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Strain Improvement through Mutation to Enhance Pectinase Yield from *Aspergillus niger* and Molecular Characterization Of *Polygalactouronase* Gene

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Abstract

Strain improvement is an important tool in commercial development of microbial fermentation processes for hyper production of enzymes. *Aspergillus niger* was isolated and identified as best pectinase producer through submerged fermentation and the same culture was subjected to mutate through physical (UV irradiation at 254 nm) and chemical (ethidium bromide, ethyl methyl sulphonate and sodium azide) mutation for enhancement of pectic acid-degrading enzymes (pectinase). Mutated *A.niger*UV radiation exposure for 60 mins yields 260 nkat of pectinase activity when compared with wild strain yielded 235.06nkat. A mutant of *A.niger* showed higher enzyme activity on treatment with ethyl methyl sulphonate (2mg/ml for 60 mins) followed ethidium bromide (6mg/ml for 60mins) treatment found to be 265 nkat and 340 nkat of pectinase activity respectively. The pectinase producer which is susceptive to sodium azide (0.1% for 30min) yields 180 nKat pectinase activity. The combined UV and EtBr treatment (60min, 6mg/ml) yielded mutant with 1.69 fold enhanced polygalacturonase production compared with wild strain. The structure of poly galactouronase gene (*pga*I) was investigated and the amino acid sequence encoding enzyme was found to have 99% similarity with other *A.niger* isolates.

Key words: *Aspergillus niger*, mutation, ethidium bromide, sodium azide, *pga*I gene sequencing.

INTRODUCTION

For commercial production of enzyme, filamentous fungi are more commonly employed than yeast and bacteria [1]. Saprophytic and plant pathogenic fungi produces various enzymes for degrading plant cell wall component, which majorly comprises pectinase, the enzyme involved in the degradation of pectin. Pectinase are the group of enzymes involved in Pectin degradation; a polysaccharide substrate found in the cell wall and middle lamella of plants [2]. Pectins are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules that are present in the Plant kingdom. Most pectic enzyme are used in the fruit processing industry. Pectic enzymes alone account for about one quarter of the world's food enzyme production [3]. The most common application of pectinase is in the food industry to extract and clarify fruit and beverages [4,5]. Pectinases are also used in industrial processes, such as in ramie fiber degumming, oil extraction, coffee and tea fermentation, and industrial wastewater treatment [6,7].

Production of Pectinase has been largely focused in *Aspergillus niger*[8]. *A.niger* is a work horse of industrial microbiology that has been subjected to different types of mutagenesis for enhanced production of pectinase. Enzymes produced from the fungi *Aspergillus*, are generally regarded as safe (GRAS) [9]. Various fruit and vegetable processing waste have been employed for the production of pectinases [10]. Due to the fact that, agricultural residues are attractive due to its low cost and abundant availability, the ability of *A. niger* isolates are tested for the production of polygalacturonase by the utilization of citrus fruit peel as substrate. Strain mutation, induction and screening techniques can improve microbes for pectinase production by using different mutagens such as nitrous acid, diethyl sulfate (DES), and ethyl methyl sulfonate. When fungi are grown with mutagens at sub-lethal concentrations, the rate of enzyme production often increases. The treatment of spores and vegetative mycelium is recognized as an effective means for generating mutants [11,12]. Exposure of fungal cells to UV have increased pectinase yield [13]. Polygalacturonase (PGA) is one of the major members of pectinases which cleaves α-1,4-glycosidic of D-galacturonic acid in pectin and it is classified into endo- and exo-polygalacturonase on the basis of the way of eliminating galacturonic acid. Seven different *pga* genes present have been already characterized [14,15]

In this paper, various mutational studies are reported with an aim to improve the enzymatic yield and molecular characterization of *pgaI* gene from soil fungi *Aspergillus niger*. Its Nucleic acid and amino acid homology are studied through sequence alignment with existing data base of *A.niger*.

MATERIALS AND METHODS

Microorganism

The high yield pectinase producer *Aspergillus niger* culture was isolated from soil collected from Porur vegetable market) and was maintained at 4°C on potato dextrose agar slants (HiMedia, India).

Fermentation media

Submerged fermentation medium of 100 ml was prepared with Wheat bran 20% and Orange peel 10%, pH 5.5 (Adjusted using acetic acid) and sterilized at 15 psi (121°C). The spore suspension (10^7 spores.ml^{-1}) was inoculated and incubated at RT for 8 days under 200 rpm agitation. The culture medium (5 ml) was centrifuged (10,000 g, 10 min, at 30°C) and supernatant was collected for further analysis.

