

Preliminary Study on Morphological Diversity of *Aspergillus niger* Strains Grown on Various Agri - waste in Relation to Enzyme Production

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Abstract

The present investigation was aimed to observe the correlation of morphology of various isolates of *Aspergillus niger* on the production of Polygalacturonase (PG) and Pectin Methyl Esterase (PME) with the concept to develop a screening procedure for high enzyme producing strains of *Aspergillus niger* grown on agri waste. The strains were isolated from orange peel, tamarind seed powder, groundnut, carrot, pomegranate peel and apple baggasse, soil, waste water and bread. The maximum enzyme activity (PG: $2.20 \pm 0.05 \mu\text{moles/ml/min}$ & PME: $0.73 \pm 0.08 \mu\text{moles/ml/min}$) was observed with the smallest spore size of *Aspergillus niger* obtained from orange peel ($5.49 \pm 2.56 \mu\text{m}$). However, no correlation of conidiophore size, height of conidial stalk on pectinase production was noted. There is no report found on the level of pectinases in strain of *Aspergillus niger* grown on tamarind seed powder which showed considerable amount of pectinases in present study.

Keywords: Polygalacturonase; Pectin methyl esterase; *Aspergillus niger*; Morphological diversity

Introduction

Pectins are high molecular weight acid polysaccharides, primarily made up of α -(1-4) linked D-galacturonic acid residues with a small number of rhamnose residues in the main chain and arabinose, galactose and xylose on its side chain [1]. Pectinase is a generic name for a family of enzymes that catalyse hydrolysis of the glycosidic bonds in the pectic polymers [21]. Pectinases include polygalacturonase (EC3.2.1.15). Pectinase is used in the extraction, clarification, filtration, and depectinization of fruit juices [10] and wines, for the maceration of fruits and vegetables, removal of the inner wall of lotus seed, garlic, almond, and peanut [1]. Pectinase plays the role of degrading pectinic substances. An important consideration for the food processing industry is the cost efficiency of pectinase production [13]. Filamentous fungi are important in industrial enzyme production, since they are able to synthesize and secrete large amounts of extra cellular proteins. The importance of morphological and physiological studies on fungi in liquid cultures has been recently reviewed [5] Fungal macro- and micro-morphology affect the rheology of the fermentation medium, thereby having a significant impact on the mixing, mass transfer and aeration processes within the bioreactor. In addition, micro-morphology may influence metabolite productivity, which may lead to lower net specific growth rate [8] or to enhanced enzyme production by strains with altered morphology [11] However, few criteria about fungal physiology and morphology in solid-state cultures have been established in the limited studies available. For instance, working with *Neurospora crassa* in solid culture defined the hyphal growth unit (total hyphal length/number of hyphal tips) as a measurement of the fungal invasive capacity [24]. In contrast, the colony radial extension rate obtained by linear regression of colony diameter versus time [7]. Black *Aspergillus*, which comprises of species belonging to *Aspergillus* section *Nigri*, are worldwide distributed and have a significant impact on modern society. Many species cause food spoilage, and several are used in the fermentation industry [6]. *A. niger* has even been granted the GRAS (Generally Regarded As Safe) status in certain industrial production processes by the Food and Drug Administration of the US government [18]. Various reports evidenced that members of the *A. niger* species complex, together with *A. carbonarius* and *A. japonicus*/

aculeatus are frequently responsible for post-harvest decay of fresh fruit (apples, pears, peaches, citrus, grapes, figs, strawberries, tomatoes, melons, etc.) and some vegetables (especially onions, garlic, and yams) [20]. Studies suggest that Black *Aspergilli* are one of the most difficult groups concerning classification and identification [3]. The taxonomy of *Aspergillus* section *Nigri* has been studied by many taxonomists, leading to various species concepts. The difficulties in species recognition within the *Aspergillus niger* "aggregate" and the fact that most of the studies carried out on black *Aspergilli* occurring on grapes lack molecular characterization of the strains perplexed the extent of their natural occurrence and species distribution on grapes and food [3]. Studies on the biodiversity of toxigenic *Aspergillus* species is useful to clarify molecular, ecological and biochemical characteristics of the different species in relation to their different adaptation to environmental and geographical conditions, and to their potential toxigenicity [19]. *A. niger* is also an important model organism for several important research areas including the study of eukaryotic protein secretion in general, the effects of various environmental factors on suppressing or triggering the export of various biomass degrading enzymes, molecular mechanisms critical to fermentation process development, and mechanisms involved in the control of fungal morphology [17,18]. The genetic diversity within *A. flavus* populations has been widely studied in relation to their potential aflatoxigenicity and morphological variants L- and S-strains. Within *A. flavus* and other *Aspergillus* species capable of aflatoxin production, considerable diversity is found.

