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Screening & Optimization of Fungal Strains for Cellulase Production

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Abstract

The hydrolysis of cellulose through the use of cellulase enzyme is one of the processes included in industrial sector for validation and processing of several other sectors. To mitigate its demand, several microbial sources, including fungus are in search mode to avail cellulase in surplus quantities. The study generally based on isolation, screening and identification of most potent fungal sources for cellulase bioproduction. In the study, out of a huge entity of 120 fungal isolate samples, through primary and secondary screening, six potent fungal isolates were selected and these were identified through microscopic and molecular characterization to be Aspergillus flavus (CSK), Aspergillus niger (K9), Aspergillus nomius (SK1) and Ganodermalucidum (K99). From the study, Aspergillus flavus was selected for cellulase production with optimizedcultural conditions like pH at 5 and temperature to 30°C with addition of lactose (1.5%) and yeast extract (1.5%) to the culture media. From the results, Aspergillus flavus provided highest proteins (0.8028 mg/ml), biomass (5.781 mg/flask), carboxymethylcellulase enzyme (114.56 IU/mL) and Filter paperase enzyme (52.56 IU/mL) at optimized conditions. The optimization methods would facilitate maximized quantity of cellulase production thereby validating the proposed method.

***Keywords:** Aspergillus flavus, Cellulase, primary screening, secondary screening, optimization.*

Introduction

The hydrolysis of cellulose is an enzymatic reaction actively requiring cellulase enzyme. However, in the industrial sectors, due to availability of commercial cellulose currently being quite expensive, several studies are going on to facilitate cellulase availability from easily available sources (like microbes). Depending upon the pattern and site of cleavage, cellulose is categorized into major subgroups namely endoglucanases, exocellulases and processive endoglucanases (Wilson, 2009). The structural classification of cellulose mostly relies on its structural diversity and varying mode of action. Most of the cellulose include three major types of enzymes such as cellobiohydrolase, β-glucosidase and β-1, 4-endoglucanase (Jecu, 2000; Teter, 2014; Obeng *et al.*, 2017). On the aspect of mode of action, the Cellobiohydrolases hydrolyze cellulose to produce soluble cellobiose; whereas the endoglucaneses are directed for internal cleavage of cellulose (Rahman *et al.*, 2018). The β-glucosidase initiates conversion of cellobiose to glucose. It mostly is a mixture of several fungal sourced cellulolytic enzymes with the presence of tunnel-like active domains at terminal side of cellulose (Teter, 2014). Most of the natural Cellulases are being produced and biosynthesised by several microorganisms like bacteria and fungi (Sharma *et al.*, 2017), especially from *Aspergillus niger, Aspergillus nidulans, Trichoderma, Stachybotrys, Chaetomium, Penicillium, Cladosporium* and *Aspergillus oryzae* (Sukumaran *et al.*, 2005; Chinedu *et al.*, 2011; Pradeep *et al.*, 2012).

Synergistic actions of the cellulolytic enzymes are reported to perfectly execute the complete hydrolysis of crystalline cellulose (Sari *et al.*, 2016). Cellulases are the third most substantial enzymes with wide economic applications. Individually or in combination these are actively incorporated in many industrial sectors of food, beverages, textiles and pharmaceutics (Jerusik, 2010; Kuhad *et al.*, 2011; Uzuner, 2019). It is also vital in production of fermentable lignocellulosics. Due to the cost expenses of cellulase enzyme production synthetically and as it has got major role in bio-production of several biological compounds (Howard *et al.*, 2003), hence studies required to be assessed in such a way so as to validate a novel technique for biosynthesis of cellulase from biological sources, especially from microbes (Rathnan *et al.*, 2012). The vitality of microbial cellulases has got enormous applications in several industrial sectorscontributing significantlyglobal enzyme sectors. For the improvisation of cellulase production rate along with maintaining its qualitymany microbial (fungal) strains are being studied (Lodha *et al.*, 2020).

