The use of enzymes like pectinases in the food processing can meet such public demands. SSF adds on to economic feasibility of the process by utilizing the low cost agricultural residues, on one hand, and on the other hand, it solves the problem of their disposal which otherwise cause pollution[7].

MATERIALS AND METHODS

Collection of samples

Decayed, rotten and fungus infected rotten ber fruit (RBF) were collected from Dharwad city fruit market. All the samples were collected in sterile polypropylene bags and stored at 4 ^oC in a refrigerator until the isolation experiments were completed.

Isolation of pectinolytic fungi

Isolation[9] was carried out in two steps *viz*. i) enrichment and ii) isolation

Enrichment

one g of rotten ber fruit sample was ground uniformly using a sterile pestle and mortar and inoculated in to 100 ml pectin broth (5% w/v yeast extract, 1 ml 0.1% v/v bromothymol blue, 0.6 ml 10% v/v $CaCl_2 \cdot 2H_2O$, pH 6.0) supplemented with 0.5% w/v pectin (Hi-Media, Mumbai) and incubated on a rotary shaker (120 rpm) at 30 ^0C for 7 days. One ml of this enriched culture was inoculated in to 100 ml of freshly prepared pectin broth supplemented with 1.0% w/v of pectin and enriched for 7 days. Several such serial transfers were made in pectin broth, each time with increasing pectin concentration up to 3.0%. The sample was finally subjected for isolation of pectinase producing fungi.

Isolation of pectinolytic fungi

After fifth week of enrichment, dilutions upto10^{-6} was made and aliquots from 10^{-4}, 10^{-5} and 10^{-6} dilutions were placed on pectin solubilising agar medium[10]. Ampicillin antibiotic @ 100 mg/ml was added to the molten and cooled medium to prevent bacterial growth. The above dilutions were plated in triplicates. The plates were then incubated at 30 ^oC for 7 days. The colonies, thus isolated, were sub-cultured 4 times on Czapek yeast extract agar (CYA) till active growth of isolates were obtained, and thereafter maintained on the Czapek yeast extract agar (CYA) media slants for further experimental work.

Primary screening for pectinase activity

Agar discs of purified fungal isolates were transferred to pectin agar medium having pectin as sole carbon source and plates incubated at 30 ^oC for 7 days. At the end of the incubation period, the plates were stained with 50mM iodine solution to view the clear zones around the colonies. Selection was done on the basis of formation of

clear zones and the corresponding zone and colony diameters were recorded and potency index values were determined[11].

Reference strain *Aspergillus niger* MTCC 1344

An active culture of *Aspergillus niger* MTCC 1344 was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, and maintained by sub culturing on the Czapek Yeast Extract Agar (CYA) medium.

Inoculum preparation

The potato dextrose broth medium was prepared, adjusted to pH 5.5 and autoclaved for 15 minutes at 121°C and cooled. A loop-full culture from sporulation medium of fungi raised on Czapecdox yeast extract agar (CYA) slants, was transferred aseptically in a laminar air flow into the conical flask (250 ml) containing 100 ml sterilized inoculum medium. It was incubated on orbital shaker (130 rpm) at 37 °C. After 72 h, the spore count in the inoculum was adjusted to $1x10^7$ spores/ml with the help of a haemocytometer[12].

Quantitative estimation of pectinolytic activity

Quantitative estimation of pectinolytic activity of selected potent isolates was done under submerged fermentation condition. One ml of freshly grown broth culture ($1x10^7$ spores/ml) of each isolate was transferred to the sterilized pectin broth medium and the flasks incubated in a shaker incubator at 250 rpm for 8 days at 30 ^{0}C.

Enzyme production

After incubation, the mycelial mass was separated by filtration and resulting supernatant was centrifuged using refrigerated centrifuge at 10,000 rpm for 10 min. and supernatant was carefully transferred to sterile containers and the samples were used for carrying out enzyme assay.