In present study effort has been made to determine possible

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relationship between morphological diversity of *Aspergillus niger* and production of Pectinases, keeping in view to develop simple methods for identification of maximum enzyme producing strains.

Material and Methods

The present investigation was started in the month of December 2009 at Department of Biotechnology, Lovely School of Sciences, Lovely Professional University, Phagwara, Punjab, India and accomplished in July 2010.

Isolation of microorganism

Aspergillus niger was isolated from various sources such as orange peel, tamarind seed powder, apple baggasse, pomegranate, carrot, groundnut, soil, waste water and bread. These samples were especially collected from the rotten fruits, grocery market of Jalandhar, Phagwara and campus of Lovely Professional University, Punjab.

Sources and Culture maintenance of microorganism for enzyme production: Nine solid substrates were used in this study such as orange peel, pomegranate peel, tamarind seed powder, carrot, groundnut, soil, waste water, apple baggasse and bread. These cultures were maintained and sub cultured under aseptic conditions on Potato Dextrose Agar (PDA) slants and stored at 4°C in the laboratory of Department of Biotechnology, Lovely professional University Phagwara, Punjab, India. The fungal cultures were grown on PDA at 30°C for 5 days. A spore inoculum was prepared by adding a loopful of spores from the slants to 5 ml (1x10⁵ cfu/ml) of sterile normal saline and shaken vigorously. 0.1 ml (v/v) of the inoculum was used to inoculate fermentation medium. The substrate medium was prepared by adding the soluble pectin at the rate of 1% in the mineral solution and 30 ml of it was taken in each 100 ml Erlenmeyer flask. The cotton plugged flasks were autoclaved at 121°C for 20 min, cooled to room temperature and inoculated with 0.1 ml of inoculum, mixed thoroughly by gently tapping the flasks on the palm of the hand and were incubated at 30°C for different incubation periods. After completion of the incubation period, the contents of the flasks were filtered through a perforated funnel and centrifuged to get the clear culture filtrate. The filtrate was employed for the estimation of the activities of pectinases.

Enzyme activity measurements

Polygalacturonase activity: To determine the polygalacturonase activity we measured the amount of reducing sugars such as galacturonic acid released from non esterified pectin, hydrolysed in the reaction mixture [16]. The reaction mixture consists of 2 ml of 1% buffered solution of poly- galacturonate in phosphate citrate buffer (pH 5.5). Further, 0.5ml of enzyme prepared was incubated at 30°C and samples were withdrawn at 0 and 30 min. The amount of reducing sugars was estimated using -Somoygi [14] method. The enzyme activity was expressed in micromoles of the reducing sugars liberated/min/ml of enzyme prepared.

Pectin methyl esterase activity: To determine the pectin methyl esterase activity we measured the increase in acidity after the hydrolysis of methyl esters of pectin from the enzymes prepared. 5ml of buffered pectin substrate was incubated with 1ml of enzyme and 2ml of samples withdrawn at 0 and 60min were titrated against 0.01N sodium hydroxide to determine the amount of acid released. The enzyme activity was expressed as the micro-equivalents of acid released/min/ml of enzyme preparation.

The enzyme activities are presented as mean ±SE of all the values.

The results of enzyme production were analysed statistically using student's t- test.

Microscopy

Morphology of fungal hyphae and conidia was assessed by direct observation of fresh mycelial samples and spore size was recorded using micrometer. The production of sporulation structures was assessed by direct observation of fresh mycelial samples.