Due to the structural diversity, hyphal heterogeneity, habitat diversity and nutritional specificity of fungus group; these are widely recommended and involved in extracellular synthesis of cellulase enzyme. In view of the biotechnological significance of cellulases, the present work was aimed at focusing on the step wise strategies by screening fungal isolates collected from different regions of Eastern Ghats of Andhra Pradesh for

production of xylanases (Naik *et al.*, 2020) for production of cellulase. Efforts were done for screening of larger collection of fungal isolates to optimize the production of cellulolytic enzymes with the best potential isolate by one factor approach at time.

Material and Methods

Collection of fungal samples

Fungal species were collected from the forests of Eastern Ghats situated in Rayalaseema, Andhra Pradesh. Fungal soils samples were collected from these spots. In total, 14 sites were identified; 8 sites from Kurnool District (Srisailam, Rudrakoduru, Mahanandi, Ahobilam, Gundlabrahmeswaram, Dornala, Atmakur and Bhairavakunta), 2 from Kadapa (Lankamalla and Idupulapaya) and 4 samples from Chittoor District (Tirupati, Bakarapet, Talakona, and Horsley hills) (Figure 1).

Isolation and culture of fungal samples

All the samples were cleaned properly followed by suspension in 0.5% spore suspension, serial dilution and culture in CMC agar media at 30 °C for 7 days. These were subcultured twice and then maintained on Czapek-Dox agar slants and MS medium and used for cellulase activity screening (based on zone of clearance and cellulase production).

