Production, Optimization and purification of isolated fungal strain *Aspergillus terreus* GU227345.1 and screening of local biomass

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Abstract:

Cellulases are an important industrial enzyme used for bioethanol production. The present research was designed to generate hypercellulase from a locally isolated strain of fungus. For this purpose, fungal strains were collected from local soil, vegetable waste, and agrowaste. The isolated strains were first morphologically identified, then genetically sequenced for further use. The highest cellulase production was achieved by optimizing physico-chemical parameters through RSM by SSF. Furthermore, cellulase was purified using $(NH_4)_2SO_4$ precipitation, dialysis, and chromatography. The results showed GU227345.1 *Aspergillus terreus* gave the highest yield (86.665 IU/min/mL) by maintaining parameters, i.e., incubation time of 4 days, temperature of (35ºC), substrate (6 g), pH (6), moisture (90%), inoculum (5 mL), (NH4)2SO4 (0.5 g), and $MgSO_4$ (0.25 g). It was further enhanced by $(NH_4)_2SO_4$ precipitation, membrane dialysis, and chromatography. The Km of cellulase was 7.375 mg/mL, and the Vmax value was 1250 mmol min^{-1} mg^{-1}.

Keywords:

Enzyme, Biowaste, Bioethanol, Cellulase, Fungal, SSF, RSM.

1 Introduction:

Brazil started operating more than 300 bioethanol production plants in 2006 with the goal of producing 15 billion liters of pure bioethanol annually. In 2008, the US quickly overtook other countries in the world in ethanol output. Germany and France produce the most ethanol in Europe, which accounts for 5% of global output. It is strongly suggested that SGB be produced and used instead of first-generation bioethanol. Globally, scientists are focusing on renewable fuel resources, such as biofuel, rather than fossil fuels. In this regard, agro-industrial wastes are more sustainable, and there is a requirement for optimization of production techniques. In the 1980s, federal and local lawmakers raised the value of ethanol and providing incentives for the country's widespread ethanol production. In the 1990s, the Kyoto

protocol introduced the use of ethanol to reduce CO emissions. An agreement between regional governmental institutions and the business sector is known as the Minnesota Model, which is also helping export-level production of bioethanol in the USA. Using cellulosic resources, ethanol manufacturing facilities were built during World War II in various nations, including Russia, China, United States, Germany, Korea and Switzerland (Bayrakci Ozdingis & Kocar, 2018). In addition, a major and concerning problem of waste management has also emerged in agriculture-based economies (Hansen & Cheong, 2019).

Lignocellulosic substrate has gained much attention globally for the past three decades as an alternate biomass to compensate for growing energy and enzyme demand (Kaar & Holtzapple, 1998). There are four major types of lignocellulosic materials: remnants of vast forests throughout the world, domestic waste, paper waste, and agricultural waste material (Balat, 2011). One decade ago, the annual production of lignocellulosic material in the world was 1x1011 tonnes, which could be converted into 2x1021 Joules of energy production (Kaushal et al., 2012).

Lignocellulose consists of three components i.e., cellulose (β-D-glucosyl homopolymer), hemicellulose (xylan, arabinans, mannans, and galectin heteropolymers), and lignin (a polyphenolic polymer) (Saleem et al., 2005). Photosynthesis helps convert hundreds of billions of metric tonnes of greenhouse gases into Lignocellulose (Znameroski & Glass, 2013).

In lignocellulosic biomass, there are two main components: cellulose and xylan, both of which constitute about 70–80% of the biomass of dry wood material. If these are broken down into their constituent parts, they will release glucose and xylose, respectively. The digestion of cellulose and xylan is carried out by cellulase and xylanase, respectively. The plant's body generally consists of 40–50% cellulose, and from the cell wall of the plant, 1010 tonnes per year of yield are harvested (Sabara et al., 2022). The cellulase market has been increasing desperately due to the significant importance of biofuels and other chemicals (Areeshi, 2022). Cellulase production should be cost-effective because it represents advanced biocatalytic properties (Bhati et al., 2021).

The enzyme used to hydrolyze lignocelluloses is cellulase. In order to produce 3.8 l of cellulosic ethanol, 100 g of enzyme must be used. Highly active, pure enzymes weighing 100 g often cost between $200 and $300 USD (Bayrakci Ozdingis & Kocar, 2018).

Enzyme industry is gaining more importance now a days, especially cellulose breaking enzyme cellulase, because cellulose is a wide spread economic resource. Fungi are used for this production because of its industrial viability (Santa-Rosa et al., 2018).

Researchers are searching new strains of fungi due to its economic importance and industrial efficiency (Santa-Rosa et al., 2018).

So, in this regard, the present research was designed to isolate indigenous fungi for screening of hyperproduction, optimization, and purification of cellulase.

2 Material and Method:

2.1 1.1 Selection of Microorganisms

Ten different fungi were collected locally from Dist. Gujrat and its vicinities, which were present on Banana tree trunk (sample A), Mulberry (Sample B), Indian Rosewood (Sample C), Lawn Grass (Sample D), Root Soil of Indian Rosewood (Sample E), Paper (Sample F), Cow Feed (Sample G), Eucalyptus (Sample H), Guava (Sample I), and Bread (Sample J).

2.1.1 Fungal Growth and Preservation

All fungi that were collected were grown separately on PDA petri-plates by spreading method; serial dilution was performed where required. PDA petri plates were placed in an incubator at 37^{o}C for 72 hours. Further fungus isolation was performed by preparing triplicate PDA slants from each fungus petri-plate using the steaking method.

2.1.2 Preparation of Inoculum

Those 11 slants that had maximum growth were selected, which were SD1, SF3, SF2, SE1, SI3, SJ1, SB3, SI2, SF1, SA3, and SC3. Inoculum of each selected fungus was prepared in a 250mL Erlenmeyer flask, adding PDA without agar and pH adjusted to 5.5 by loop transfer aseptically. Inoculum at 37oC for one week on an orbital shaker (with 120 rpm to achieve turbidity and homogeneous growth). Beads were added to each flask to prevent mycelium growth. Inoculum was harvested for further use after one week and contained isolated spores of different fungi in each flask.

2.1.3 Bradford for protein estimation

Bradford assay for protein identification was used for the quantification of proteins (Kielkopf et al., 2020).

2.1.4 Cellulase assay

Miller's DNS assay for finding the Enzyme activity of cellulase (Miller, 1959).

2.2 Substrate screening

The agro-industrial substrate was collected from the local areas of Gujrat, Pakistan, which are Citrus maxima, Cydonia oblonga, Mangifora indica, Erythrophleum suaveolens, Saraca asoca, Ficus benghalensis, Litchi chinensis, Triticum, Tripidium bengalense, Psidium guajava (Wheat straw, Guava leaves, Ashoka leaves, orange peel, Banyan leaves, Sarkanda leaves, Mango leaves, lichi leaves, Indian Rosewood leaves, and Quince leaves). Waste was dried in the sunlight, then crushed and ground into fine powder form; furthermore, all substrates were stored in airtight plastic jars. On each substrate, all fungal inoculums were applied one by one. The fungus and substrate that produced high cellulase activity were selected. The schematic diagram for selection and screening is given in Figure 1.

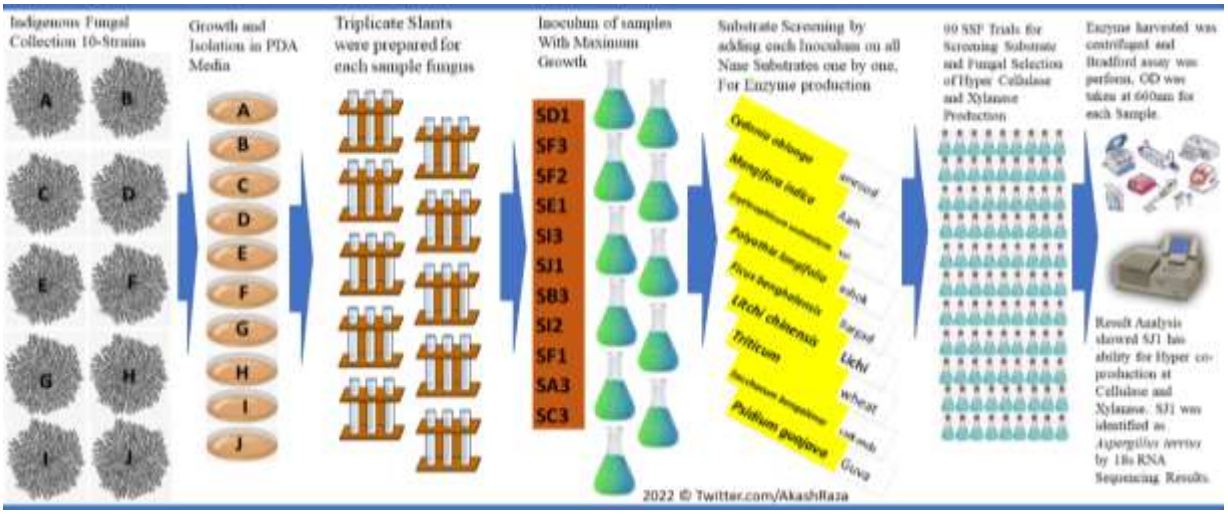


Figure 1: Fungal selection and substrate screening

2.3 RNA Sequencing Method

The primers NS1 5' (GTA GTC ATAA TGC TTG TCT C) 3'and NS8 5'(TCC GCA GGT TCA CCT ACG GA) 3' were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30µL reaction mixture by using a *EF-Taq* (Solgent, Korea) as follows: activation of Taq polymerase at 95ºC for 2 minutes, 35 cycles of 95ºC for 1 minute, 55ºC and 72ºC for 1 minutes each were performed, finishing with a 10-minute step at 72ºC. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). The sequencing reaction was performed using a PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit. The DNA samples containing the

extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95ºC for 5 minutes, followed by 5 minutes on ice, and then analyzed by an ABII Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The novel sequence obtained was Submitted to GenBank.