Polygalacturonase (PG) assay

Polygalacturonase (PG) assay was measured by determining the amount of reducing groups released according to the method described[13,14] and reducing sugar (galacturonic acid) was estimated by Dinitrosalicylic acid (DNSA) method[15]. One unit of Polygalacturonase (PG) is defined as the amount of enzyme that releases one ¼ mol of galacturonic acid/ml/min under the assay conditions.

Pectin Lyase Assay

Pectin lyase was assayed by measuring the increase in absorbance at 550 nm according to method[16]. One unit of activity is the amount enzyme causing a change in absorbance under the conditions of assay.

Pectin esterase (PE) activity assay

The PE activity was estimated by the method[17]. One enzyme unit was expressed as amount of enzyme required to produce carboxylic groups to be titrated by one micro–equivalent of NaOH and the activity was expressed as ¼ moles of carboxylic acid produced / ml.

Morphological and cultural study of the fungal isolates

Pectinase producing potent fungal isolates were identified on the basis of colony morphology, cultural and microscopic features. The isolates were inoculated on to plates containing Czapek yeast-extract (CYA) agar medium and incubated at 30 ^{o}C for 8 days[18]. The macroscopic variables analyzed included colony type, obverse and reverse mycelium color, degree of sporulation, and the presence or absence of exudates. The microscopic characteristics of the mycelia were examined in a lactophenol aniline blue stain[19]. The slide was observed under a compound microscope equipped with an image analyser (Motic Images plus 2.0, Digital microscopy software).

Molecular characterization

DNA extraction by modified CTAB extraction method[20]. The most potent fungal isolate was grown on potato-dextrose agar (PDA) at 30 ^{o}C for three days and 150 mg of fungal mycelia was scraped and centrifuged to collect the mycelium which was frozen in liquid nitrogen and ground to a fine powder using a sterilized pestle and mortar. The frozen mycelium (50-100 mg) was taken in 1.5 ml microfuge tubes and 500¼l of pre warmed (60 ^{o}C) TES lysis buffer (100 mM Tris pH 8.0; 10 mM pH 8.0; 2 % SDS), and 50¼g of proteinase K were added to the ground material, incubated at 60 ^{0}C for 60 min. A volume of 140¼l of 5M NaCl and 64 ¼l of 10% (w/v) of CTAB were added to the suspension and incubated at 65 ^{0}C for 10 min. DNA was extracted by adding equal volume of chloroform: isoamylalcohol (24:1), centrifuged at 13,000 rpm for 10 min. DNA was precipitated by adding 6 volume of cold isopropanol and 0.1 volume of 3M sodium acetate pH 5.2 and maintained at -20^{0}C, centrifuged and washed twice with 70 % ethanol suspended in 100¼l TE buffer (10 mM Tris pH 8.0; 1mM EDTA pH 8.0). RNA was

digested by adding 10 mg/ml of RNAse A and incubated at 37^0 C for 45 min and stored at -20 ^{0}C for further use.

Polymerase chain reaction

The ITS region was amplified with the primers ITS1 (5'-TCCGTTG GTGAACCAGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). Amplification was done with gradient annealing temperatures at 50 ^{o}C, 50.7 ^{o}C, 51.7^{o}C, 53.1 ^{o}C, 55.2 ^{o}C, 56.6 ^{o}C, 57.6 ^{o}C, and 59.0 ^{o}C. Amplification was performed in 100 ml of reaction mixture containing 50 pico mol of primers, 2.5 units *Taq* DNA polymerase, 200 mM of each dNTP, 10 µl of 10 X PCR buffer and 0.2 mg of template DNA and subjected to PCR in a thermal cycle with an initial denaturation step for 3 min at 95 ^{o}C, followed by 30 cycles of annealing for 40 sec at 55.2 ^{o}C, extension for 40 sec at 72 ^{o}C and denaturation for 40 sec at 94^{o}C before a final extension step for 5 min at 72 ^{o}C. The reactions were performed on PalmCycler, gradient thermal cycler with a palm top computer interface (Corbett Life Sciences (QIAGEN) Mortlake, Australia) using a program[21].

