ZKG INTERNATIONAL ISSN: 2366-1313

UV Mutagenesis for the Enhanced Production of Polygalacturonase by *Aspergillus niger strain RA401*

K. Venkata Naga Rajeshwari[1], S. Suma[*1]

[1] Department of Biochemistry, Chaitanya (Deemed to be University),

Kishanpura, Hanumakonda, Telangana, India-506001

*E-mail:sumareddy197420@gmail.com

Abstract:A potent technique for the industrial development of microbial fermentation systems is strain improvement. *Aspergillus niger strain RA401*which had previously been recognized as polygalacturonase producers underwent the efficient mutagenesis and selection process known as random screening. The design and application of a classical mutation and selection method for the enhanced production of pectic acid-degrading enzymes utilized a physical method (ultraviolet irradiation at 254 nm). To create mutants descended from *A. niger strain RA401*, three mutation cycles were carried out. In solid-state fermentation, polygalacturonase enzyme production of the mutants was compared to that of their wild types. Mutant UM1028, which had 2.35 times more polygalacturonase activity than the wild strain of *A. niger strain RA401,* produced the greatest polygalacturonase activity after three mutation cycles. Accordingly, the production of polygalacturonase was 2.35 times higher than it was in a wild strain. The development of a traditional mutation and selection technique was suggested as a promising tool by the study's findings.

Keywords Strain improvement. Mutagenesis. *Aspergillus niger*. Solid-state Fermentation, Polygalacturonase

1.0 Introduction:

For the normal manufacturing of commercial enzymes like proteases, cellulases, and pectinases, large-scale fermentation processes employ bacteria. The overproduction of natural products, the incorporation of cheap and sophisticated raw materials, or the shortening of the fermentation period could all improve the economics of such operations. One strategy used to target the enhancement of fermentation economics is the improvement of microbial strains [4]. The production strain must be continuously improved if a fermentation industry is to become and remain competitive. Industrial strain development relies on modifications to the microbial DNA

sequence, which can be accomplished by genetic engineering, genetic recombination, or mutation techniques. Mutagenesis is used in conventional strain enhancement methods, followed by screening or selection. These traditional techniques for strain improvement have a long history. So long as product requirements are met and regulatory notification is full, strains produced through non-recombinant techniques or traditional strain improvement are generally regarded as less substantial process alterations [22]. Induced mutation is the intentional creation of novel genotypes, whereas spontaneous mutation occurs accidentally. The genetic material is exposed to physical or chemical substances, referred to as mutagens, to induce mutations. N-methyl-N′-nitro-N-nitrosoguanidine (NTG), ethyl methanesulfonate (EMS), hydroxylamine (NH2OH), nitrous acid (HNO2), and ultraviolet (UV) radiation are some of the traditional mutagens used for strain improvement (Demain and Adrio 2008; Parekh et al. 2000). DNA changes are brought on by each mutagen in a distinct way. Additionally, a mutagen may cause several lesion types. The majority of mutagenic substances alter DNA in some way, either by breaking DNA strands or by deleting, adding, transposing, or substituting bases. For instance, DNA pyrimidine dimerization and cross-links are brought about by UV irradiation mutation [22].

Improved carbohydrate-active enzyme synthesis by fungi has also been successfully achieved using traditional strain development techniques [25];[3];[10];[1]. Pectinolytic enzymes like polygalacturonase (PG), which are widely employed in the food sector, such as for the extraction and clarifying of fruit juices or in winemaking, are mostly produced by filamentous fungi [16]; [21].

Aspergillus sojae has the ability to produce pectinase in both fermentation systems, as previously shown in research [9];[11]. Additionally, providing specific PG overproducing mutants created as a result of the work described here for studies focusing on PG titers and fermentation process parameter optimization showed that the process could be successfully developed in submerged or solid-state culture [5];[8].