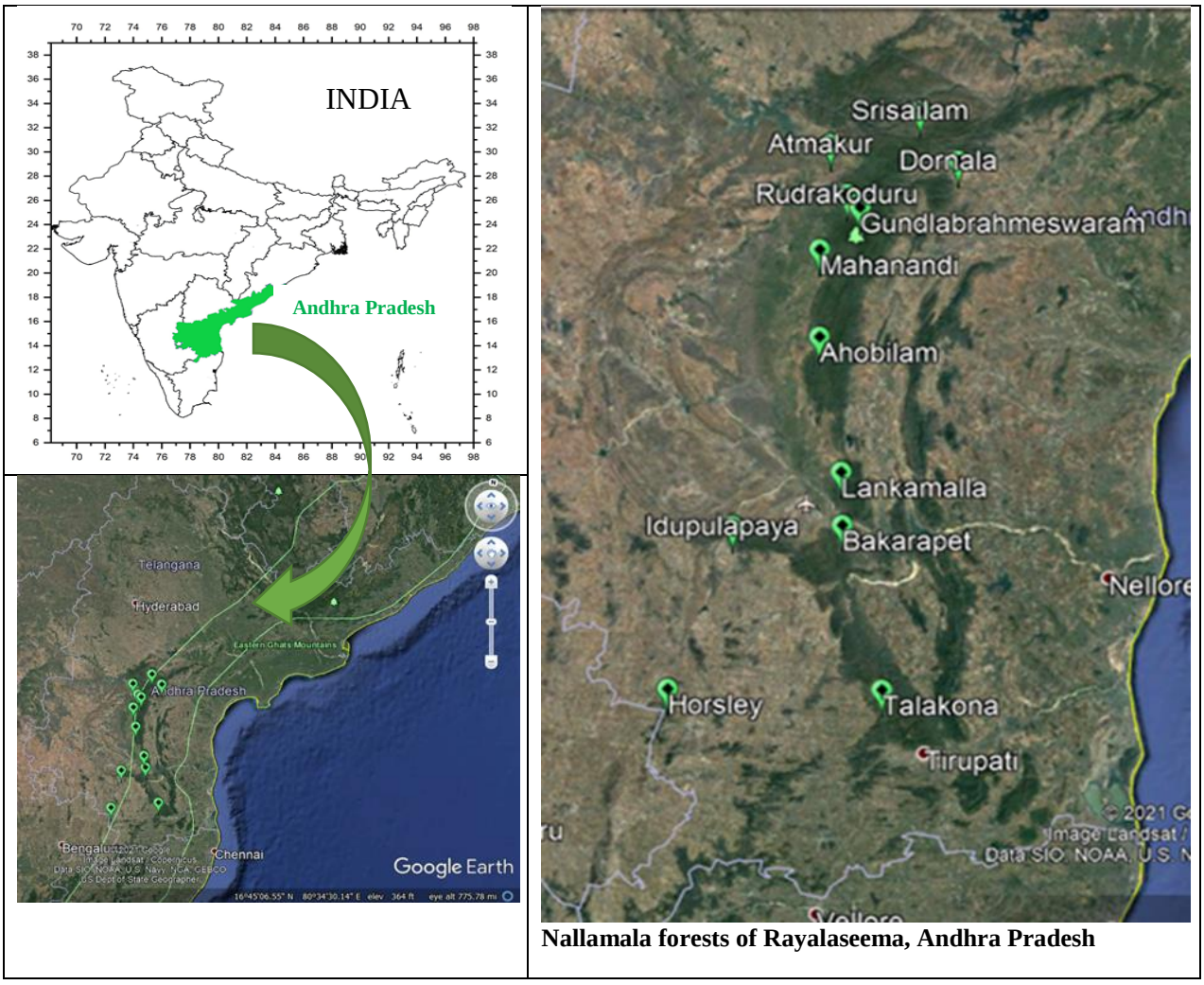

Figure 1: Fungal sample collection sites in Nallamala forests of Rayalaseema, Andhra Pradesh, India.

Primary screening

The medium used in this study for screening for cellulase enzyme production through submerged fermentation of fungal isolates is mineral salts amended with 1% carboxylmethyl cellulose medium (CMC-MSM). The agar plug of 5-day old culture was placed at MSM plate center and incubated at 30 °C for five days. Then the plates were flooded with 10-15 ml of 0.01% Congo red solution for 15 min followed by destaining with 1% NaCl for 20 min (Bradner *et al.*, 1999) and observed for clear zone around fungal colony.

Secondary screening

Secondary screening was conducted with the isolates having zone of clearance > 7.6 cm in primary screening. Cellulase production was further checked in submerged fermentation on MSM liquid medium with 0.1% CMC at 30 °C and 100 rpm for seven days. In view exhibition of maximum cellulase activity by the fungal isolates on MSM amended with CMC (Reddy *et al.*, 2015), broth of fungal isolates were withdrawn after seven days of growth and checked for cellulase and carboxymethylcellulase activity. These were also evaluated through microscopic analysis and molecular characterization.

Results and Discussion

Primary screening of fungal samples

A total of 120 fungal isolates (30 collected samples and 90 sustained samples) were subjected for primary screening process with estimation of zone of clearance for cellulose degradation (Supplementary data). Out of 120 isolates 36 fungal

isolates were selected considering the maximum zone of clearance (in cm). These selected fungal strains were termed as preselected fungal isolates (Supplementary data). Amongst these, 14 isolates were again selected with zone of clearance through indexing method (Mano *et al.*, 1993; Ribeiro *et al.*, 2014) given in (Table 1; Figure 2).

Figure 2: Selected 14 fungal isolates through primary screening

Secondary screening of fungal samples

Biochemical Identification

Cellulase production in submerged fermentation

The primarily selected 14 fungal isolates were further screened for secondary stage to narrow down the best suited fungal strains for cellulase synthesis on MSM with CMC under submerged fermentation. For this instance the quantity of cellulolytic enzymes (Carboxymethylcellulase, Filter paperase and β–glucosidase) by these fungal isolates were validated after 7th day of growth. Fungal isolates in submerged fermentation yielded Carboxymethylcellulase ranged from 32-91 IU/ml, Filter paperase ranged from 15-48 IU/ml and of β–glucosidase from 1.2-5.9 IU/ml in the current experiment. When trends in formation of three cellulolytic enzymes are examined, it is clear that except for Carboxymethylcellulase, the other two enzymes (Filter paperase and of β–glucosidase) did not show any distinct trend (Table 2).