Results

The study was conducted with nine substrates including orange peel, pomegranate, tamarind seed powder, carrot, groundnut, soil, waste water, apple baggasse and bread. The strong effect of spore size on the production of PG was clearly observed (Table 1 and 2, Figure 1). The present investigation revealed highest amount of PG (2.29±0.05) and PME (0.73±0.08) production on orange peel amongst the nine agro waste sources by *Aspergillus niger* with smallest spore size (5.49±2.56µm) (Table 1, Figure 1, Figure 2c and 3c). Isolated colonies of strains from orange peel were observed with 4±0.7cm conidiophore size and 8±0.3cm height of conidial stalk (Figure 2c and 3c). However the maximum height of conidial stalk (11±0.9cm) was observed in case of pomegranate with the conidiophore size of 5±0.5cm., showing isolated colonies. The spore size 6.25±2.86µm was comparatively more than the strain grown on orange peel hence showed subsequent reduction in the level of PG and PME (1.18±0.04; 0.38±0.02µmoles/ml/min) (Table 1 & 2, Figure 1). Highest amount of PG and PME activity of orange

S.No.	Isolates	Spore Size(µm)	PG (µmoles/min/ml)	PME (µmoles/min/ml)
1.	Orange peel	5.49±2.56	2.29±0.05	0.73±0.08
2.	Tamarind seed powder	5.65±2.93	1.76±0.03	0.37±0.05
3.	Groundnut	5.80±2.10	0.95±0.06	0.44±0.04
4.	Carrot	6.12±3.16	1.65±0.20	0.36±0.09
5.	Pomegranate peel	6.25±2.86	1.18±0.04	0.38±0.02
6.	Apple baggasse	8.45±2.90	0.62±0.07	0.16±0.03
7.	Soil	5.53±2.50	2.19±0.06	0.11±0.06
8.	Bread	7.00±1.9	0.76±0.06	0.24±0.06
9.	Waste water	8.50±2.43	0.62±0.03	0.18±0.05

Table 1: Quantitative estimation of pectinase production in relation to its spore size by *Aspergillus niger*.

Sr. No.	Source	Colony formation	Conidiophores size	Height of conidial stalk
1	Apple baggasse	Dense and crumpled	4±0.4cm	10±0.7cm
2	Pomegranate peel	Isolated and not crumpled	5±0.5cm	11±0.9cm
3	Orange peel	Isolated and not crumpled	4±0.7cm	8±0.3cm
4	Tamarind seed powder	Isolated and crumpled	1.5±0.3cm	5±0.3cm
5	Carrot	Isolated and slightly crumpled	2±0.8cm	6±0.6cm
6	Groundnut	A few Isolated and crumpled	3±0.3cm	8±0.7cm
7	Soil sample	Small and Dense	1.8±0.3cm	7±0.3cm
8	Waste water	Isolated and slightly crumpled	1.6±0.2cm	7±0.7cm
9	Bread	Small and Dense	1.7±0.2cm	3±0.4cm

Table 2: Qualitative estimation of colony, conidiophores size and their height of *Azospirillum niger*

peel, was followed by PG and PME activity of tamarind seed powder ($1.76 \pm 0.03 \mu\text{moles/ml/min}$, $0.37 \pm 0.05 \mu\text{moles/ml/min}$) and soil sample ($2.19 \pm 0.06 \mu\text{moles/ml/min}$, $0.11 \pm 0.06 \mu\text{moles/ml/min}$) with spore size of 5.65 ± 2.93 and $5.53 \pm 2.50 \mu\text{m}$ respectively. Least value of PME was observed in case of soil sample. The size of conidiophores of soil strain ($1.8 \pm 0.3 \text{ cm}$) was comparatively less than orange peel grown strain ($4 \pm 0.7 \text{ cm}$). The spread of colonies was isolated and crumpled in the strains grown on tamarind seed ($1.5 \pm 0.3 \text{ cm}$, $5 \pm 0.3 \text{ cm}$) and groundnut ($3 \pm 0.3 \text{ cm}$, $8 \pm 0.7 \text{ cm}$) with large variation in size of conidiophores and height of conidial stalk (Figure 2 and 3). The pattern of colonies of strains on apple baggasse and waste water was dense and isolated respectively. However, level of pectinases and other parameters studied were near in both types of strains (Table 1 & 2, Figure 1) indicated minimum level of PG and maximum spore size in case of waste water grown strain ($8.50 \pm 2.43 \mu\text{m}$). The strain grown on carrot showed isolated and slightly crumpled colonies with the dimension of $2 \pm 0.8 \text{ cm}$ of conidiophores and $6 \pm 0.6 \text{ cm}$ height of conidial stalk. The spore size was $6.12 \pm 3.16 \mu\text{m}$ with PG and PME activity measured as 1.65 ± 0.20 and $0.36 \pm 0.09 \mu\text{moles/ml/min}$ (Table 1, Figure 1).