2.4 Optimization nutritional and physio-chemical factors in SSF by RSM

Selected substrate for the fermentation process, which was carried out by following RSM and optimized through factorial design. Two RSM tables were designed for performing trails with the following parameter ranges with the help of Minitab software version 20.2.0.

RSM-1 was designed with the following factors and their ranges: incubation (2–6 days), temperature (24-25ºC), substrate (2-10 g) and pH (4-8).

RSM-2 was designed with the following factors and their ranges: moisture (30–90%), inoculum (0.5–5 mL), $(NH_4)_2SO_4$ (0.5–3 g), and $MgSO_4$ (0.05–0.25 g).

All substrate was autoclaved at 120ºC for 30 minutes and weighed. Trails were conducted in laminar flow. After inoculation, flasks were incubated at the physico-chemical conditions of the RSM model designed through Minitab (version 20.2.0).

After getting optimum values, the RSM-1 four parameters were fixed, and RSM-2 trials were performed to get the optimum values of moisture, inoculum, $(NH_4)_2SO_4$, and $MgSO_4$.

2.4Enzyme extraction

After SSF enzyme was extracted. 100 ml of distilled water was added in each flask. The muslin cloth was used for this purpose, and the filtrates were transferred into falcon tubes and centrifuged at 15000 rpm for 15 minutes to attain a supernatant from dissolved biomass.

2.6 $(NH_4)_2SO_4$ precipitation and dialysis

The crude enzyme extracted was precipitated and then dialyzed using standard procedure according to the steps mentioned in Figure 2.

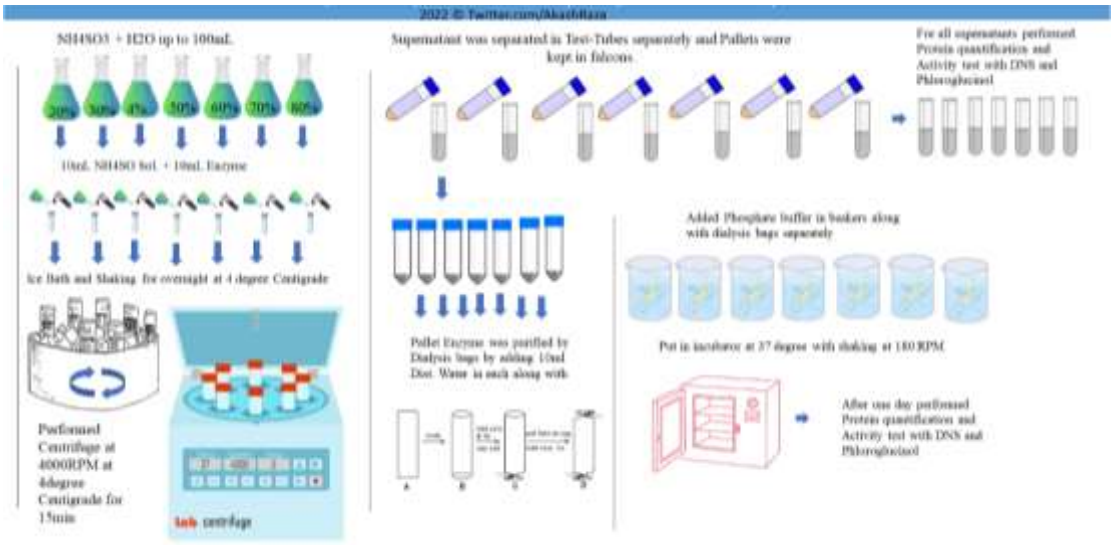


Figure 2: Enzyme production and dialysis by (NH4)2SO4

2.7 Chromatography

Enzymes obtained after (NH4)2SO4 precipitation and dialysis were subjected to chromatography according to the Pickart method with some modifications as per the requirements for separation and purification. In this step, a chromatography column was setup, 5 gram of silica gel were added to 100 mL of water, autoclaved for 15 minutes, and then allowed to rise to room temperature for 20 minutes. Separately, a chromatography column was setup by adding a cotton plug and 2-centimetre sand, then silica gel solution was poured into the column and left for two days to settle. Then washed the column with phosphate buffer, pH 7. Then added 2 mL of dialyzed enzyme. Eluted with phosphate buffer. Took 30 fractions of 1.5 mL each after 3 minutes in Eppendorf (Pickart & Thaler, 1975). The schematic diagram is given in Figure 3.

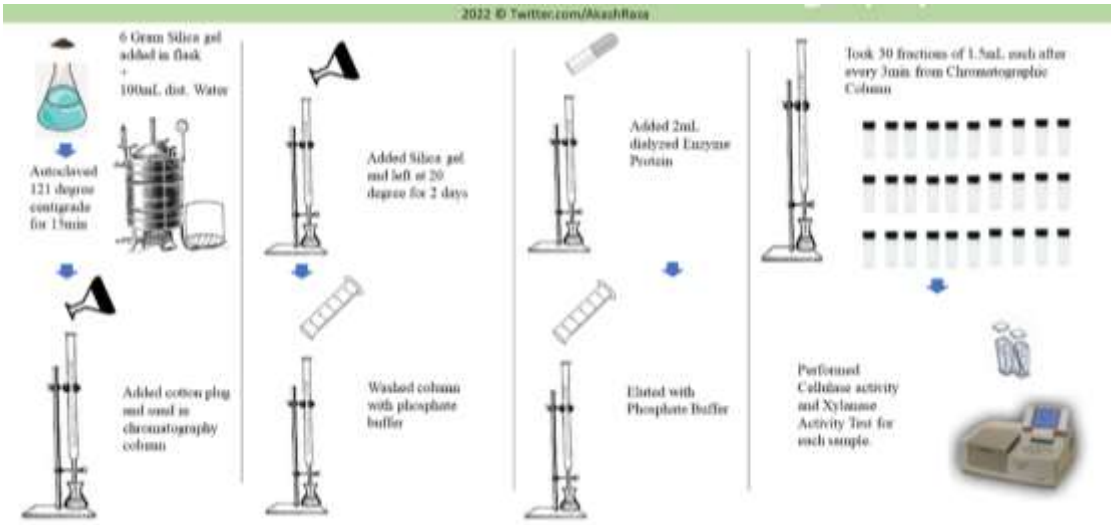
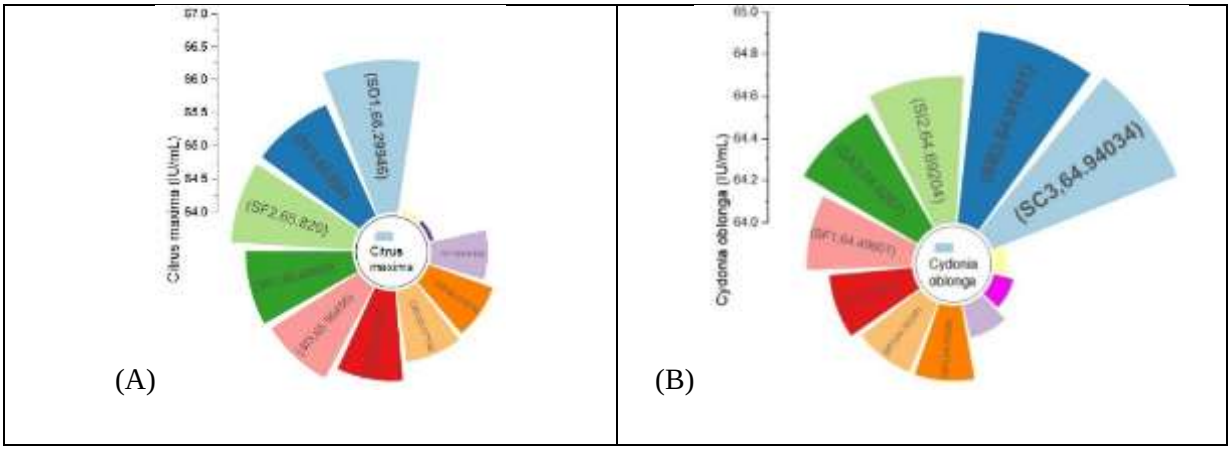