Calculation of Evaluation indexing

Furthermore the enzymatic data were validated for calculation of Evaluation Indexing (EI) (Mano *et al.*, 1983). The average EI values validated the suitability of the concerned enzyme (s) with a value of > 50. For Carboxymethylcellulase (CMCase) the EI value of all 14 fungal isolates is more than 50 and hence considered for secondary selection. Similarly for Filter paperase (FPase), J5, SG3, K99, CSK, K9 and SK1 showed higher EI values and selected through secondary screening. Finally for for β–glucosidase (BGL), J5, Q12, F3, SG3, K99, CSK, K9 and SK1 showed higher EI values and selected through secondary screening (Table 2). Hence from this instant the above mentioned 6 fungal isolates were used for further screening.

Impact of extracellular protein, biomass and pH

The formation of extracellular protein and biomass was found to be the highest with fungal isolate, CSK. along with isolates namely SG3, K99, K9 SK1 and J5. Higher protein content was corroborated with higher cellulase producing rate as depicted in Table 2. Impact of change on culture media pH on the biochemical estimation of fungal isolates were validated and the results depicted the fungal isolates namely CSK, SG3, K99, K9, SK1 and J5 produced higher proteins and biomass within the pH range of 5.24-5.54 (Table 2).

Table 1: Primary screening of fungal samples (Zone of clearance through indexing method).

Sl. No.	Isolates	Cellulolytic enzymes (IU/ml)			EI Values		pH	Protein (mg/ml)	Biomass (mg/flask)
		Endoglucanase	Exoglucanase Cellulase	β-glucosidase	Average	SD (±)			
1	J5	68	32	4.2	53.3	2.476	5.54	2.825	0.624
2	Q12	64	28	3.8	51.2	1.39	6.59	2.142	0.4869
3	B41	68	22	2.9	47.4	2.928	5.51	2.984	0.5972
4	F3	65	23	3.6	49.3	3.108	7.52	2.732	0.5346
5	M1	66	21	2.4	46	3.924	5.02	3.723	0.5629
6	G8	53	18	2.1	44.4	5.222	4.62	1.629	0.4925
7	J3	56	15	1.8	42.9	6.62	5.03	1.367	0.591
8	G2	34	16	1.4	42.3	7.051	6.27	2.73	0.0862
9	P1	32	19	1.2	42.7	7.246	7.62	1.217	0.064
10	SG3	71	31	3.9	52.4	1.533	5.54	3.88	0.6789
11	K99	79	37	4.8	56.2	4.774	5.54	4.625	0.724
12	CSK	91	48	5.9	62	9.722	5.24	4.949	0.8199
13	K9	88	43	5.1	58.7	6.987	5.54	4.889	0.7897
14	SK1	74	39	4.9	57	5.499	5.54	4.725	0.7084

In screening procedures for competent fungal isolates, collection of fungal soil samples and their primary and secondary screening have become standard (KrushnaNaik *et al.*, 2018). Apart from screening of fungi through standard cellulolytic enzyme methods, other procedures such as application of evaluation index are practiced to further support the screening process. Evaluation indexing ads support scoring method. If more than one character is to be examined, it is highly difficult in judging on individual trait or character. In such case, the method of selecting organism based on cumulative or collective characters is more important.

Microscopic identification

In the present study, the data on secondary screening of successful fungal isolates from the preliminary screening process, analyzed for evaluation index are reported. Further, the successful fungal isolates in the second screening are identified through microscopic, molecular taxonomy and phylogenic procedures.