Discussion

Microbial production of pectinases has been extensively studied [23,5] in actinomycetes [12] *Aspergillus flavus* and *Aspergillus sp.* [22]. The morphological form of the cultivated fungus is an important factor in the production of pectinases. In submerged culture the morphology of filamentous microorganisms varies between two extreme forms, pellets and free filaments depending on culture conditions and the genotype of the strain. According to many reports, mycelial morphology is crucial to the process of fermentation, not only in relation to the shape of the hyphae themselves and the aggregation into microscopic clumps (micro-morphology), but also in the pelleted form of growth (macro-morphology). Studies revealed, free mycelia are required for increased productivity as the production of penicillin from *Penicillium chrysogenum* [9] Regarding pellet morphologies as a prerequisite for increased productivities, methodologies for pellet production for several microorganisms have been suggested [1]. In the present study PG activity was found reduced with the increase in spore size (Table 1, Figure 1) that might be attributed to less availability of substrate and type of carbon source. However, the mechanism by which the spore morphology acts on the production of pectic enzyme is not known. On the other hand PME activity was more or less similar with increase

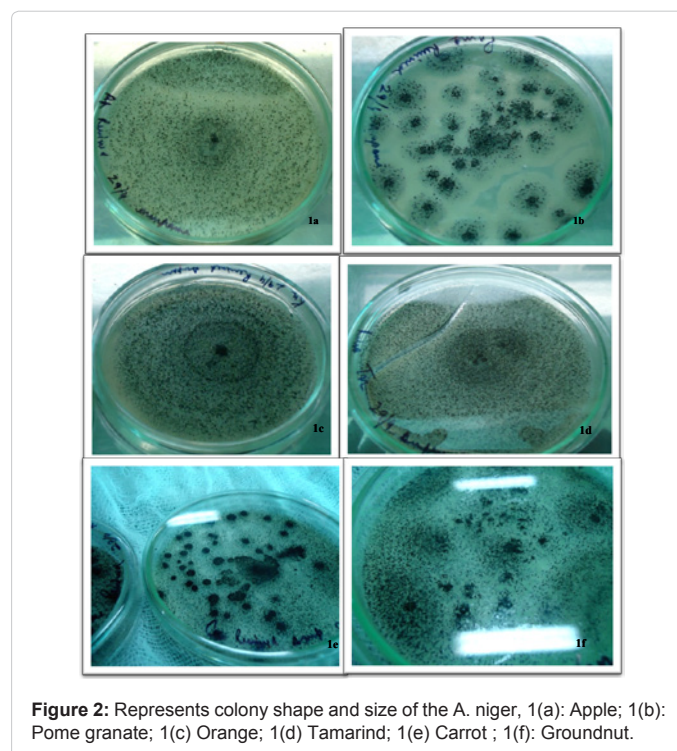


Figure 2: Represents colony shape and size of the *A. niger*, 1(a): Apple; 1(b): Pomegranate; 1(c) Orange; 1(d) Tamarind; 1(e) Carrot; 1(f): Groundnut.