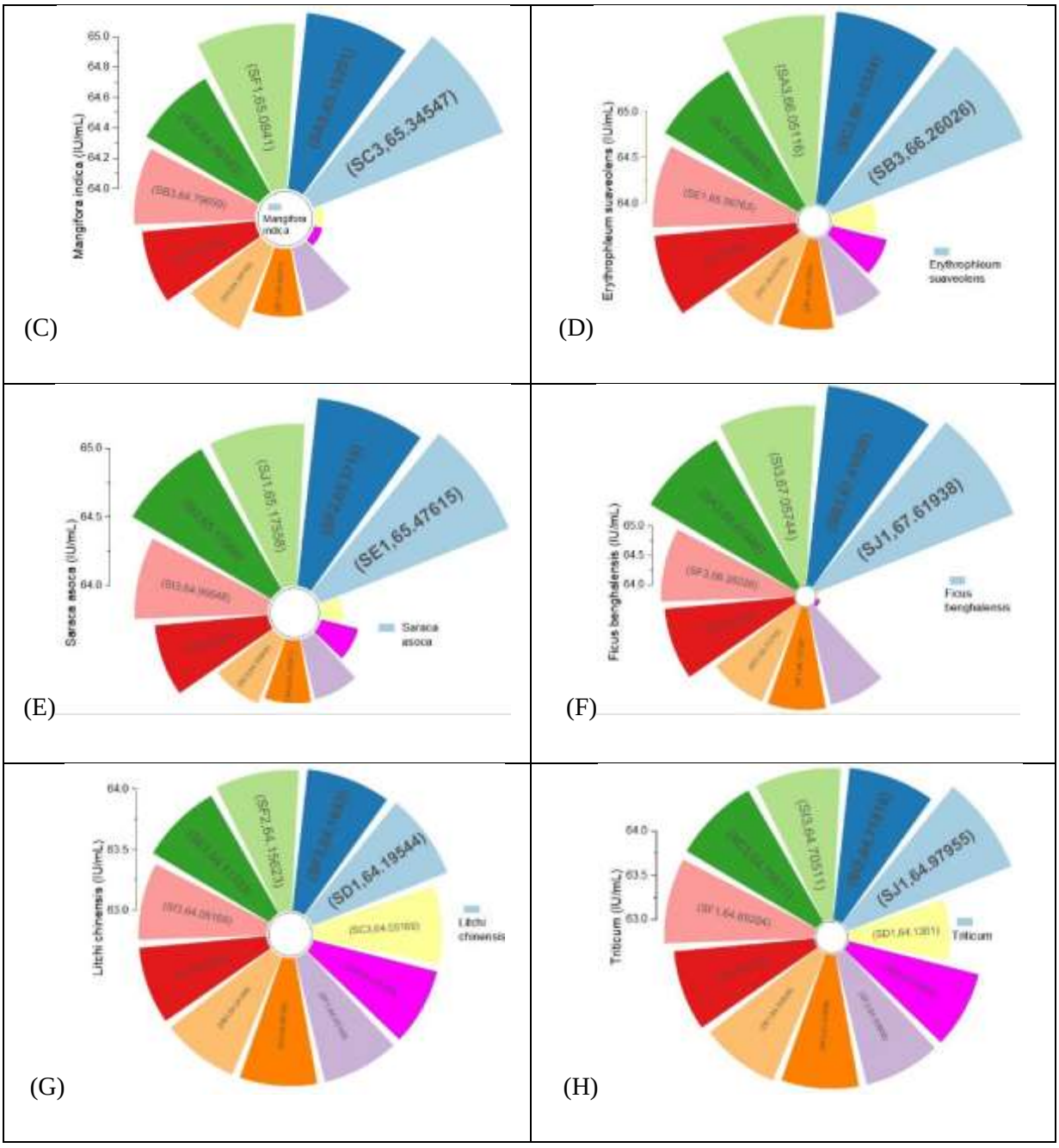
Figure 3: Gel filtration column chromatography

3 Results and Discussions

3.1 Substrate Screening and Fungal Isolation

Hypercellulase-producing substrates and fungi were screened through enzyme activity.




(C)
Mangifera indica (IU/mL)
65.0
64.8
64.6
64.4
64.2
64.0
(SF1,65.06411)
(SC3,65.34547)
Mangifera indica
(D)
Erythrophleum suaveolens (IU/mL)
65.0
64.5
64.0
(SA3,66.05116)
(SB3,66.26026)
Erythrophleum suaveolens
(E)
Saraca asoca (IU/mL)
65.0
64.5
64.0
(SJ1,65.17558)
(SE1,65.47615)
Saraca asoca
(F)
Ficus benghalensis (IU/mL)
65.0
64.5
64.0
(SI3,67.05744)
(SJ1,67.61938)
Ficus benghalensis
(G)
Litchi chinensis (IU/mL)
64.0
63.5
63.0
(SF2,64.15623)
(SD1,64.19544)
Litchi chinensis
(H)
Triticum (IU/mL)
64.0
63.5
63.0
(SI3,64.70511)
(SJ1,64.97955)
(SD1,64.12811)
Triticum

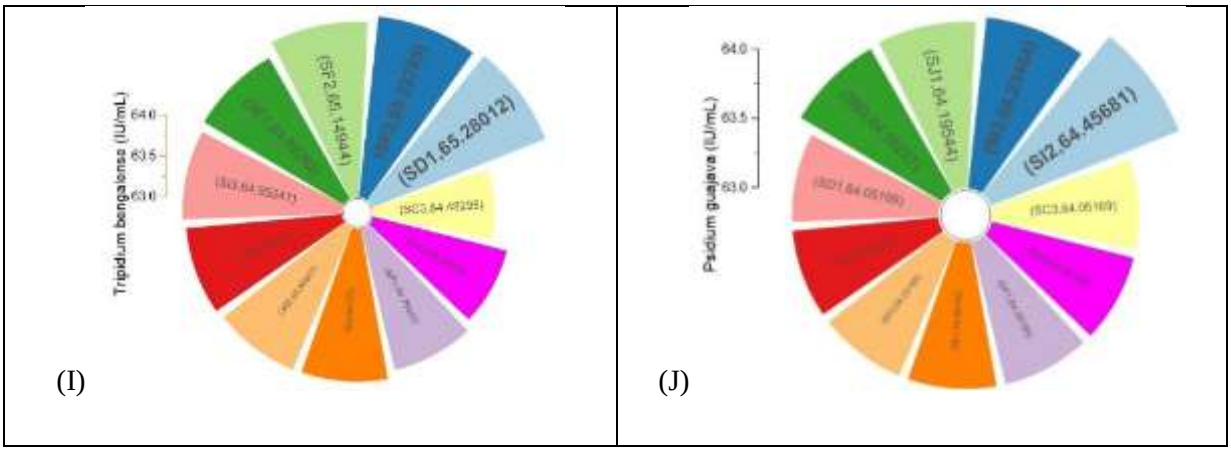


Figure 4: Substrate and fungal screening for cellulase production (A) ***Citrus maxima*** **(B)** ***Cydonia oblonga*** **(C)** ***Mangifora indica*** **(D)** ***Erythrophleum suaveolens*** **(E)** ***Saraca asoca*** **(F)** ***Ficus benghalensis*** **(G)** ***Litchi chinensis*** **(H)** ***Triticum*** **(I)** ***Tripidium bengalense*** **(J)** ***Psidium guajava***

Results showed that *Ficus benghalensis* produced the maximum Cellulase content; the fungus that produced this maximum value of cellulase content was SJ1, which was later identified by molecular tools.

Morphological identification showed that SJ1 is a species belonging to the genus *Aspergillus*. Molecular sequencing was performed later for the complete identification of this novel cellulase hyperenzyme-producing sub-species.

In previous research by Shanmugapriya (2012), the highest enzyme activity was recorded at a pH of 6 using yeast extract as a nitrogen source and glucose as a carbon source at 40 ^{o}C for 48 hours. Enzyme activity of CMC and coir waste were used as substrates. It was noted that enzyme activity increased in the pH range of 5 to 8 when used as a substrate. The values were discovered to decline with additional incubation. The highest enzyme activity was seen when yeast extract was used as the nitrogen source and xylose was used as the carbon source at pH 7 at 50 ^{o}C for 48 hours with sawdust as the substrate (Shanmugapriya et al., 2012).