Mutation by physical agents

UV mutagenesis

The cell suspensions of different dilutions were prepared 10^{-6},10^{-7},10^{-8}. The induction of mutation was carried out by exposure to UV germicidal lamp (256 nm) kept at a distance of 50 cm for time intervals ranging from 10–90min, [16,17]. After irradiation, the plates were wrapped in aluminum foil and were kept in dark. 0.1ml of cell suspension was inoculated into Potato dextrose agar plates. About 45 single cell colonies were isolated from those plates and screened for pectinase activity. The fungal isolate which has maximum zone of clearance (UV9) was inoculated in production medium and incubated at RT for 7 days.

Mutation through chemical agents

Ethidium bromide (Etbr), Ethylmethylsulphonate (EMS), Sodium azide mutagenesis

The mutant fungal strains were developed by taking 2 ml of cell suspension in 6 test tubes and one was marked as control and kept aside. The remaining tubes were incubated with 2-10 mg/ml of EtBr for 60 min at RT. The cells were centrifuged at 3000 rpm for 10 min and washed with sterile phosphate buffer pH (7.0). 0.1ml of washed cells was spread on the potato dextrose agar (PDA) plates and incubated for about 48hrs. The single cell colony was screened for pectinase activity. The maximum pectinase producer was identified and its spore suspension (10^7 spores.ml^{-1}) was inoculated in production medium. The above procedure was followed to obtain with EMS (2-10mg/ml for 60 min)[18,19] and Sodium azide (0.1%) with varying incubation time (10-60min)

Combination of physical and chemical mutagenesis

The spore suspension containing (10^7 spores.ml^{-1}) colony forming units (cfu)/ml was spread on the potato dextrose agar plates. The single cell colony was isolated and inoculated in 2ml PDB and incubated overnight at 30 °C. The induction of mutation was carried out by exposure to UV germicidal lamp (256 nm) kept at a distance of 50 cm for time interval of 60 min followed by the mutation with EMS (2mg/ml for 10 min). The treated cells were washed to remove excess mutagen and 0.1ml of cell suspension was spread plated on PDA. The single cell colonies were then inoculated in PDB and the spore suspension containing (10^7 spores.ml^{-1}) was inoculated in production medium. The induction of UV mutation was carried out first and followed by the mutation with EtBr (6mg/ml for 10min) and with Sodium azide (0.1% for 10min).

Determination of pectinolytic activity

The quantitative assay for pectinase was made possible using Citrus pectin (0.1%) as the substrate through Di-nitrosalicylic acid method (DNS method) [20]. The tubes containing 1 ml of substrate were incubated at 37 ºC for 3 min followed by addition of 1 ml of enzyme extract. The mixture was incubated for 15 min at 37 ºC. 1 ml of DNS reagent and added into the tubes and seethed for 10 min and cooled immediately by adding 3ml of Distilled H_2O. The reaction mixture was centrifuged at 10,000 rpm for 5 min. The absorbance was read at 540 nm using a UV/Vis spectrophotometer (UNICO). One unit of the pectinolytic enzyme was defined as the amount of enzyme that catalysed the formation of 1µmol galacturonic acid under the assay conditions.

Isolation of RNA

The RNA was isolated by using MACHEREY- NAGEL Nucleospin RNA extraction kit

The overnight frozen cells from deep freezer were taken for RNA isolation.350µl of buffer RA1, 3.5 µl β- mercaptoethanol was added to cells and vortexed vigorously to lyse the cells. The clear lysate was filtered through the filter, placed in the collection tube (2ml) and the mixture was added and centrifuged to 1min at 11,000xg. Then the filtrate was transferred to the new 1.5ml micro centrifuge tube and 350µl ethanol (70%) was added to the homogenate lysate and mixed by vortexing. Then the lysate was added to the column and centrifuged for 30s at 11,000Xg. The column was placed in the new tube and 350µl of membrane desalting buffer added in the column and centrifuged at 11,000xg for 1min to dry the membrane. 95µl of DNase was applied to the reaction mixture directly onto the center of the silica membrane of the column. The tube was incubated at room temperature for 15min. 200µl of RAW2 buffer was added to the column and centrifuged 30s at 11,000xg.The column was transferred to the new collection tube.600µl of RA3 buffer was added to the column and centrifuged for 30s at 11,000xg. The flow through was discarded and the column was placed in the tube.250µl of RA3 buffer was added to the column and centrifuged for 2min at 11,000 xg to dry the membrane completely. The column was placed into nuclease free collection tube and RNA was eluted with 60µl of RNase- free water and centrifuged at 11,000xg for 1min. The RNA was confirmed by Formaldehyde agarose gel.