All the 6 fungal cultural isolates were verified and identified through morphological characterization to validate their structural position through microscopic identification on light microscope with attached d-winter 200 digital camera. Each isolates were treated with lactophenol blue stain prior to visualization under microscope for clear identification (Figure 3). The identification was based upon colony morphology and structure of conidia and conidiophores. From the photographic plates, fungal isolates (CSK, K9, SK1, K99, SG3 and J5) were as *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius*,*Ganodermalucidum*, *Parmotremacrinitum*, and *Fusarium* sp. respectively (Figure 3).

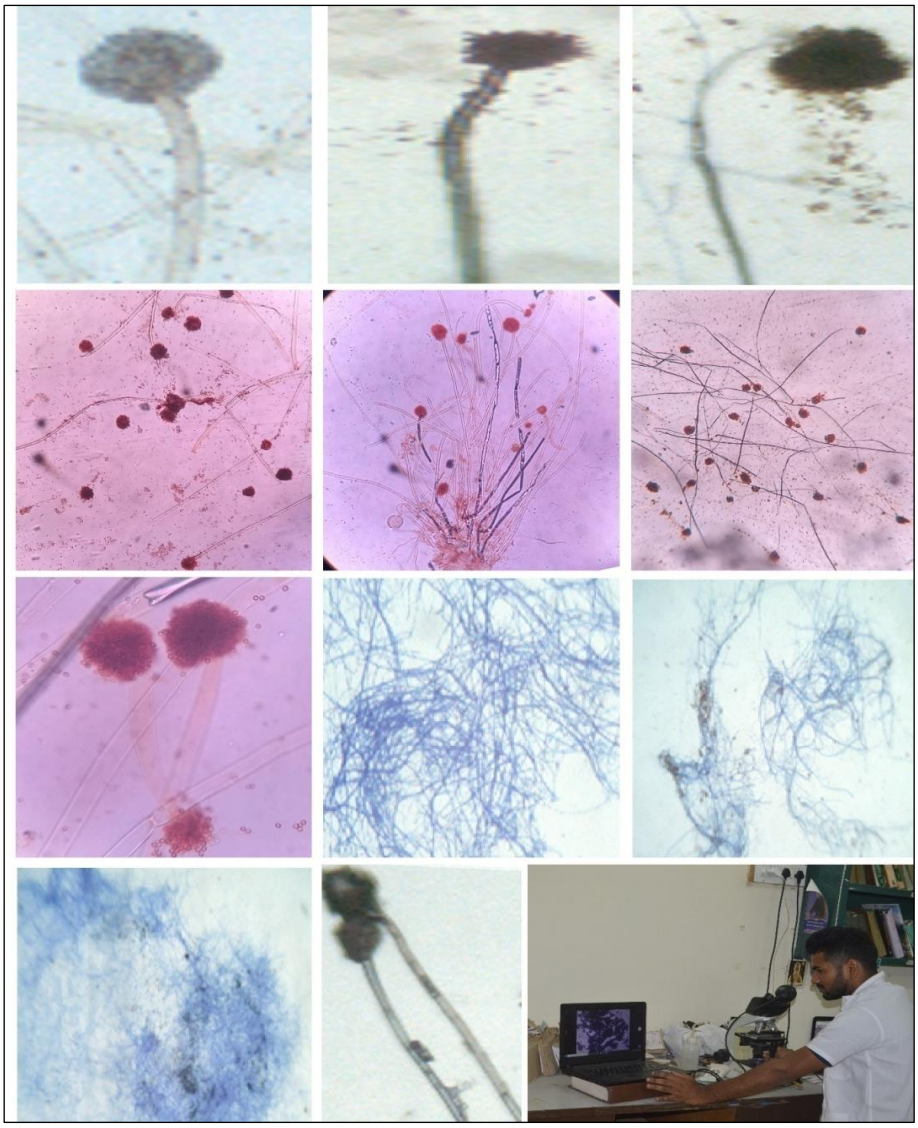

Figure 3: Microscopic photographs of fungal isolates.