In Shanmugapriya's work, cellulase activity is found in carboxymethylcellulose, sawdust, and coir waste. The crude sample's specific enzyme activity was discovered to be 4.32 U/mg. With bovine serum albumin as a reference, the protein contents of the crude sample were ascertained (Shanmugapriya et al., 2012).

Narra used different lignocellulosic substrates, including rice straw, zingivo grass, wheat straw, jowar straw, cotton stalk, and bajra straw, for SSF. It was discovered that every substrate supported sizable

endoglucanase production. However, rice straw produced the highest cellulase activity (33.12 U/g) (Narra et al., 2014).

3.2 Results of 18S RNA Sequencing for Fungal Identification

18S RNA Sequencing was performed, and the results revealed that the hyper-enzyme-producing species screened was *Aspergillus terrius.* The submitted sequence to NCBI for GenBank's registered accession number is ON753798 as given in Table 1. Following are the results of RNA sequencing and the NCBI report on sequence submission.

Table 1: NCBI GenBank accession number of fungal strain

#Accession	Sequence ID	Release Date
ON753798	SJAKASHRAZA_contig_1	Dec 31, 2022

Table 2: 18S rRNA service report of fungal identification

18S rRNA service report

Order Number : HC00434701
Sample name : SJAKASHRAZA_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
NS1 5' (GTA GTC ATA TGC TTG TCT C) 3'	NS1 5' (GTA GTC ATA TGC TTG TCT C) 3'
NS8 5' (TCC GCA GGT TCA CCT ACG GA) 3'	NS8 5' (TCC GCA GGT TCA CCT ACG GA) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
GU227345.1	Aspergillus terreus	1694	7	1668	98	3057	0.0	1660/1662	99

Kingdom	Family	Genus	Species
Eukaryota	Aspergillaceae	Aspergillus	Aspergillus terreus

Molecular identification was carried out to ascertain the specie isolate. A phylogram of GU227345.1 *Aspergillus terreus* obtained using the Neighbor-Joining Tree method.

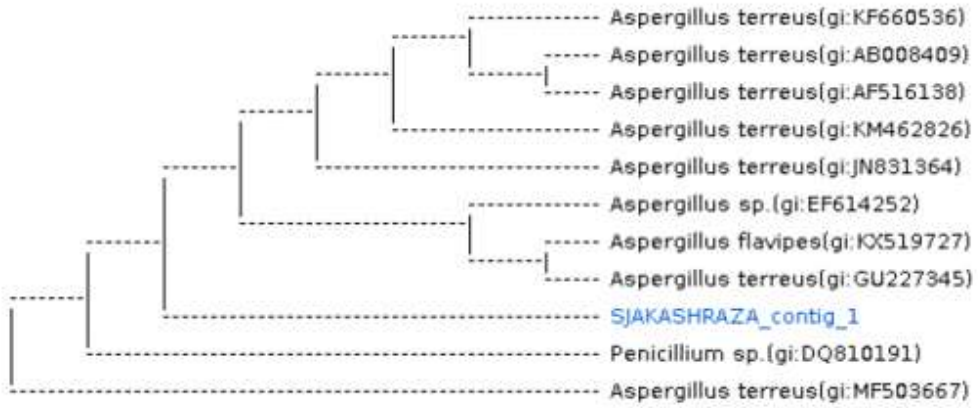


Figure 5: phylogram of GU227345.1 *Aspergillus terreus* obtained using the Neighbor-Joining Tree method

During research by lokhande, same selected isolates underwent an enzyme test. The maximum exoglucanase (2.22 IU/ml), endoglucanase (2.02 IU/ml), and -glucasidase (1.97 IU/ml) activity was found in *Aspergillus niger* ABS 228S. (Lokhande & Pethe, 2016).

In Narra research, Cellulolytic microbes were screened from soil, compost, deteriorated rice straw, and other sources. The degraded rice straw was used to isolate the celluloytic strain that was employed in this investigation. On cellulose agar plates, the isolated fungal strain displayed a distinct zone. The strain was identified as *A. terreus* based on morphological and genetic analysis (NCBI GenBank Accession Number: KF971363) (Narra et al., 2014). After microscopic and molecular revealed that the LBKURCC293 isolate was *Aspergillus* sp. which is a specie that is frequently used because of its fermentation ability a variety of biomass and conditions in which bacteria and yeast cant survive (Novianty et al., 2022).

Previous research had been effective in isolating thermophilic Aspergillus fumigatus isolates that produced cellulase and other extracellular enzymes such as lignocellulolytic and cellulolytic. According to reports, even *Aspergillus* species and their consortia may survive in very contaminated soil conditions and produce lengthy hydrocarbon-decomposing enzymes (Novianty et al., 2022).

3.3 Optimization nutritional and physio-chemical factors in SSF by RSM

Once SJ1, which is *Aspergillus,* was identified as an enzyme-producing species and *Ficus benghalensis* was selected as a hyper-enzyme-producing lignocellulosic substrate, The fungus and substrate were optimized for nutritional and physio-chemical factors by performing SSF according to RSM in two steps. The following results were obtained.

Firstly, RSM was applied for parameters like temperature, substrate, pH, and incubation time. After optimization, further parameters were optimized. The ANOVA table of the first RSM indicated that the data is significant with an R-Square value above 98%. The regression equation drawn from the data is given below; furthermore, a surface 3D graph plot also helps to compare the interaction between multiple parameters.

Table 3: Analysis of variance

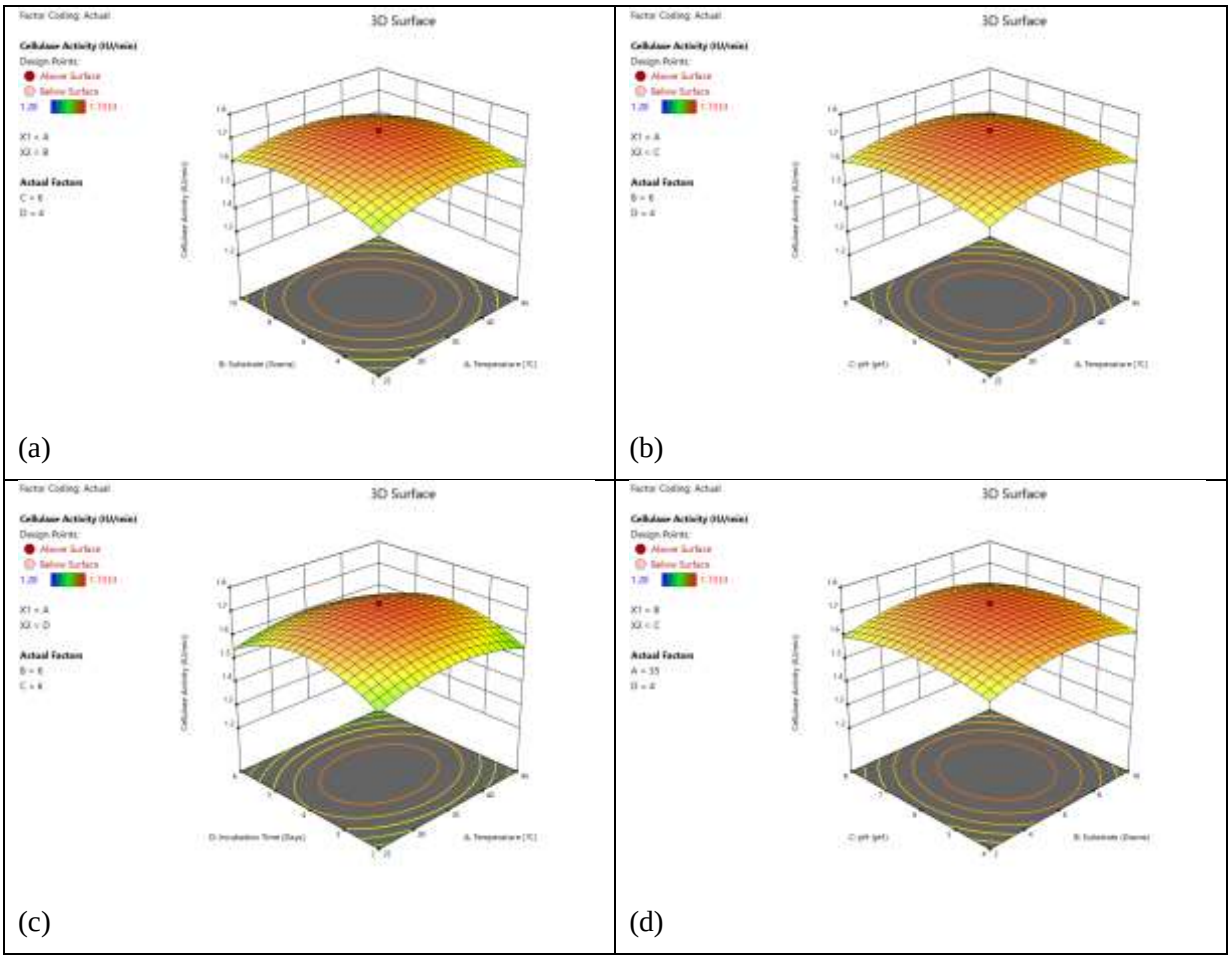
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	14	963.019	68.787	68.24	0.000
Linear	4	6.672	1.668	1.65	0.213
Temperature	1	0.164	0.164	0.16	0.692
Substrate	1	4.819	4.819	4.78	0.045
pH	1	0.109	0.109	0.11	0.747
Incubation time	1	1.580	1.580	1.57	0.230
Square	4	951.907	237.977	236.08	0.000
Temperature*Temperature	1	242.003	242.003	240.08	0.000
Substrate*Substrate	1	222.765	222.765	220.99	0.000
pH*pH	1	135.287	135.287	134.21	0.000
Incubation time*Incubation time	1	681.152	681.152	675.74	0.000
2-Way Interaction	6	4.440	0.740	0.73	0.630
Temperature*Substrate	1	3.230	3.230	3.20	0.094
Temperature*pH	1	0.376	0.376	0.37	0.551
Temperature*Incubation time	1	0.013	0.013	0.01	0.912
Substrate*pH	1	0.035	0.035	0.03	0.855
Substrate*Incubation time	1	0.781	0.781	0.77	0.393
pH*Incubation time	1	0.005	0.005	0.01	0.942
Error	15	15.120	1.008		
Lack-of-Fit	10	12.810	1.281	2.77	0.136
Pure Error	5	2.310	0.462		
Total	29	978.140			