Synthesis of cDNA Synthesis

cDNA was prepared using total RNA (Transcriptor first strand cDNA synthesis, Roche, Switzerland). The template- primer mixture (20µl) which contains total RNA 8µl; Oligo dT 1µl; Water 4µl; RT reaction buffer 4µl; Protector RNase inhibitor 0.5µl; Deoxynuclotide mix 10mM 2µl; Reverse Transcriptase 0.5 µl was prepared. The template primer mixture was denatured by heating the tube for 10min at 65˚C in the thermal block cycler with a heated lid (to minimize evaporation). The tube was cooled with ice and the remaining components were added to the mix. After adding all components, the reagents were mixed in the tube gently and incubated the RT reaction for 60min at 50˚C. Reverse transcriptase activity was inactivated by heating to 85˚C for 5 min. The reaction was terminated by placing the tube on ice. The PCR condition was maintained at initial denaturation (94ºC for 5 minutes), annealing (55ºC for 45 Secs), extension (72ºC for 1min) and final extension (72ºC for 5mins).

RESULT AND DISCUSSION

Obtaining enriched strain by physical mutagen (UV) was the choice of study. When deoxyribonucleic acid (DNA) is exposed to UV light (254nm), the most frequent DNA damage or lesions, results at dimers of any two adjacent pyrimidine bases. UV radiation is a potent mutagen [21] and is found to be best for the improvement of strains like *Aspergillus niger* for maximum production of various enzymes [22]. The results of this study reveal increased yield from mutant (UV9) compared to control strain. Figure 1 describes the pectinase production of mutated strain when exposed to various time intervals 10-90min (uv rays 254nm). The survivability of fungal cells are also depicted (Figure-2a, 2b). These findings are in accordance with the results reported [23,24]. Wild strain produces enzyme activity of 235.06nkat while the mutagenised strain yields 260nkat when exposed to UV for 60 min. Among the 25 mutant isolated UV9 is validated to be the superior strain.There is an increased interest in utilization of this fungi for the production of acid pectinases which has wide application in the fruit processing industry.

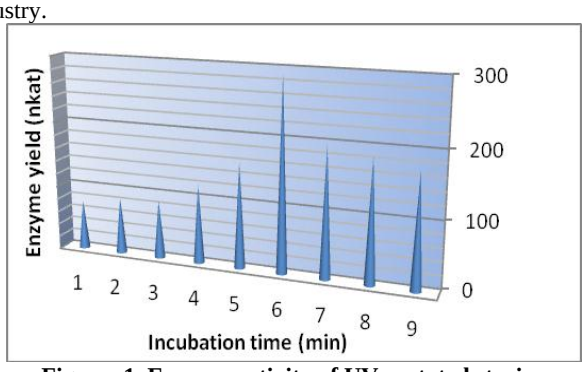

Figure- 1 Enzyme activity of UV mutated strains

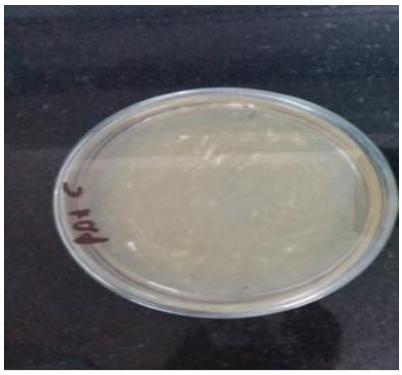

Figure- 2a Control plate

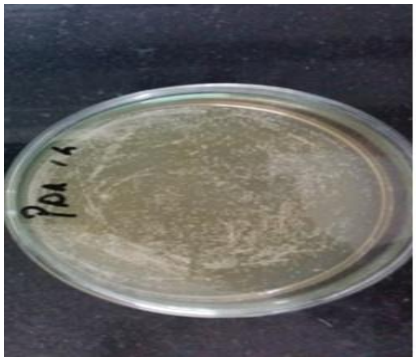

Figure- 2b UV mutation (60 min)