Recombinant DNA technology's more recent advancements enable precision mutagenesis to create desired phenotypes. However, without a fundamental comprehension of the organism, it is impossible to approach genetic engineering utilizing recombinant DNA techniques intelligently. Importantly, strains produced using conventional techniques are not considered genetically modified organisms (GMO), in contrast to more current recombinant DNA-based technology.

ISSN: 2366-1313

This removes a significant barrier to their acceptability by regulatory authorities and consumers. The conventional approach of phenotypic optimization is well-established in the food industry because to the "generally recognized as safe" (GRAS) categorization and ease of selection [6]. UV mutagenesis is widely regarded as harmless, despite the fact that both chemical and UV mutagenesis can be employed to produce mutagenesis.

The work presented here highlights the use of an optimized mutation and screening methodology to the standard strain engineering approach for increased PG production by *A. niger strain RA401* in solid-state fermentation (SSF). The findings of this study also allow for a comparison of the pectinolytic enzyme sets found in wild and mutant strains.

2.0 Materials and Methods:

2.1 Microorganism

In a previous study, *Aspergillus niger strain RA401*, the most potent fungal strain with pectinolytic activity, was isolated and identified using molecular techniques. It was obtained from decayed fruit and vegetables in Warangal and Hanamkonda City, and it was used as the parent (wild) strain [13].

2.2 Fungal suspension

Aspergillus niger strain RA401 isolated fungal cells were cultured for 48 hours before being scraped with an inoculating loop in a laminar air hood to create the spore suspension. This fungal solution was used in investigations on strain development**.**

2.3 Strain improvement technique

2.3.1 Mutagenesis by UV irradiation: Eleven test tubes containing the previously mentioned spore suspension (10^7 spores/mL in acetate buffer, pH 5.0) were used; ten of the test tubes were exposed to UV light (a germicidal lamp with a 254 nm wavelength) above the spore suspension at a distance of 17 cm for durations ranging from 5 minutes to 60 minutes, and one test tube (the control) was left in the dark. After that, 0.1 milliliters of the UV-treated spore suspension was injected into 20 milliliters of modified pectin potato dextrose agar-coated Petri plates. The colony-forming unit was employed to ascertain the survival rate. For further incubation, colonies were retained at 30 °C, and mutants were visually selected for sporulation. Mutants were created by isolating colonies from a single conidial spore. We further selected the mutants by reproducing them on a screening media. On the basis of the identification of an enhanced hydrolysis zone, "zone mutants" are chosen. Mutants' increased PG production was verified in

ISSN: 2366-1313

the SSF. The mutant strain with the highest PG activity was chosen for further mutation treatment. We repeatedly exposed spores to UV rays. Mutations required three rounds to produce a mutant, but mutant UM1028 required an additional two cycles of UV radiation treatment because it came from the most prolific Polygalacturonase producer found in the wild.

The spore solution was mutagenically treated under semi-darkened conditions, exposed to UV light in a box, and stirred with a magnetic bar using a modified version of the method reported by De Nicolás-Santiago et al. (2006) [7]. The treated samples were incubated for 30 minutes at room temperature in the dark after being placed on ice for 5 minutes to halt photoreactivation repair [12].

2.3.2 Solid-state fermentation and Enzyme production:

Using 15 g of banana peels as a source of pectin and 10 ml of a mineral salt solution made up of $(NH_4)_2SO_4$ 0.14%, K_2HPO_4 0.6%, KH_2PO_4 0.20%, and $MgSO_4.7H_20$ 0.01%, SSF was carried out in 250 mL culture flasks. The pH was brought down to 5 using a 0.2 N HCl solution (sterilized for 20 min at 121 °C). The SSF medium was added with the inoculum from the mutant strain and incubated at 28°C for 24, 48, 72, 96, and 120 hours. The inoculum was adjusted to 1 106 spores per gram dry weight. At intervals of 24, 48, 72, 96, and 120 hours, 50 cc of sterile, distilled water that had been filtered was added to the flaks. The mycelium was separated from the solid medium using centrifugation at 4 °C and 3,000 g for 20 min. Protein content and enzyme activity in the supernatant were both examined.