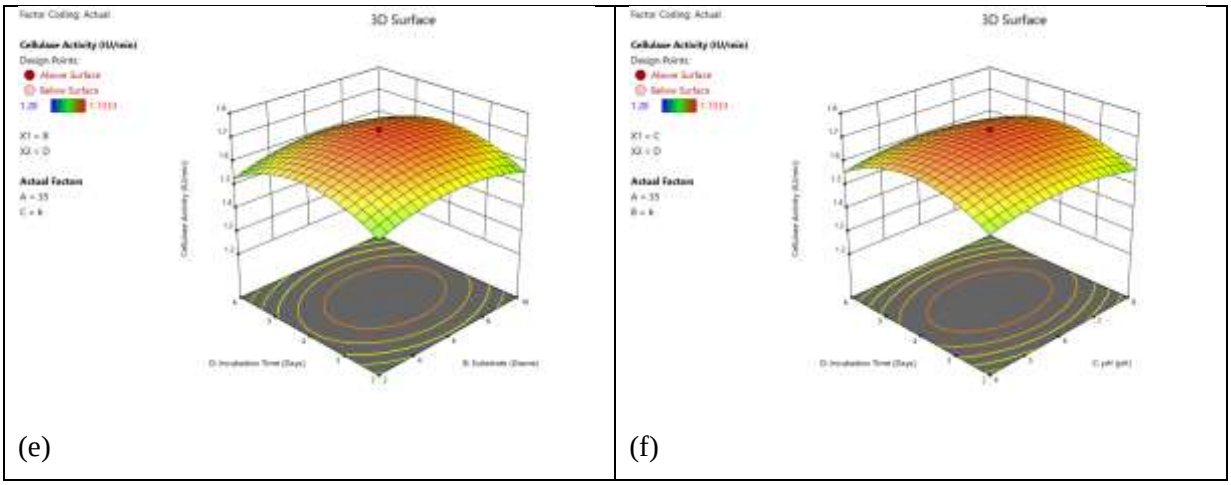
R^2=98.45%

Regression equation in uncoded units

Cellulase Activity = -0.32 + 2.179 Temperature + 2.567 Substrate + 6.914 pH + 9.595 Incubation time - 0.02970 Temperature*Temperature - 0.1781 Substrate*Substrate - 0.5552 pH*pH - 1.2458 Incubation time*Incubation time - 0.01123 Temperature*Substrate - 0.0077 Temperature*pH + 0.0014 Temperature*Incubation time - 0.0058 Substrate*pH + 0.0276 Substrate*Incubation time + 0.0046 pH*Incubation time

After getting the statistical analysis report of the results, the 3D Surface plots were made with the help of Design Expert software Version 13.





(e) (f)

Figure 6: 3D Surface plots of cellulase activity (a) temperature vs. substrate (b) temperature vs. pH (c) temperature vs. incubation time (d) substrate vs. pH (e) substrate vs. incubation (f) pH vs. Incubation

After trials of RSM, when comparisons between Temperature and Substrate were performed for maximum Cellulase activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when pH was fixed at 6 and for 4 Days of incubation, the maximum value was obtained at 25 ^{o}C and for 2 grams of substrate.

After trials of RSM, when comparisons between Temperature and pH were performed for maximum Cellulase activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when the substrate was fixed at 6 grams for 4 Days of incubation, the maximum value was obtained at 25^{o}C and 8 pH.

After trials of RSM, when comparisons between Temperature and Incubation were performed for maximum Cellulase activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when Substrate was fixed at 6 grams and at 6-pH, the maximum value was obtained at 25^{o}C and for 6 days, as well as at 45^{o}C of substrate and 2 days of incubation.

This might be due to the fact that as the temperature increases, it takes a smaller number of days for complete breakdown by the fungal enzyme.

After trials of RSM, when comparisons between Substrate and pH were performed for maximum Cellulase activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when temperature was fixed at 35 ^{o}C and for 4 days of incubation, the maximum value was obtained at 8 pH and for a 2-gram substrate.

After trials of RSM, when comparisons between Substrate and Incubation were performed it showed that at 35°C temperature and 6 pH, after 6-days, and 10g substrate the maximum value was obtained, similarly at 2g of substrate and for 2-Days of incubation maximum activity was observed.

After trials of RSM, when comparisons between pH and Incubation were performed for maximum Cellulase activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that at 35°C temperature, 4 pH and 6g substrate for 6 days, the maximum value was, as well as at 8-pH and 2-Days of incubation.

The pH of the medium was initially between 4 and 4.9, and while the yield steadily increased, cellulase activity declined as the pH rose, and mycelium development, while adversely affected, was observed at high temperatures. The ideal culture conditions for the cellulase yield were reported with endophytic fungi R4 from Taxus cuspidata at 34°C, 77 h, and an initial pH of 5. The predicted response values of Fpase, CMCase, and GLase activity were 1.47 U/mL, 5.28 U/mL, and 6.66 U/mL, respectively (H. Li et al., 2021). The greatest enzyme production for *A. sydowii* occurred on the sixth day of SmF at 100 rpm (Matkar et al., 2013). The ideal pH for *A. sydowii* to produce cellulase was 5.5. FPA (0.99 IU/ml), endoglucanase (0.89 IU/ml), and exoglucanase (2.03 IU/ml) had the highest enzyme activity at pH 5.5. The ideal temperature for the synthesis of cellulase was 40 °C. FPA, endoglucanase, exoglucanase, and b-glucosidase had respective concentrations of 0.95 IU/ml, 0.39 IU/ml, 1.65 IU/ml, and 6.01 IU/ml. At 45 °C, activity decreased by 13%, while at 50 °C, it decreased by 25%. At pH 6.0, the highest level of b-glucosidase activity (8.40 IU/ml) was found (Matkar et al., 2013).

Maximum activity was reported by *Aspergillus niger* ABS228 at the optimal culture conditions of 40°C and pH 6 on the fifth day of growth. 1% carboxymethylcellulose was also used as a substrate concentration (Lokhande & Pethe, 2016).

A. terreus performed confirmatory research using solid-state fermentation. Endoglucanase activity (141.29 U/g) was achieved at a substrate concentration of 5 g, a pH of 5.0, inoculation with 3.2 mL of spores, and a moisture ratio of 1:7 (w/v) (Narra et al., 2014).

Cellulase production by the isolate LBKURCC293 was shown to be possible at 50 °C under 96 hours of incubation. After 96 hours of incubation, the specific cellulase activity was determined to be 8.0×10^{-3} IU/mg protein (Novianty et al., 2022).

In the case of *Aspergillus fumigatus*, the ideal conditions for CMCase formation were 48.6 °C, 1.6% (w/v), pH 2.0, and 8.5 days of processing. This combination thus produced a projected maximal CMCase activity of 97.06 IU/mL (Saroj et al., 2021).

3.3.2 Second RSM results of cellulase activity

Second RSM was designed for Moisture, Inoculum, Ammonia source and Magnesium source.