Enzyme production by mutant, treated with chemicals (EMS and EtBr) is interpreted (figure-3). EMS is an alkylating agent that induces point mutations by A-T transition to G-C. [25].*A.niger*is incubated in EMS of varying concentration (2-10 mg/ml for 60 min). Among 20 different mutants screened, the enzyme activity of EMS12 is found to be 265nkat, which is higher than the control which yields about 235nkat. Minjares-Carranco et al., [26] have already reported that this mutagen can be used to increase the activity of pectinase. Among 25 different mutant analyzed EtBr8 (6mg/ml for 60 mins) yields 340 nkat of pectinase, which is 1.44 times higher than the wild strain. The mutant is found to be stable even after many generations. This is the first report ensuring that EtBr acts as a positive mutagen which increases the pectinase yield of 1.44 times in *A.niger*.

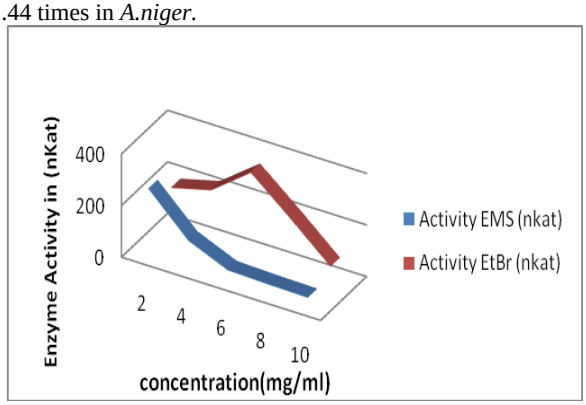

Figure- 3 Effect of EMS and EtBr mutation in enzyme activity of *A.niger*

The effect of Sodium azide (0.1%) treatment with different incubation time (10-60 mins) on the Pectinase producer is shown in Figure-4. The enzyme activity of the mutant (SA6) is significant and calculated to be 180nkat when incubated at 0.1% (30 min). But yield from SA6 is low compared to the original strain (235nkat). Hence, Sodium azide mutation is found to have a negative feedback on pectinase production in the isolated strain.

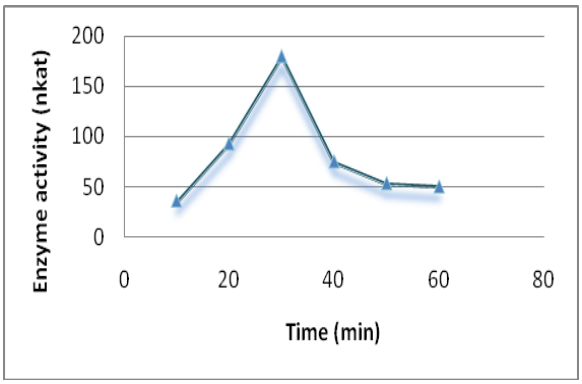

Figure-4 Effect of Sodium azide (SA) mutation

Physical and chemical mutagens are combined to have an effective mutant for increased productivity. Synergistic effects of physical and chemical mutagen have also been reported by other researchers [27,28]. The mutant raised by the combined mutation of UV (60 min) and EtBr (6mg/ml) produces enzyme activity of 400nkat which is about 1.69 fold higher activity compared with the wild strain, Whereas the mutant raised due to the combined effect of UV(60min) and EMS (2mg/ml) and the mutant exposed to UV (60min) and Sodium azide (0.1%) yield 230nkat and 240nkat respectively.

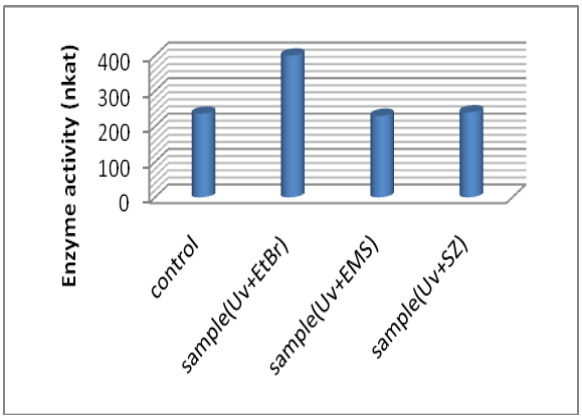

Figure- 5Effect of combined physical and chemical mutation.