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplified product using 1.5 per cent agarose in 1X TAE **(**Tris Acetate EDTA) buffer, 0.5 µg ml^{-1} of ethidium bromide, and loading buffer (0.25% bromophenol blue in 40% sucrose). Four µl of the loading dye was added to 20 µl of PCR product and loaded to the agarose gel. Electrophoresis was carried at 65 V for 1.5 h. The gel was observed under UV light and documented using gel documentation unit.

DNA Sequencing

DNA sequencing and sequence analysis

PCR products generated using the primers ITS1/ITS4 was used as a template for DNA sequencing, The PCR eluted product was sequenced through outsourcing. Samples were sent to Chromous Biotech Pvt ltd, Bangalore-560092, India. The sequences obtained were subjected to *In silico* BLAST (Basic Local Alignment Search Tool) analysis and compared with sequences available in the NCBI database after the ntBLAST search results (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the *In silico* analysis, molecular identification of pectinolytic fungal isolate was confirmed.

Solid state fermentation (SSF)

Citrus peels were collected from different fruit juice centers, located in Hubli and Dharwad city and used for SSF studies. Ten g of citrus peel in a flask was mixed with adequate quantity of sterile water to maintain the moisture content along with the mixture of salt solution but no pectin is added. The pH of substrates was adjusted accordingly with either 0.01 M HCl or 0.01 M NaOH and autoclaved for 15 minutes at 121°C and cooled. The spore concentration was adjusted to 1x10^7 spores/ml. From this suspension, 1ml was withdrawn and inoculated into each flask and incubated at different temperature (^{o}C). Four fermentation periods of 2nd, 4th, 6th and 8th day were given to each treatment to arrive at the best period of fermentation under pre-optimized conditions. For proper aeration, flasks were intermittently shaken.

In order to estimate the polygalacturonase, pectin lyase and pectinase esterase, samples were drawn by adding adequate quantity (1:4) of distilled water to fermented citrus peel and properly mixed; the mixture thus obtained was gently shaken and filtered using a sterile cheese cloth. The resulting supernatant was centrifuged using refrigerated centrifuge (4^o C) at 10,000 rpm for 10 min. and supernatant was again filtered using whatman no. 1 filter paper and carefully transferred to sterile containers and the crude enzyme samples were stored at 4 ^{o}C and used for further studies. Four different pH levels (3.0, 4.0, 5.0, 6.0 and 7.0) of the medium were evaluated for the maximum enzyme production. The pH of substrates was adjusted accordingly with either 0.01 M HCl or 0.01 M NaOH. The temperature range from 20 to 50 ^{0}C was maintained in the BOD incubator throughout the incubation period.

RESULTS AND DISCUSSION

Screening of isolates for pectinolytic activity

Five fungal isolates were selectively isolated by enrichment method using rotten ber fruit. The fungal cultures were primarily screened by disc plate method and the potency index values are furnished in Fig.1.

It was observed that the isolate RBF96 produced the highest potency index (3.25), based on the clear halo zone formation (Fig.2), which was

much higher than the reference strain *Aspergillus niger* MTCC1344 (2.99).

This plate assay screening method, used in the study, has been widely employed for screening of pectinase enzyme [22,] and the results are in agreement with the earlier reports[11]. Different enzymes produced by these isolates namely polygalacturonase (PG), pectin lyase (PL) and pectin esterase (PE) were quantified under submerged fermentation conditions. Results in Fig. 3 show that the highest production of polygalacturonase (189.33 U/ml), pectin lyase (251 U/ml) and pectin esterase (276.67 U/ml) respectively, was observed in the isolate RBF96, which was even greater than those produced by *A. niger* MTCC1344. Similar results have been reported earlier in *Aspergillus* sp [23, 24].

On the basis of these screening experiments, the isolate RBF96 was found to be the most potent isolate and hence was selected for further experiments.