Table 4: Analysis of variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	14	7547.48	539.11	118.92	0.000
Linear	4	2664.89	666.22	146.96	0.000
Moisture	1	157.55	157.55	34.75	0.000
Inoculum	1	492.69	492.69	108.68	0.000
(NH4)2SO4	1	1439.89	1439.89	317.62	0.000
MgSO4	1	574.75	574.75	126.78	0.000
Square	4	2111.91	527.98	116.46	0.000
Moisture*Moisture	1	843.93	843.93	186.16	0.000
Inoculum*Inoculum	1	457.50	457.50	100.92	0.000
(NH4)2SO4 *(NH4)2SO4	1	1289.11	1289.11	284.36	0.000
MgSO4*MgSO4	1	285.59	285.59	63.00	0.000
2-Way Interaction	6	2770.67	461.78	101.86	0.000
Moisture*Inoculum	1	1301.65	1301.65	287.13	0.000
Moisture*(NH4)2SO4	1	444.98	444.98	98.16	0.000
Moisture*MgSO4	1	0.36	0.36	0.08	0.781
Inoculum*(NH4)2SO4	1	149.88	149.88	33.06	0.000
Inoculum*MgSO4	1	286.15	286.15	63.12	0.000
(NH4)2SO4 *MgSO4	1	587.66	587.66	129.63	0.000
Error	15	68.00	4.53		
Lack-of-Fit	10	63.12	6.31	6.47	0.026
Pure Error	5	4.88	0.98		
Total	29	7615.48			

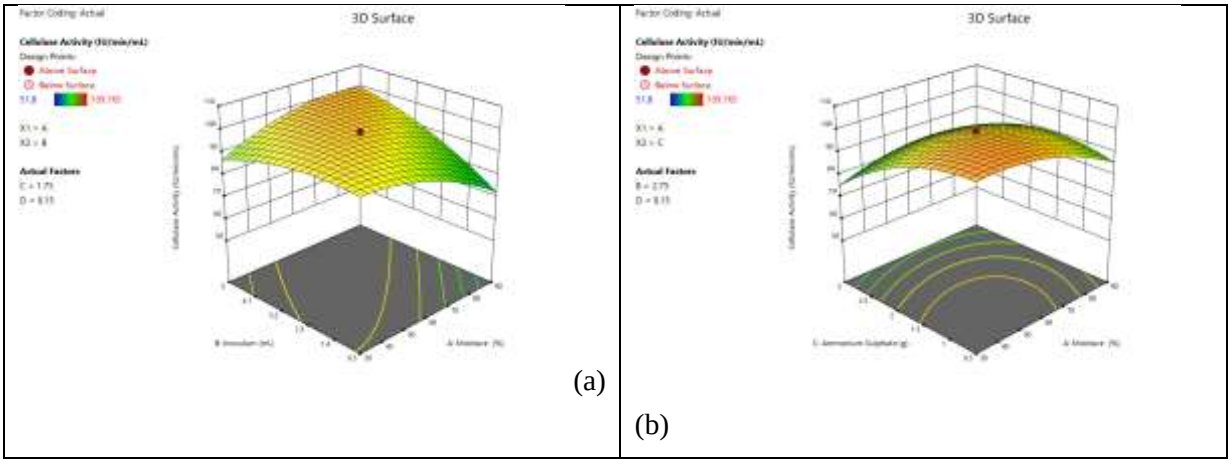
R^2 = 98.45%

Regression equation in uncoded units

Cellulase Activity = 89.80 + 0.0331 Moisture - 6.290 Inoculum + 5.00 (NH4)2SO4 + 175.9 MgSO4 - 0.006163 Moisture*Moisture - 0.8067 Inoculum*Inoculum - 4.388 (NH4)2SO4 *(NH4)2SO4 - 322.7 MgSO4*MgSO4 + 0.13362 Moisture*Inoculum + 0.1406 Moisture*(NH4)2SO4 + 0.050 Moisture*MgSO4 + 1.088 Inoculum*(NH4)2SO4 + 18.80 Inoculum*MgSO4 - 48.48 (NH4)2SO4 *MgSO4

Analysis report shows that the results were significant with R-Square value above 99%.

After getting statistical analysis report of results, the 3D Surface plots were made with the help of Design Expert software version 13.



(a) (b)

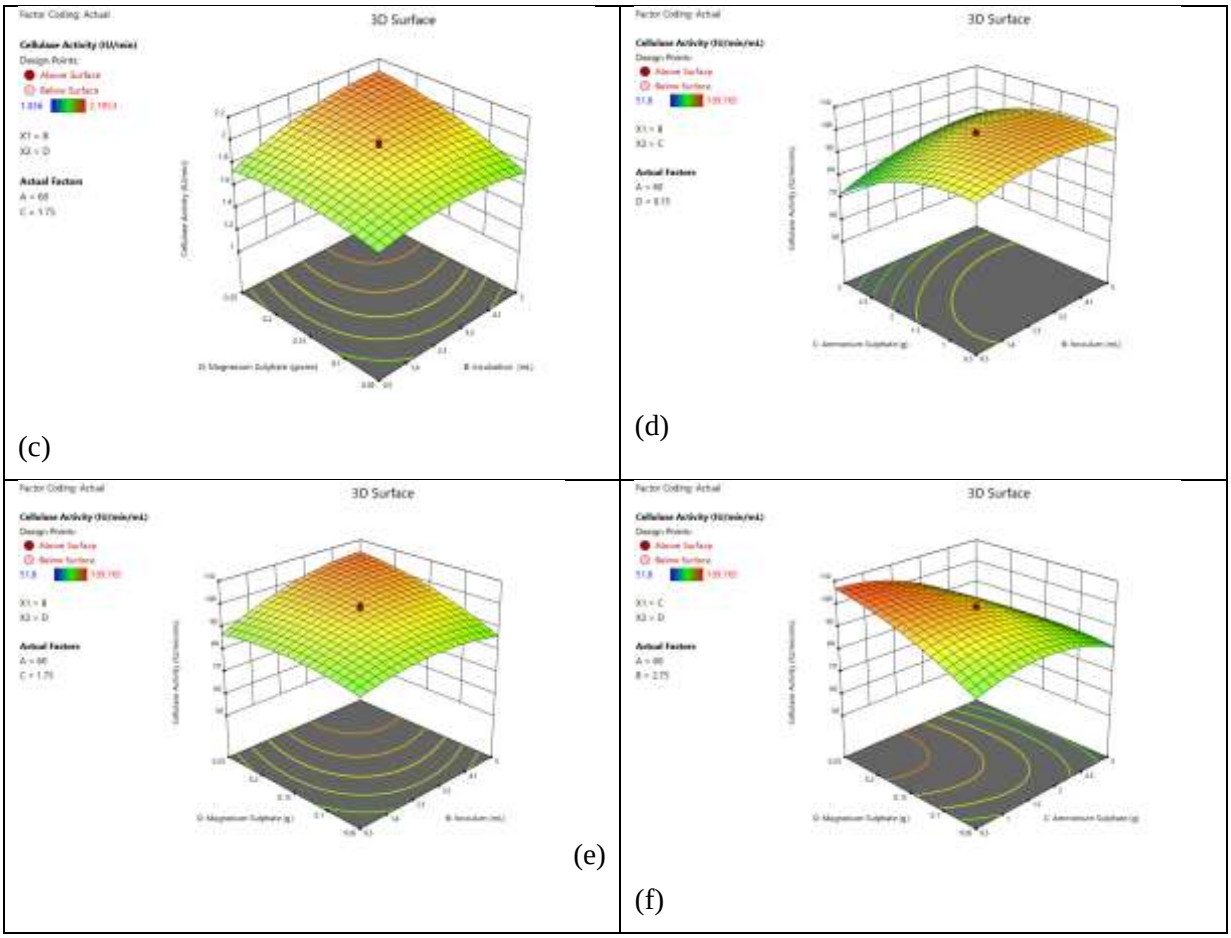


Figure 7: 3D Surface plots of cellulase activity (a) moisture vs. inoculum (b) moisture vs. $(NH_4)_2SO_4$ (c) moisture vs. $MgSO_4$ (d) inoculum vs. $(NH_4)_2SO_4$ (e) inoculum vs. $MgSO_4$ (f) $(NH_4)_2SO_4$ vs. $MgSO_4$.

After trials of RSM, when comparisons between Moisture and Inoculum were performed for maximum Cellulase Activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when (NH4)2SO4 was fixed at 1.75 grams and $MgSO_4$ at 0.15 grams, the maximum value was obtained at 30% moisture level and with an inoculum size of 0.5 mL, as well as at 90% moisture level and with an inoculum size of 5 mL.

After trials of RSM, when comparisons between Moisture and $(NH_4)_2SO_4$ were performed for maximum Cellulase Activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when Inoculum was fixed at 2.75 mL and $MgSO_4$ at 0.15 grams, the maximum value was obtained at a 30% moisture level and with 0.5 grams of $(NH_4)_2SO_4$.