In order to isolate *pga*I gene total RNA was extracted using MACHEREY- NAGEL Nucleospin RNA extraction procedure (Figure-6) and cDNA was generated using (Transcriptor first strand cDNA synthesis, Roche, Switzerland). The cDNA of *pgaI gene* wasfound to have1102 bp which encoded a protein of 288 amino acid. The evaluated results of Blast search using the isolated *pga*I gene showed that the length was very similar to that of other reported *A.niger* isolates [29]. Nevertheless, the sequence similarity searches (Figure-7) laid out that *pgaI* sequence of *A.niger* isolate shared 99% homology with polygalactouronase gene of *A.niger* (X58892, AM 269981, XM 001389525) [30]. The results of blast search (Figure-8) using the deduced protein sequence displayed 100% homology with *A.niger* (XP001389562, 1NHCA)

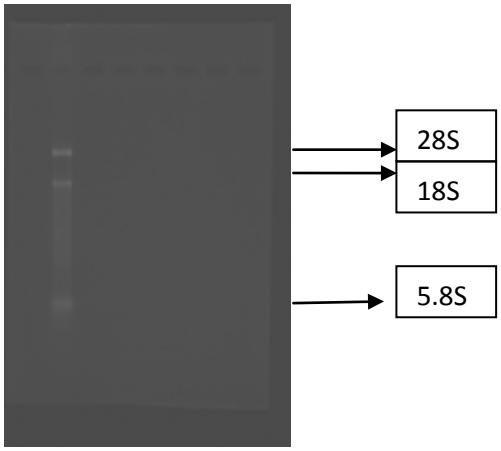

Figure-6 Isolation of total RNA from *A. niger*

Results of Blast search for nucleotide sequence

Table - 7 Nucleotide sequences producing significant alignment

Description	Max score	Total score	Query cover	E value	Ident	Accession
*Aspergillus niger*pgaI gene for polygalacturonase (PGI)	1958	1958	100%	0.0	99%	X58892.1
*Aspergillus niger*contig An01c0340, genomic contig	1953	1953	100%	0.0	99%	AM269981.1
*Aspergillus niger*CBS 513.88 endopolygalacturonase C, mRNA	754	1748	89%	0.0	99%	XM_001389525.1
*Aspergillus niger*strain M1 polygalacturonase (pgaI) mRNA, partial cds	715	1670	89%	0.0	97%	GQ251519.1
Penicillium olsonii pg1 gene for polygalacturonase, exons 1-2	556	556	79%	3e-154	79%	AJ243521.1
Aspergillus oryzae strain PO endo-polygalacturonase (pga) gene, complete cds	536	536	79%	4e-148	78%	KF499033.1
Aspergillus oryzae RIB40 DNA, SC023	536	536	79%	4e-148	78%	AP007157.1
*Aspergillus oryzae*pgaB gene for polygalacturonase B, complete cds	536	536	79%	4e-148	78%	AB007769.1
Aspergillus aculeatus mRNA for polygalacturonase 2 (pg2 gene)	475	942	74%	8e-130	88%	AJ581481.1
Aspergillus flavus NRRL3357 extracellular polygalacturonase, putative, mRNA	399	399	38%	5e-107	84%	XM_002376646.1
Aspergillus oryzae RIB40 endopolygalacturonase C, mRNA	388	388	38%	1e-103	83%	XM_001820901.2
Neosartoryafischeri NRRL 181 extracellular polygalacturonase, putative (NFIA_102450) partial mRNA	298	298	33%	2e-76	81%	XM_001266656.1
Aspergillus niger strain SC323 endo-polygalacturonase mRNA, complete cds	276	486	73%	9e-70	80%	KP265703.1
*Aspergillus niger*endogalacturonase A (pgaA) mRNA, complete cds	270	486	74%	4e-68	79%	KF157661.1