2.3.3 Screening medium

The pectinolytic effect was further established using the well diffusion approach. A well 10 mm in diameter was aseptically made using a cork borer on a potato dextrose agar plate with 1% (w/v) pectin enrichment. Each well received 100 l of culture filtrate before being allowed to incubate at 30 °C overnight. To detect pectinolytic activity, the substrate utilization zone around the wells was studied using a KI solution [26].

2.3.4 Enzyme Assay: The 3,5-dinitro salicylic acid (DNS) assay was used to measure the release of reducing groups from Polygalacturonic acid in order to assess the exo-Polygalacturonase activity [20]; [19].

ISSN: 2366-1313

2.3.5 Protein Estimation: Using Lowry's method [18], we estimated the total protein content of the cell-free filtrate using bovine serum albumin as a reference.

2.4: Optimization of Mutagenesis by UV light:

To maximize the amount of Polygalacturonase produced by the mutant strain, first optimization of mutagenesis at various time intervals, at various distances from UV light and at various temperatures was done.

The aim of UV light treatment optimization was to achieve a desired survival percentage of 50–60% by using mutagenesis. The parameters for each element were then optimized after three factors were tested in two steps. In the initial screening process, the effect of distance from UV light in centimeters (10, 15, and 20), incubation time (0.5–3 h), and temperature (16–30 °C) on the survival rate was examined. To attain the desired survival rate, the investigated fungal strain required a shorter (in cm) distance from UV radiation, a longer incubation time, or a higher incubation temperature. Furthermore, temperature had a greater influence on mutagenesis. As a result, the optimization step's temperature range was narrowed, and factor values were optimized at various temperatures (Table 1).

Table 1 Screening Factors for Mutagenesis

Experiment Number	Factors		
	Distance from the UV lamp in cm	Time in Min	Temperature in ^{o}C
1	10	5	30
2	15	10	25
3	20	15	30
4	15	20	30
5	15	25	30
6	15	30	30
7	15	35	20

ISSN: 2366-1313

8	20	40	25
9	15	45	30
10	15	50	30
11	15	55	25
12	15	60	30

2.5 Purification of Crude Enzyme produced by selected mutant strain:

2. 5.1 Ammonium sulfate precipitation: The cell-free filtrate (100 ml) was brought to 50% saturation by mixing with ammonium sulfate (Sigma) slowly with gentle agitation and allowed to stand for 24 h at 4°C. After the equilibration, the precipitate was removed by centrifugation (5000 rpm at 4°C for 15 min). The obtained precipitate was dissolved in 50 ml of 0.05 M sodium acetate buffer.

2.5.2 Dialysis for Desalting:

According to [15], dialysis was used to desalt the precipitate. The 10 cm dialysis bag became functional after a rinse in distilled water. The bag's one end was tightly tied before the generated precipitate was introduced. The dialysis bag's other end was tightly knotted to prevent any leaks. To get rid of ions and low molecular weight substances that can harm the enzyme's ability to function, the dialysis bag was then suspended in a beaker of 0.05 M sodium acetate buffer. Dialysis at 4oC was used to desalt the protein fraction with Exo-PG activity overnight.

2.5.3 Gel Filtration Chromatography: The dialyzed enzyme fraction was then further purified using gel filtration chromatography. A vertical glass tube chromatography column of Sephadex G-100 (Particle size, 40–120) was created and calibrated in accordance with Ajayi's recommendations [2]. 30 ml of the enzyme concentration was put onto the Sephadex G-100 column before being eluted with 0.05 M sodium-acetate buffer at a flow rate of 20 ml/h.

A spectrophotometer was used to determine absorbance (absorbance at 280) after fractions (5 ml each) were collected. Each fraction's polygalacturonase activity was investigated. The fractions with higher enzyme activity were pooled for additional examination.