Morphological and cultural characteristics of the fungal isolates for identification

All the five pectinolytic fungal isolates were identified based on colony characters, rate of growth on Czapek yeast-extract (CYA) agar medium (Fig.4) at different incubation temperatures and microscopic features. The details of the results are furnished in Table 1. Micro photograph showing morphological characteristics of pectinolytic fungi RBF96 is shown in fig 5. The most potent isolate RBF96 was identified as *Aspergillus niger*, RBF95 and RBF99 as *Aspergillus fumigatus and* the other two isolates RBF98 and RBF100 as *Aspergillus oryzae*. This identification was done by referring to the Hand Book of Soil Fungi[25]. Pectinase production by *Aspergillus niger*[26], *A. oryzae*[27] and *A. fumigatus*[11] have already been reported.

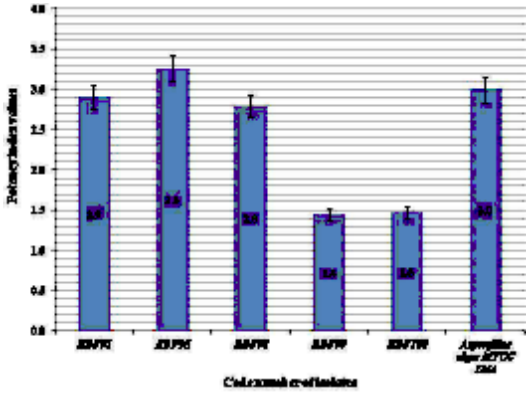

Fig.1. Pectinase potency index of the fungal isolates

Molecular identification of RBF96 by PCR amplification of ITS region

Identification included comparison of their polymerase chain reaction (PCR) amplicons of the ITS1–5.8S–ITS4 ribosomal DNA region, followed by nucleotide sequence analysis. A band revealing ITS rDNA amplification of pectinolytic fungal isolate RBF 96 is shown in (Fig. 6).

The detailed results of partial sequencing of PCR amplified ITS rDNA region are given in Table 2. *In silico* BLAST analysis of the obtained nucleotide sequences (query length 297 bp) in the NCBI Database confirmed RBF96 as *Aspergillus niger* with maximum identity of 100 % to the NCBI GenBank accession number KC119204.1. The molecular approach adopted here is similar to earlier report[28] on the fungal diversity of fresh olive (*Olea europaea* L.) fruits, olive paste (crushed olives) and olive pomace (solid waste). Optimization of culture conditions for pectinase production using citrus peel as substrates for solid state fermentation (SSF)

Effect of moisture content

Moisture content plays a significant role in SSF as it gives a good comparison between water availability and substrate swelling. It is also significant as both oxygen availability and its diffusion depend on this. The results (Fig.7) have clearly indicated that the highest

Fig. 2. Clear halo zone formation by *Aspergillus niger* RBF96 on pectin agar media indicating pectinolytic activity

Table 1. Identification of the pectinase positive fungal isolates based on colony morphology, growth characters and microscopic features

Code number of isolates	Colony characteristics on Czapek Yeast extract Agar (CYA)	growth on Petri plate (90 X 15 mm dia)	Rate of Growth at temp °C			Microscopic features	Probable fungi
			30	40	50		
RBF95	Rapidly spreading, colonies are colorless to dull blue green, reverse - colorless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densly crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	*Aspergillus fumigatus*
RBF96	Moderately growing colonies with carbon black colored conidia; reverse-colorless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, longer conidiophores, phialides biseriate	*Aspergillus niger*
RBF98	Rapidly spreading, colonies are olive yellow to old gold , reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 10 days	+	+	_	Conidial heads radiate, conidiophores erect, thin walled and long, vesicles sub globose, phialides uniseriate in smaller heads, conidia elliptical, sclerotia dark coloured.	*Aspergillus oryzae*
RBF99	Rapidly spreading, colonies are colorless to yellow, reverse - colorless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densly crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	*Aspergillus fumigatus*
RBF100	Rapidly spreading, colonies are olive yellow to old gold and finally shades of yellow brown, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 10 days	+	+	_	Conidial heads radiate, conidiophores erect, thin walled and typically long, vesicles sub globose, phialides uniseriate in smaller heads, conidia elliptical, sclerotia often produced and dark coloured.	*Aspergillus oryzae*