Results of Blast search for Amino acid sequence

Table -8 Amino acid sequence alignment

Description	Max score	Total score	Query cover	E value	Ident	Accession
endopolygalacturonase C [Aspergillus niger CBS 513.88]	570	570	100%	0.0	100%	XP_001389562.1
Chain A, Structural Insights Into The Processivity Of Endopolygalacturonase I From Aspergillus niger	568	568	100%	0.0	100%	1NHC_A
polygalacturonase [Aspergillus niger]	567	567	100%	0.0	98%	GAQ36232.1
polygalacturonase [Aspergillus luchuensis]	565	565	100%	0.0	98%	GAT23311.1
polygalacturonase (PgaI) [Aspergillus kawachii IFO 4308]	563	563	100%	0.0	98%	GAA87322.1
polygalacturonase [Aspergillus niger]	551	551	100%	0.0	97%	ACS44814.1
hypothetical protein ASPNIDRAFT_141677 [Aspergillus niger ATCC 1015]	*532*	*532*	*100%*	0.0	*95%*	*EHA26932.1*
Full=Polygalacturonase II; Short=PG-II; AltName:	512	512	97%	0.0	90%	Q70HJ4.1
extracellular polygalacturonase, putative [Aspergillus fischeri NRRL 181]	481	481	100%	4e-169	82%	XP_001266657.1
probable endopolygalacturonase A [Aspergillus lentulus]	481	481	100%	6e-169	81%	GAQ08488.1
polygalacturonase [Aspergillus fumigatus var. RP-2014]	478	478	100%	7e-168	81%	KEY81513.1
probable endopolygalacturonase A [Aspergillus udagawae] extracellular polygalacturonase [Aspergillus fumigatus	478	478	100%	7e-168	81%	*GAO83226.1*
polygalacturonasepgaI [Aspergillus ruber CBS 135680]	426	426	99%	3e-147	78%	EYE90976.1
RecName: Full=Polygalacturonase; Short=PG; AltName:	425	425	96%	8e-147	74%	O42824.1
endopolygalacturonase C [Aspergillus nomius NRRL 13137]	422	422	99%	6e-146	70%	XP_015404808.1
polygalacturonase I precursor [Aspergillus terreus NIH2624]	417	417	96%	1e-143	78%	XP_001214169.1
Putative Endopolygalacturonase I [Penicillium brasilianum]	415	415	96%	5e-143	73%	CEJ57944.1
endopolygalacturonase C [Aspergillus oryzae RIB40]	411	411	96%	2e-141	71%	XP_001820953.1
extracellular polygalacturonase [Aspergillus rambellii]	410	410	97%	2e-141	74%	KKK12990.1
polygalacturonase B [Aspergillus oryzae]	408	408	96%	3e-140	71%	BAA34782.1
extracellular polygalacturonase, putative [Aspergillus clavatus NRRL 1]	405	405	97%	5e-139	75%	XP_001272239.1
RecName: Full=Polygalacturonase 1; Short=PG 1; AltName: Full=Pectinase 1; Flags: Precursor	402	402	97%	9e-138	70%	Q9Y834.1
endo-polygalacturonase [Aspergillus niger]	398	398	100%	3e-136	71%	AJD09825.1
Full=Pectinase A; AltName: Full=Polygalacturonase A; Flags:	398	398	100%	3e-136	71%	Q9P4W4.1
Precursor						
AltName: Full=Pectinase A; AltName:	396	396	100%	1e-135	70%	Q8NK99.1
probable endopolygalacturonase A [Aspergillus niger]	394	394	100%	6e-135	70%	GAQ43403.1
putative polygalacturonase [Penicillium oxalicum 114-2]	394	394	93%	7e-135	70%	EPS32977.1
Pc22g20290 [Penicillium rubens Wisconsin 54-1255]	394	394	96%	1e-134	69%	XP_002565929.1
Full=Pectinase A; AltName: Full=Polygalacturonase A; Flags:	394	394	100%	2e-134	70%	Q9P358.1
Precursor						
endogalacturonase A [Aspergillus niger]	393	393	100%	3e-134	70%	AGV40780.1
endopolygalacturonase C [Aspergillus niger CBS 513.88]	392	392	98%	5e-134	71%	XP_001398000.2
Glycoside hydrolase, family 28 [Penicillium griseofulvum]	399	399	97%	4e-133	68%	KXG54083.1
polygalacturonase [Aspergillus flavus]	386	386	96%	1e-131	69%	AAA85280.1

CONCLUSION

The current work on enhancement of pectinase production from *Aspergillus niger*has been achieved through strain improvement by mutation. During the course of search, this work has confirmed that mutation through chemical and physical agents provides higher enzyme yields than wild type. This report emphasizes, for the first time, that *Aspergillus niger* treated with UV and EtBr simultaneously, results in higher pectinase production using submerged fermentation under the optimal conditions. This enzyme is an ecofriendly tool of nature that is being used extensively in wine industry, food industry, paper industry etc. However scale up studies are required for commercial production.

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