Molecular characterization

However the isolate J5 was already being identified by Ramanjaneyulu (2015) and Ramanjaneyulu and Reddy (2016) already and hence was excluded, where as other 5 isolates were further screened and identified through molecular characterization (Sambrook *et al.,* 1989) through gene sequencing (Table 2) at Skanda Life Sciences Pvt., Limited, Bangalore and YaazhXenomic Laboratory, Coimbatore, Tamil Nadu. The five fungal isolates were identified on the basis of sequencing of Internal Transcribed Spacer (ITS) of ribosomal DNA (rDNA) (Romanelli *et al.,* 2014) and nucleotide sequences of these isolates as deposited at NCBI with accession numbers, MW776724 (for CSK), MW815505 (for K9), MW527405 (for K99), MW8543331 (for SK1). Furthermore, phylogenetic analysis was carried out with top 15 matches of the sequences by neighbor joining method (MEGA 6.0). The phylogenetic relationship was obtained by comparing the ITS region of rDNA sequences of the selected strains against the top 15 sequences obtained from NCBI-BLAST database through multiple alignment in Clustal W2 and Clustel Omega of *Molecular Evolutionary Genetics Analysis 6.0v* (MEGA 6.0v) (Borman *et al.,* 2008; Nilsson *et al.,* 2009; Fuerst *et al.,* 2015).

Table 2: Identified fungal cultures and their deposited accession No. in NCBI.

Sl. NO.	Code of Isolate	Name given to isolate	Accession No.
1	CSK	*Aspergillus flavus*	MW776723
2	K9	*Aspergillus niger*	MW815505
3	SK1	*Aspergillus nomius*	MW854331
4	K99	*Ganodermalucidum*	MW527405

From both the primary and secondary screening, amongst the remaining selected four fungal isolates, *Aspergillus flavus* (CSK) was selected for further optimization processes.

Optimization of cellulase production

Many of the microbes were reported to be capable of degrading cellulose, some of them produce significant amount of extracellular enzymes. Fungi are mostly favored than others due to versatile substrate utilization with penetration ability. Fungi like *Scopulariopsis, Trichoderma, Verticillium, Cladosporium, Penicillium, Aspergillus, Stachybotrys,* and *Chaetomium* have been validated for cellulase formation. Amongst all, *Trichoderma* and *Aspergillus* are frequently studied, in response to industrial and agricultural purposes (Chinedu *et al.,* 2011; Pradeep *et al.,* 2012; Singh *et al.,* 2019; Lodha *et al.,* 2020). Several studies have been carried out till now regarding optimization of different criteria for cellulase production by the application of different fungal isolates (Ahmed *et al.,* 2018).Exploiting the fungal material for cellulases started during 1980 and continued to various other specialized branches of investigation. The initial one refers to survey, collection and isolation of potential fungal isolates, studies on improving the cellulolytic enzymes, optimization of enzyme production and induction of mutation in promising fungal isolates for improving the strain(s) (Goukanapalle *et al.,* 2020). On this context the optimization of production of cellulase by *Aspergillus flavus* through providing suitable inputs such as environments, substrates etc, was carried out.

The optimized cultural conditions with related environmental factors play a pivotal role in better culture growth. The major components of production of bioactive compounds through cultural condition requires optimized parameters such as pH, time duration, carbon and nitrogen sources and temperature (Polyanna *et al.,* 2011). On this aspect the all total seven physic-chemical parameters were set to optimize its cellulase production rate. These were temperature, pH, carbon and nitrogen source, agitation speed, inoculum size and surfactants.