ISSN: 2366-1313

2.5.4 SDS PAGE for analytical electrophoresis

In a (10 x 8 cm) device, SDS-PAGE was performed to determine the molecular weight of the isolated enzyme. Electrophoresis was carried out in a vertical slab gel apparatus in a parallel lane using a 5% (w/v) polyacrylamide stacking gel and a 12% (w/v) resolving gel in Tris/glycine buffer (pH 8.3) and a molecular weight marker (14KDa-188KDa) [17]. To see the protein band, use the Coomassie Brilliant Blue stain.

3. Results & Discussion:

3.1 Strain Improvement Technique:

3.1.1 Improvement of strain by UV irradiation:

Table 2 displays the Polygalacturonase synthesis shown by mutants derived from UV irradiation of *A. niger strain 401* at various time points.

Table 2 Screening Factors for Mutagenesis and polygalacturonase activity

Experiment Number	Factors			Polygalacturonase activity in U/g	SD
	Distance from the UV lamp in cm	Time in Min	Temperature in ^{o}C		
Wild	Nil	Nil	30	1280	20
1	10	5	25	1220	10
2	15	10	30	1240	40
3	20	15	40	1210	20
4	15	20	30	1290	40
5	15	25	30	1300	20
6	15	30	30	1320	10
7	15	35	20	1260	20

ISSN: 2366-1313

8	20	40	25	1280	10
9	15	45	30	1340	40
10	15	50	30	1350	10
11	15	55	25	1350	20
12	15	60	30	**1370**	**10**

When the experimental data were analyzed, the linear model showed no issues with fit. The spore survival rate from the wild strain UM1028 to the mutant strain demonstrated high model quality. All three of the analyzed parameters had a substantial impact on the spore survival rates of both fungal strains, and both strains achieved the desired survival rate of 50–60% within the chosen factor range.

The following factors were taken into consideration for UV mutagenesis based on the above results for screening the factors for mutagenesis, as given in Table 3. The time of UV exposure in minutes is plotted against enzyme activity in Figure 1.

Table 3 Selected Variables and Factor Parameters for Optimization to Improve Survival Rate

Microorganism	Factor	Parameters
UM1028	Distance in cm	15
	Incubation Time min	30-60 mins
	Temperature in ^{0}C	25 to 30