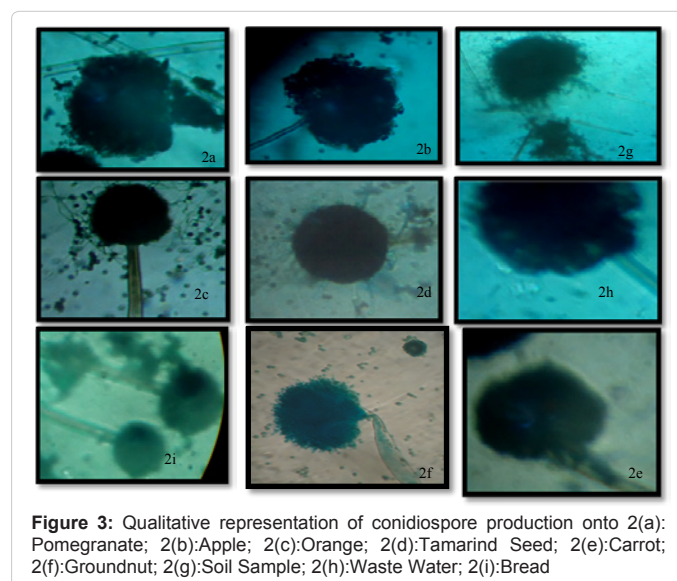


Figure 3: Qualitative representation of conidiospore production onto 2(a): Pomegranate; 2(b): Apple; 2(c): Orange; 2(d): Tamarind Seed; 2(e): Carrot; 2(f): Groundnut; 2(g): Soil Sample; 2(h): Waste Water; 2(i): Bread

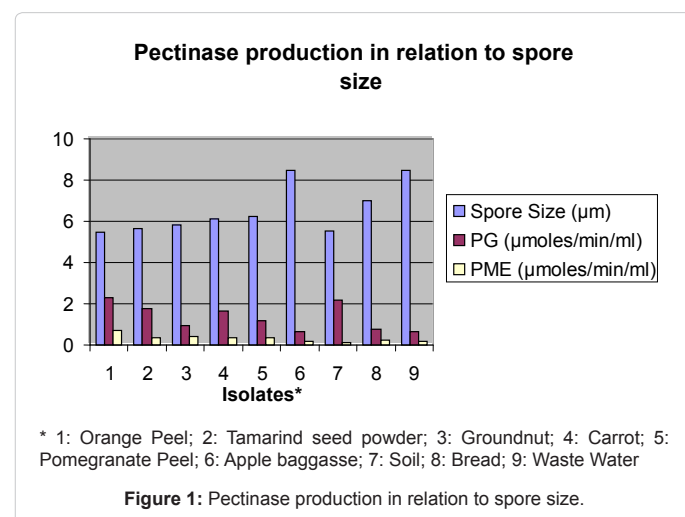


Figure 1: Pectinase production in relation to spore size.

in spore size (Table 1, Figure 1) and there is no such correlation of PME production with the spore morphology. The colonies were almost small and dense in soil and bread isolates. Reports on the preferred morphology are often contradictory since each one of the two extreme forms such as pellets and filaments have their own characteristics concerning cell physiology, growth kinetics, nutrient consumption and broth rheology [16] As per study, drawback of pelleted suspensions is that cell growth- and consequent metabolic activity-occurs at the surface only, where contact with oxygen and medium nutrients is adequate, while the cells inside the pellets respond to a very different environment. Further into pellets, mass transfer limitation gradually occurs and cells become subjected to autolysis [15]. Only a fraction

of the mycelium takes part in biosynthesis of metabolites when this requires for example elevated dissolved oxygen concentrations. Pellet formation results in striking effects on polygalacturonidase production by *A. niger* and its synthesis correlate well with the particular morphological type. The more compact the pellet, the greater the enzyme synthesis. Regardless the medium used, an increase of almost two orders of magnitude in the polygalacturonidase concentration and production rates between the diffuse mycelium and pellets was observed. Similar increases were observed in α -galactosidase synthesis by mycelial pellets of *Mortierella vinacea* and nikkomycin production by *Streptomyces tendae* [4]. Such phenomena were related to diffusion limitations in pellets, which either reduce the extent of catabolic repression in pellets or limit the oxygen supply preventing thus an oxidative inactivation of specific sets of enzymes, as well as to the existence of additional factors, such as gradients of metabolic products in pellets serving as biological signals (modulators). The colony formed by the strains from tamarind seed powder, groundnut, carrot and waste water was isolated and slightly crumpled with slight variation in size of conidiophores and height of conidial stalk. The increase in size of spores caused depletion in PG and PME except groundnut where the spore size did not affect much on the concentration of pectinases. It appears that the enzyme synthesis related to mycelium structure is independent of the mechanism which regulates the level of pectinase synthesis within a specific morphological structure. Altogether, the relationship between physiology, morphology and enzyme production, if any, is poorly understood.

Conclusion

Our results using *A. niger* from various agro wastes, showed enhanced enzyme production and somewhat linear relationship between enzyme production (Pectinases) and morphology (spore size). However, there is a scope for further study on optimization of the conditions for increased production of pectinases from agrowastes and development of markers for preliminary screening of strains containing significant amount of pectinases.

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