After trials of RSM, When comparisons between Moisture and $MgSO_4$ were performed for maximum Cellulase Activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when Inoculum was fixed at 2.75 mL and $(NH_4)_2SO_4$ at 1.75 grams, then maximum value was obtained at 80% moisture level and with 0.05 grams of $MgSO_4$, as well as at 30% moisture level and with 0.25 grams of $MgSO_4$.

After trials of RSM, when comparisons between Inoculum and $(NH_4)_2SO_4$ were performed for maximum Cellulase Activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when Moisture was fixed at 60% and $MgSO_4$ at 0.15 grams, the maximum value was obtained at Inoculum size 0.5 mL with 0.5 grams of $(NH_4)_2SO_4$, as well as at Inoculum size 5mL and with 0.5 grams of $(NH_4)_2SO_4$.

After trials of RSM, when comparisons between Inoculum and $MgSO_4$ were performed for maximum Cellulase Activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when Moisture was fixed at 60% and $(NH_4)_2SO_4$ at 1.75 grams, the maximum value was obtained at Inoculum size 3 mL and with 0.05 grams of $MgSO_4$, as well as at Inoculum size 5 mL and with 0.25 grams of $MgSO_4$.

After trials of RSM, when comparisons between $(NH_4)_2SO_4$ and $MgSO_4$ were performed for maximum Cellulase Activity, according to RSM results, and the results were displayed in a 3D surface plot, it showed that when Moisture was fixed at 60% and Inoculum at 2.75 grams, the maximum value was obtained for 0.5 grams of $(NH_4)_2SO_4$ and 0.25 grams of $MgSO_4$.

Smaller flocs would spread more readily and avoid developing a pelleted shape. Additionally, a larger inoculum size aids in the generation of enzymes and quick colonization. 10% (v/v) of the production medium, or 3.18 106 spores/ml, was the ideal inoculum size. FPA, endoglucanase, exoglucanase, and b-glucosidase had respective concentrations of 1.04 IU/ml, 0.96 IU/ml, 2.56 IU/ml, and 8.42 IU/ml. The impact of the nitrogen supply on the synthesis of cellulase differed.

Sodium nitrate caused the greatest increase in endoglucanase and exoglucanase activity, increasing it by 9% in comparison to peptone as the only nitrogen source. Peptone and sodium nitrate both had an identical impact on b-glucosidase activity, although urea could only increase it by 1%. The activity of the enzyme was unaffected by triammonium citrate. With various nitrogen sources, little difference was seen in the quantities of the cellulase components. All of the enzyme components had greater specific activities when exposed to sodium nitrate, whereas b-glucosidase had higher specific activities when exposed to urea. (Matkar et al., 2013).

In the search for the enhancement of cellulase production in 2022, it was reported that at 65% moisture, the maximum production was obtained from SSF medium (Srivastava et al., 2022). Another study that was performed in 2022 for cellulase production from *Aspergillus niger* showed that maximum cellulase production was obtained at 75% moisture content under SF (Santos, 2022).

Research on the production of cellulase from *Aspergillus* species showed that production can be enhanced by an increase in the size of the inoculum (Sulyman et al., 2017). Another study showed that maximum production is obtained at inoculum size (20% v/w) (Fadel et al., 2021).

Al naser research came up with the finding in 2022 that enhanced cellulase production could be obtained with a 0.35% $(NH_4)_2SO_4$ concentration (El-Naser Zohri et al., 2022). Parveen's research in 2020 showed that maximum cellulase production could be achieved at 0.5% w/v $(NH_4)_2SO_4$ and $MgSO_4$ supplement medium (Parveen et al., 2020).

3.4 Results of $(NH_4)_2SO_4$ precipitation and Dialysis

When crude enzyme was precipitated with $(NH_4)_2SO_4$ and Dialysis was performed, the pallets were collected after centrifugation, and the Cellulase activity was assessed with a DNS assay. The results are shown in Figure 8.

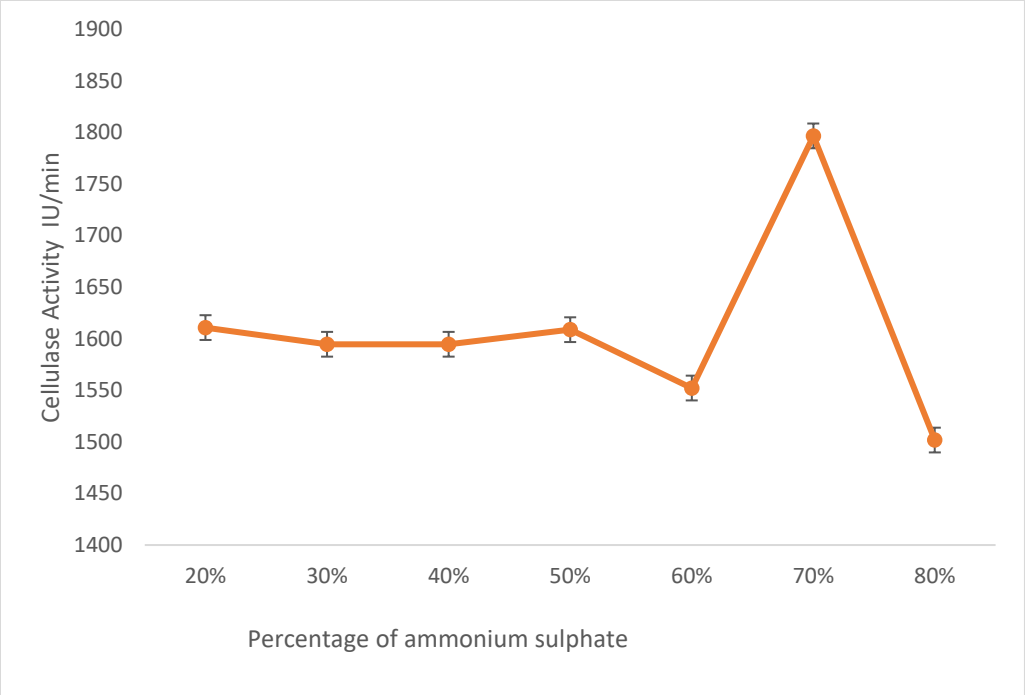


Figure 8: Cellulase activity after precipitation of enzyme with $(NH_4)_2SO_4$ for purification

Cellulase comparison showed that the maximum value was obtained at 70% $(NH_4)_2SO_4$ precipitation for the pallet.

The dialyzed sample of the crude sample had 6.57 U/mg as specific activity, whereas the $(NH_4)_2SO_4$ precipitated sample had a specific activity of 6.73 U/mg (Shanmugapriya et al., 2012). The enzyme was mostly separated present in F2 fraction of acetone fractionation with purification of 6.27-fold and enzyme recovery percentage was 60.37%. Dialyzed enzyme specific activity was greater than crude. Results show that the Sepharose-4B chromatographic column was effective. (Elshafei et al., 2009).

3.5 Results of Gel-Chromatography

The chromatography results of purified cellulase shown in Figure 9.

3.5.1 Results of Cellulase Activity

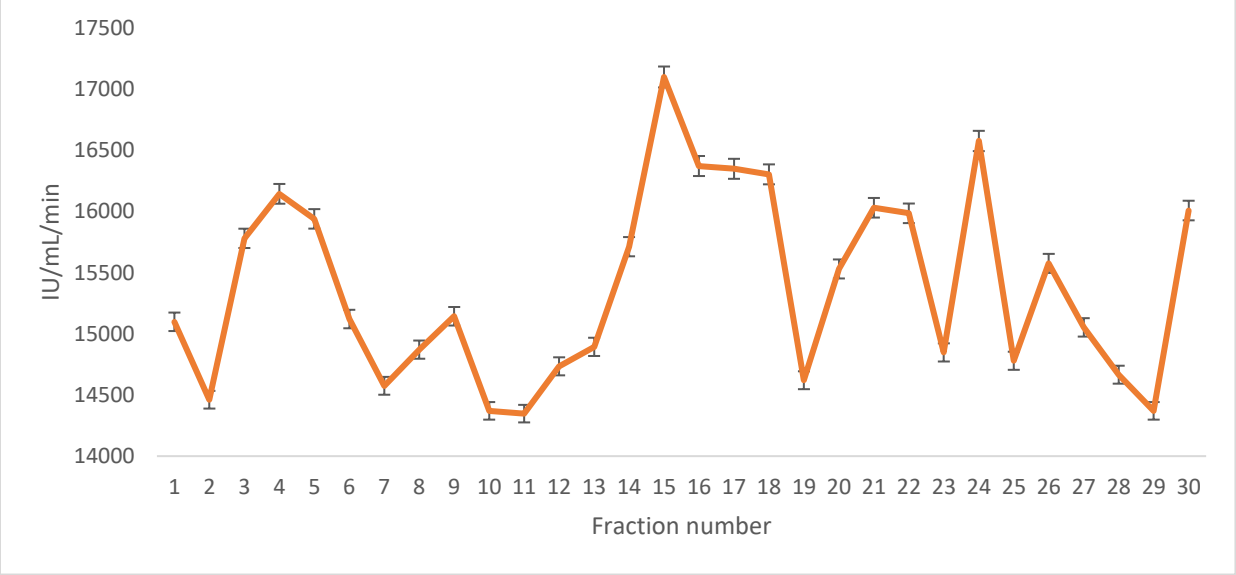


Figure 9: Cellulase activity after gel chromatography of enzyme for purification

Cellulase activity results show that fraction 15 contained the maximum cellulase enzyme purified from the chromatographic column. Crude cellulase was centrifuged to generate a clear supernatant, which was then concentrated. Using Sephacryl S-200, the concentrated enzyme was then separated and purified. Starting about the 25th fraction, endoglucanase activity was dispersed throughout different fractions. In the last fractions (48–61), a low-molecular-weight endoglucanase was detected. Endoglucanase that has undergone purification was found to have a specific activity of 12.69 U/mg and a purification fold of 3.49 (Narra et al., 2014).