ISSN: 2366-1313

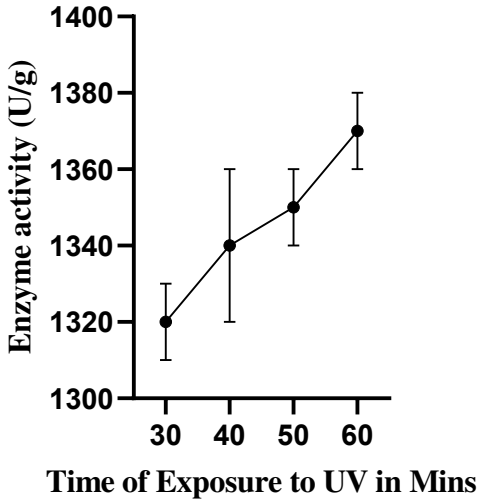


Fig. 1 Time of Exposure to UV Vs Enzyme Activity

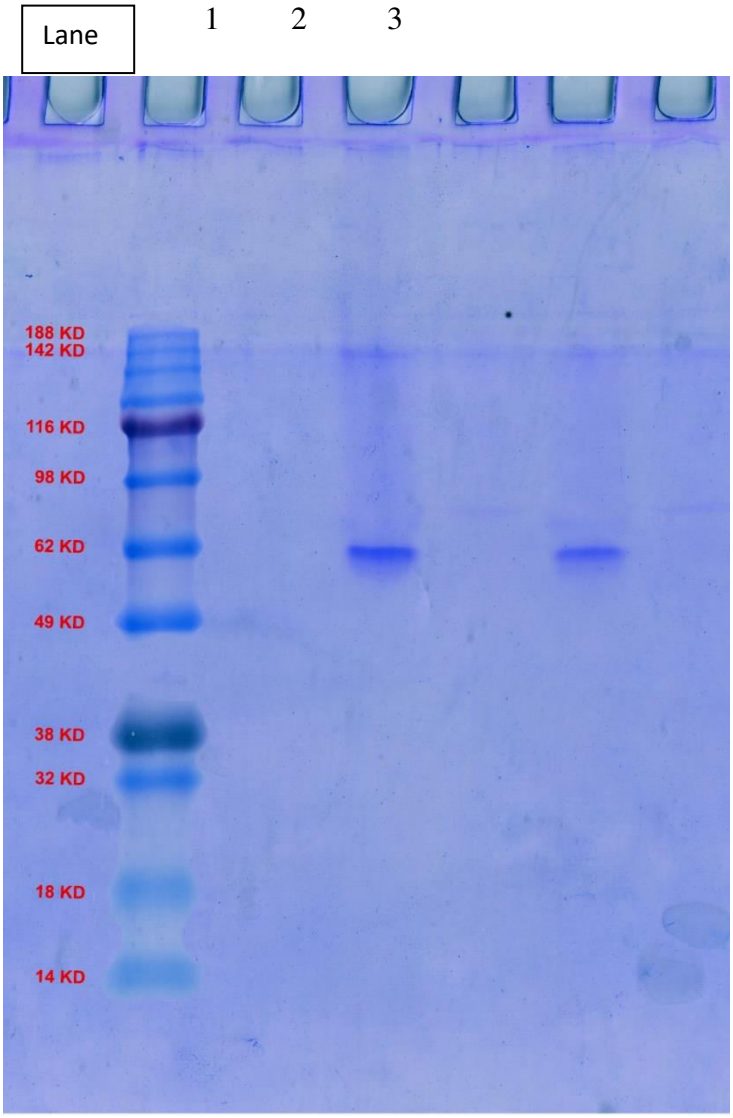
Figure 2 UV mutagenesis of *Aspergillus niger strain RA401* at various time intervals of UV exposure.

3.2 Purification of Polygalacturonase from selected mutant strain and its' comparison with wild strain *Aspergillus niger strain RA401*

ISSN: 2366-1313

The outcomes of the purification processes are summarized in Table 4. The results demonstrated that Ammonium Sulphate Precipitation and One-Stage Gel Filtration using Sephadex G100 homogeneously purified Polygalacturonase produced by selected mutant strain. The specific activity in ammonium sulfate salting out (50% saturation) was 27.40 U/mg protein, and 35% of the precipitation proteins were recovered with a purification fold of 4.70. The yield and purification fold from Sephadex G100 gel filtration was 16.84% and 7.32, respectively. The production profile of the mutant strain, *Aspergillus niger UM1028* was compared with the wild strain *Aspergillus niger strain RA401* shown in Table 4.

Fig 3: SDS-PAGE for HPLC fraction



Lane 1 Marker Lane 3UM1028 HPLC fraction

Lane 2 Wild HPLC fraction

ISSN: 2366-1313

Table 4 Production profile of PG mutant strain UM1028 in comparison with wild strain

Strain	Purification step	Protein(mg) (A)	Enzyme activity (Units) (B)	Specific activity (U/mg) C= B/A	Fold Purification D= C/C1	% Yield E= (B/B1)*100
Wild strain	Crude	450	1800	4.0	-	
UM1028	Crude	650	3680	5.84	-	-
Wild strain	Ammonium Sulphate (50%)	36.68	560	15.26	3.82	31.11
UM1028	Ammonium Sulphate (50%)	36.78	1282	27.40	4.70	35
Wild strain	Gel filtration (Sephadex G100)	12.25	220	17.96	4.5	12.23
UM1028	Gel filtration (Sephadex G100)	14.50	620	42.7	7.32	16.84
Wild strain	HPLC	6.0	180	30	7.5	10
UM1028	HPLC	8.0	460	57.5	9.85	12.5

Wild strain C1: the value of C in row 1 (i.e., 4.0); B1: the value of B in row 1(i.e. 1800).