Table 2. *In silico* BLAST analysis of potent pectinolytic fungal isolate ITS rDNA sequences for molecular characterization

Isolate desig--nation	Matching GenBank Accession number	NCBI BLAST search description for the sequences producing significant alignments	Author and year of submission	Maximum score	Query cover	E value	Maximum Identity	Sequence identification
RBF96	KC119204.1	*Aspergillus niger* strain KAML02, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Amutha and Lavanya (2012)	549	100 %	5e-153	100 %	*Aspergillus niger*

ITS1 (Internal transcribed spacer 1) rDNA partial sequence of the pectinolytic fungal isolate RBF96 is given below
ATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCC
CGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAG
CGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGG
ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

polygalacturonase (733.88 U/gram per dry weight of substrate), pectin lyase (308.67U/gds) and pectin esterase (431.67 U/gds) activities were produced at 65 % moisture level.

It was observed that as the moisture level was increased, there was a drastic decrease in all the three enzyme production. The reason being,

Fig.3. Quantitative determination of pectinase activity by different fungal isolates under submerged fermentation

Fig. 4. *Aspergillus niger* RBF96 growth on Czapek Yeast Extract Agar (CYA) media

Fig. 5. Micro photograph showing morphological characteristics of pectinolytic fungi RBF96

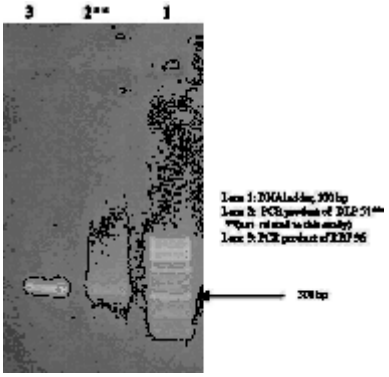

Fig. 6. Bands revealing ITS rDNA amplification of pectinolytic fungal isolate RBF 96

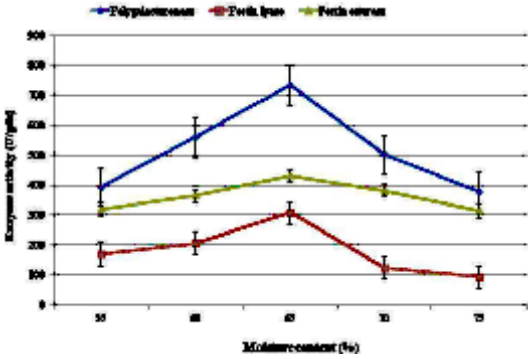

Fig.7. Effect of moisture content in citrus peel on pectinase production by *Aspergillus niger* RBF 96 under solid state fermentation

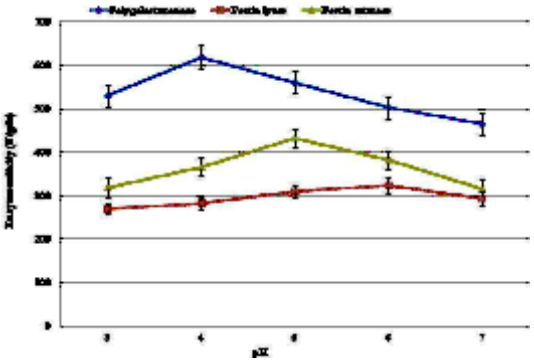

Fig.8. Effect of pH on pectinase production by *Aspergillus niger* RBF 96 using citrus peel under solid state fermentation

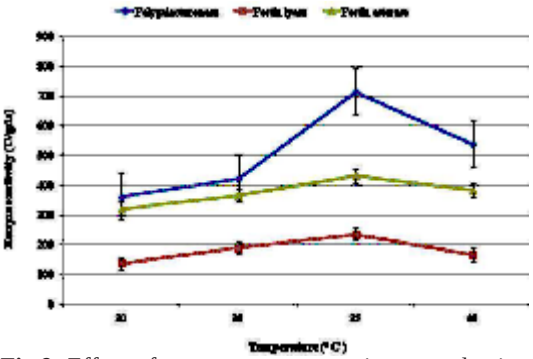

Fig.9. Effect of temperature on pectinase production by *Aspergillus niger* RBF 96 using citrus peel under solid state fermentation