The purified enzyme was most active between the pH ranges of 3.6 and 5.0, with a temperature optimum of 50 °C. Its activity peaked at pH 4.8. After 150 min at 50 °C, the enzyme still had 99% of its maximal activity. The isolated enzyme lost activity after retaining 90% activity for 1 hour and more than 75% activity for 2 hours at 60 °C. At 70 °C, the enzyme was quickly inactivated, and after 30 minutes of incubation, barely 10% of its initial activity remained (Narra et al., 2014).

The addition of $CaCl^2$ increased the enzyme's thermostability from 150 minutes to 240 minutes at 50 °C and from 60 minutes to 120 minutes at 60 °C, respectively. Between pH 3.6 and pH 5.0, the enzyme maintained more than 95% of its activity; however, at pH 6.0, just 45% of its activity was still present. The enzyme maintained 90% activity for 60 minutes at pH 3.6 and 240 minutes at pH 4.8. After 60 and 120 minutes of incubation at pH 4.0, the enzyme preserved 70% and 50% of its initial activity, respectively (Narra et al., 2014).

The endoglucanase activity was increased by 38 and 35%, respectively, by the addition of $CaCl_2$ and $ZnSO_4$. While SDS, $CuSO_4$ and $MgSO_4$ had no impact. Additionally, KCl, NaCl, and $MnSO_4$ showed 50–70% suppression of endoglucanse activity, whereas EDTA and DTT had no impact. The lack of a DDT effect on enzyme activity clearly suggests that there are no disulfide bonds in the vicinity of the active site. Sodium hypochlorite, in general, is an oxidising agent, and Ag^2 ions have a considerable binding affinity for free sulphydryl groups in proteins and permanently limit the enzyme activity. Endoglucanse activity is not inhibited by the addition of EDTA, proving that the enzyme does not require divalent metal ions to function. The salting out of the substrate from the enzyme's active site as a result of a change in the solution's ionic strength may be the cause of KCl and NaCl's inhibitory effect on enzyme activity. The addition of Ca^2 ions increased the endoglucanse activity in the reaction test, which may be primarily the result of Ca^2 ions' interactions with the negatively charged amino acid residues aspartic acid and glutamic acid (Narra et al., 2014).

As a crucial factor in the commercial use of the enzyme, the impact of storing pure cellulase was examined. The enzyme demonstrated remarkable storage stability; after 5 months at 4 °C, it still maintained about 100% of its initial activity; however, there was a slight decline (3.5%). Up to 45 days at room temperature (30 to 35 °C), no appreciable activity decrease was observed (Narra et al., 2014).

A Lineweaver-Burk double reciprocal plot was used to obtain the Michaelis constant KM and maximum velocity Vmax using CMC as the substrate. In the results, low Km (7.375 mg ml-1) and high Vmax (1250 mmol min-1 mg-1) values demonstrated strong affinity for CMC substrate as shown in figure 10.

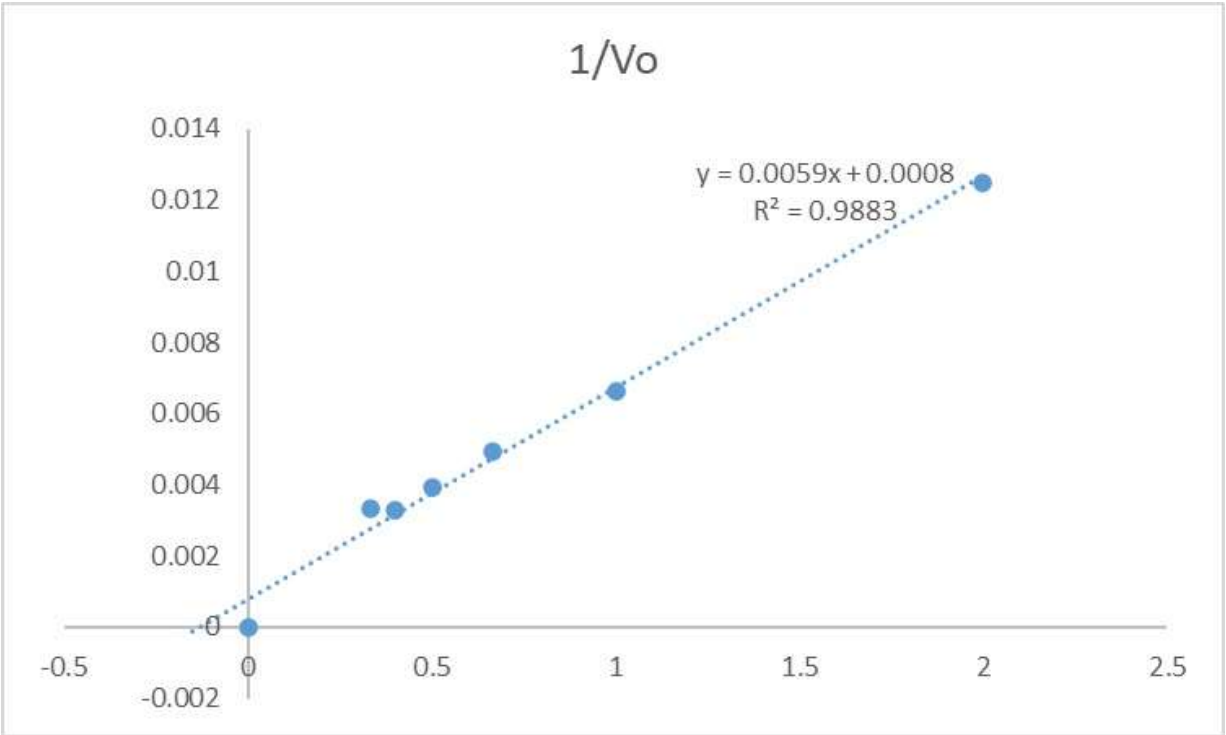


Figure 10: Lineweaver Burk double reciprocal plot of CMC

In Narra's work, the endoglucanase from *A. terreus* showed 16.15 mmol min^{-1} mg^{-1} as Vmax value and a 12.01 mg ml^{-1} as Km value. Low Km and Vmax values demonstrated strong affinity of enzyme for substrate (Narra et al., 2014).

Fungi produced other enzymes in addition to cellulase that's why protein content was high. Pure cellulase showed more activity than crude one. Purification is required for removing other protein contents (Novianty et al., 2022).

4 Conclusion

During the Production of cellulase, maximum production was achieved from Ficus benghalensis. The cellulase exhibited maximum substrate specificity for Ficus benghalensis compared to *Citrus maxima, Cydonia oblonga, Mangifora indica, Erythrophleum suaveolens, Saraca asoca, Litchi chinensis, Triticum, Tripidium bengalense* and *Psidium guajava*. The fungal screening showed that strain SJ1 which was later identified as *Aspergillus terrus* was most productive. It was identified by 18S RNA sequencing, and the sequence was published at NCBI with accession number GU226345.1. It was further optimized by the RSM method, which showed that enhanced production of 86.665 IU/min/mL could be achieved at the following parameters: incubation for 4 days, temperature (35ºC), substrate (6 grams), pH (6), moisture (90%), inoculum (5 mL), $(NH_4)_2SO_4$ (0.5 grams) and $MgSO_4$ (0.25 grams). Which could be further enhanced by $(NH_4)_2SO_4$ precipitation, membrane dialysis, and chromatography. The Km of the cellulase enzyme, was 7.375 mg/mL, and Vmax value was 1250 mmol min-1 mg-1. The enzymes could be further characterized and immobilized.

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