UM1028 C1: the value of C in row 1 (i.e., 5.84); B1: B in row 1(i.e. 3680).

4.0 Discussion:

Any microorganism may typically be treated with chemical or physical mutagens to isolate phenotypes that overproduce a product, but for this to be successful, the mutagenic treatment must be optimized [24]. The current study to improve UV mutagenesis focused on a survival rate between 50 and 60%. This range was selected because, according to Bos (1987), a modest mutagen dose should be taken into account when considering the disruption of the genetic background.

The preferred approach for creating PG hyperproducing mutants of *A. niger UM1028* was treatment by repeated UV irradiation.

Although the filamentous fungus *Aspergillus carbonarius*, the top producer of fungal pectinase (480 U/g) in SSF, already produced higher Polygalacturonic acid degrading exoenzyme activities than *A. sojae ATCC 20235*, the current study showed an increase in enzyme yields for commercial considerations of PG production by *A. niger.*

Pre-selection concentrated on morphological characteristics of sporulation. As a result, the production of mutants that could produce enough spores for inoculation was guaranteed. It was possible to find the desired mutants with increased pectinase activity measured as clear zones on screening media by choosing "zone mutants" in the second step of the screening technique. Polygalacturonic acid (sodium salt) was used to identify mutants with enhanced PG activity.

It is necessary to develop overproducing strains specifically for the corresponding fermentation system. In order to select mutants with higher PG production in SSF, the third screening stage concentrated on doing so. Only mutants acquired after the third mutation cycle were additionally examined for enzyme production in SSF and the findings of this study showed that mutants with enhanced PG production in SSF during the third mutation cycle.

Pectinase production by *A. sojae ATCC 20235* and *A. sojae CBS 100928* in SSF was previously compared, and the results showed that the latter produced more enzymes[11]. The current findings show that in solid-state culture, *A. niger UM1028* also developed increased polygalacturonase activity on comparison with wild strain *Aspergillus niger strain RA401* [14].

The improvement in PG production by *A. niger strain RA401* using traditional strain improvement techniques was demonstrated in the current experiment. This led to the creation of mutants with elevated pectinolytic activity, which may be used in biotechnological methods to produce polygalacturonase in either solid-state environment. Additionally, PG produced by chosen mutant plays a significant role in the breakdown of complex plant polysaccharides, which has the potential to be important industrially for specialized applications, such as in food processes using preparations that are primarily PG active.

ACKNOWLEDGEMENTS: The authors would like to thank Dr. Mukesh Pasupuleti, Principal Scientist, Division of Microbiology, and CSIR-CDRI for his constant support and encouragement throughout the research

References:

1. Adsul MG, Bastawde KB, Varma AJ, Gokhale DV (2007) Strain improvement of *Penicilliumjanthinellum* NCIM 1171 for increased cellulase production. Bioresour Technol 98:1467–1473
2. Ajayi AA, Olutiola PO, Fakunle JB. Studies on polygalacturonase associated with the deterioration of Tomato (*Lycopersiconesculentum Mill*) fruits infected by Botryodiplodiatheobromae PAT. Science Focus, 2003; 5:68-77
3. Antier P, Minjares A, Roussos S, Raimbault M, Viniegra-González G (1993) Pectinase-hyperproducing mutants of *Aspergillus niger C28B25* for solid-state fermentation of coffee pulp. Enzym Microb Technol 15:254–260
4. Barrios-González J (2012) Solid-state fermentation: physiology of solid medium, its molecular basis and applications. Process Biochem 47: 175–185
5. Buyukkileci AO, Tari C, Fernandez-Lahore M (2011) Enhanced production of exo-polygalacturonase from agro-based products by *Aspergillus sojae*. BioResources 6:3452–3468
6. Crook N, Alper HS (2012) Classical strain improvement. In: Patnaik R (ed) Engineering complex phenotypes in industrial strains, 1st edn. Wiley-AIChE, Hoboken, pp 1–34
7. De Nicolás-Santiago S, Regalado-González C, García-Almendárez B, Fernández FJ, Téllez-Jurado A, Huerta-Ochoa S (2006) Physiological, morphological, and mannase production studies on *Aspergillus niger* uam-gs1 mutants. J Biotechnol 9:51–60
8. Demir H, Göğüş N, Tari C, Heerd D, Fernandez Lahore M (2012) Optimization of the process parameters for the utilization of orange peel to produce polygalacturonase by solid-state fermentation from an *Aspergillus sojae* mutant strain. Turk J Biol 36:394–404
9. Gögus N, Tari C, Oncü S, Unluturk S, Tokatli F (2006) Relationship between morphology, rheology and polygalacturonase production by *Aspergillus sojae ATCC 20235* in submerged cultures. Biochem Eng J 32:171–178
10. Hadj-Taieb N, Ayadi M, Trigui S, Bouabdallah F, Gargouri A (2002) Hyperproduction of pectinase activities by a fully constitutive mutant (CT1) of Penicillium occitanis. Enzym Microb Technol 30:662–666
11. Heerd D, Yegin S, Tari C, Fernandez-Lahore M (2012) Pectinase enzyme-complex production by *Aspergillus spp* in sold-state fermentation: a comparative study. Food Bioprod Process 90:102–110
12. Heerd, D., Tari, C. & Fernández-Lahore, M. Microbial strain improvement for enhanced polygalacturonase production by *Aspergillus sojae* . *Appl Microbiol Biotechnol* **98**, 7471–7481 (2014). https://doi.org/10.1007/s00253-014-5657-z
13. K. Venkata Naga Rajeshwari, Sanikommu Suma (2023). ENZYMATIC PROFILING, ISOLATION, AND IDENTIFICATION OF *ASPERGILLUS NIGER*. *Eur. Chem. Bull. 2023,12(Special Issue 1), 781-788*
14. K. Venkata Naga Rajeshwari, Sanikommu Suma (2023). Partial purification and Characterization of Exo-Polygalacturonase produced by Aspergillusnigerstrain RA401 using Solid state Fermentation. BioGecko; Vol 12 Issue 02 2023 ISSN NO: 2230-5807:2256-2269.

ISSN: 2366-1313

15. Karthik LJ, Gaurav K, Bhaskara Rao KV. Screening of pectinase producing microorganisms from agricultural waste dump soil. Asian Journal of Biochemistry and Pharmaceutical Research. 2011;1(2): 329-37.
16. Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. Bioresour Technol 77: 215–227
17. LAEMMlI U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 1970: 680-685.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.,**1951**. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193 (1), 265–275.
19. Martins, E. S.; Silva, D.; Da Silva, R.; Gomes, E. Process Biochem. 2002, 37, 949.
20. Miller, G. L.; Anal. Bioanal. Chem.1959, 31, 426
21. Naidu GSN, Panda T (1998) Production of pectolytic enzymes—a review. Bioprocess Eng 19:355–361
22. Parekh S, Vinci VA, Strobel RJ (2000) Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54:287–301
23. Patnaik R (2008) Engineering complex phenotypes in industrial strains. Biotechnol Prog 24:38–47
24. Sarkar S, Saunders G (1999) The role of genetics in the effective utilisation of filamentous fungi. IUPAC
25. Smith DC, Wood TM (1991) Isolation of mutants of Aspergillus awamori with enhanced production of extracellular xylanase and β- xylosidase. World J Microbiol Biotechnol 7:343–354
26. Tariq, A., & Latif, Z. (2012). Isolation and biochemical characterization of bacterial isolates producing different levels of polygalacturonases from various sources. Afr J Microbiol Res, 6(45), 7259-7264.