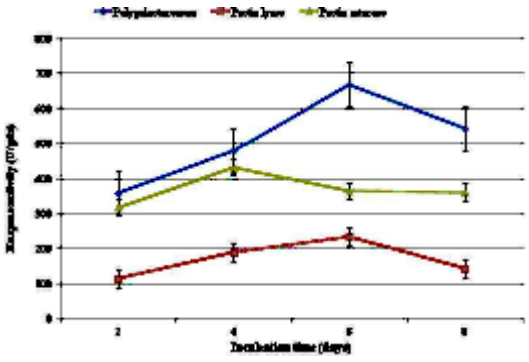

Fig.10. Effect of incubation time on pectinase production by *Aspergillus niger* RBF 96 using citrus peel under solid state fermentation

the high moisture percentage resulting in low substrate porosity which, in turn, prevented oxygen penetration, whereas low moisture content may lead to poor accessibility of nutrients resulting in hampered microbial growth, these findings are similar to the earlier reports [29,30,31].

Effect of pH

The pH of a medium is extremely important if optimal productivity is to be achieved. The pH of a medium influences growth rate in a manner similar to its influence on enzyme activity. The initial pH of the growth medium was varied from 3.0 to 7.0 for finding out the optimum pH for maximum pectinase activity. Results (Fig.8) have shown that the maximal activity of polygalacturonase (618.28 U/gds) was at pH 4.0, while that of pectin esterase (431.67 U/gds) and Pectin lyase (308.67U/gds) at 5.0 and 6.0 respectively.

In general, as the pH of the growth medium was increased, the enzyme production got decreased. Similarly, in an earlier studies also, pH 4.0, 5.0 6.0 was found to be optimum for extracellular PG, PE and PL activities in *Aspergillus niger* URM4645 under SSF using forage palm[32] as the

substrate. *Aspergillus niger* DMF45 under SSF using deseeded sunflower head[24].

Effect of temperature

Aspergillus niger RBF96 was cultured under various temperature conditions. Data shown in Fig. 9 indicate that the maximal activity of polygalacturonase (712.47 U/gds), pectin lyase (233.43/gds) and pectin esterase (431.67 U/gds) were observed at 35 ^{o}C. As the temperature was increased, drastic decrease in all the three enzyme production was noticed. Cell growth varies as a function of temperature.

The actual temperature at which a particular organism grows depends on its psychrophilic, mesophilic or thermophillic nature. Within the growth range, growth rate increases with increasing temperature until a maximum is reached above which it falls of rapidly due to increase in microbial death. Heat generation due to metabolic activities of the microorganisms, at higher temperature is often fatal because, a large part of the enzymes produced during the fermentation may be heat denatured at the end of the process[29, 33].

Effect of incubation time

For testing the optimum incubation time for maximum pectinase production, the fermentation was carried out upto 8 days and the enzyme activities were examined every two days and the results are furnished in Fig. 10. Maximal activities of polygalacturonase (667.96 U/gds) and pectin lyase (234.1 U/gds) were observed on 6th day after incubation, while pectin esterase (431.67 U/gds) activity was on 4th day after incubation. These results are in agreement with the earlier reports[23, 24, 26]. Growth of fungi in any medium varies according to physical and chemical environmental factors. Producer strains need to have shorter fermentation cycles as this would be cost effective. Cell doubling increases with increasing organic complexity.

CONCLUSION

The native fungus *Aspergillus niger* RBF96, isolated from rotten ber fruit proved to be an efficient pectinase producer. Under SSF conditions, it produced maximum activities of different types of pectinase enzyme on citrus peel, which, if not disposed off properly, could cause environmental problems. Hence, large scale production of pectinase using citrus peel can be exploited, after corroboration with field scale optimization.

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