Effect of temperature and initial pH

The impact of change in temperature and initial pH on culture of *Aspergillus flavus* grown on MSM amended with CMC at different range on cellulase production can be monitored through regular monitorial of certain biochemical parameter alterations like protein and biomass content and/or Cellulolytic enzymes production (carboxymethylcellulase (CMCase), Filter paperase (FPase) and β-Glucosidase (BGL)) by *Aspergillus flavus.* The different temperatures taken were 25, 30, 35, and 40°C at 5°C intervals; whereas for initial pH, range is in between 4-6.5 at 0.5 intervals. Amongst all the temperature range at 30°C the highest synthesis of proteins (5.302 mg/ml), biomass (0.8965 mg/flask), carboxymethylcellulase enzyme (111.92 IU/mL), Filter paperase enzyme (51.05 IU/mL) and β-Glucosidase (5.91 IU/mL) was observed. Similarly when the initial pH ranges were optimized, at pH 5, the highest synthesis of proteins (0.8028 mg/ml), biomass (5.781 mg/flask), carboxymethylcellulase enzyme (114.56 IU/mL), Filter paperase enzyme (52.56 IU/mL) and β-Glucosidase (6.75 IU/mL) was observed (Table 3). All these parameters indicated mesophilic nature of the prospective organism.

Table 3: Effect of temperature and initial pH on cellulase production by *Aspergillus flavus.*

Biochemical parameters		**Temperature (°C)**				**Initial pH**					
		25	**30**	**35**	**40**	**4**	**4.5**	**5**	**5.5**	**6**	**6.5**
Protein (mg/ml)		4.432	**5.302**	4.164	3.913	0.3455	0.5676	**0.8028**	0.4569	0.367	0.2346
Biomass (mg/flask)		0.2346	**0.8965**	0.2334	0.1008	3..761	4.854	**5.781**	3.982	3.918	2.212
Cellulolytic enzymes (IU/mL)	CMCase	88.79	**111.92**	89.13	75.05	79.43 ± 0.52	98.45± 0.21	**114.56 ± 0.52**	98.56± 0.78	88.45± 0.83	81.76± 0.91
	FPase	42.11	**51.05**	45.37	23.82	32.45 ± 0.43	44.67 ± 0.21	**52.56 ± 0.64**	49.56 ± 0.51	41.45 ± 0.12	23.89 ± 0.82
	BGL	2.9	**5.91**	4.3	1.1	2.95 ± 0.12	3.14 ± 0.49	**6.75± 0.82**	4.86 ± 0.18	2.57 ± 0.44	1.62 ± 0.26

Effect of Carbon and Nitrogen source

The effect of supplementation of external additives as carbon source on cellulase production in *Aspergillus flavus* was studied. Carbon source such as maltose, sucrose, lactose, galactose, cellulobiose, dextrose and glucosein the study have shown different results. Biomass, proteins and cellulase secretion was observed on the 7th day of incubation (Table 4). Yields of cellulolytic enzymes were found to be superior with addition of lactose (1.5%) to the culture media. Therefore, lactose proved to serve as the best additional carbon source in enhancement of yields of cellulolytic enzymes; whereas maltose, cellobiose and glucose reduced the secretion of cellulolytic enzymes.

Table 4: Effect of Carbon and Nitrogen source on cellulase production by *Aspergillus flavus.*

Sl. No.	Carbon source	Protein (mg/ml)	Biomass (mg/flask)	Cellulase (IU/ml)			Nitrogen source	Protein (mg/ml)	Biomass (mg/flask)	Cellulase (IU/ml)		
				CMCase	FPaper	BGL				CMCase	FPase	BGL
1	Maltose	3.789	0.3453	98.56	41.45	5.15	Peptone	4.744	0.5745	79.43	33.45	3.91
2	Sucrose	4.678	0.4566	110.56	47.34	5.15	Beef extract	5.545	0.7894	98.45	41.56	5.13
3	**Lactose**	**5.821**	**0.9808**	**117.45**	**52.56**	**6.46**	**Yeast extract**	**6.165**	**0.9567**	**119.47**	**54.56**	**6.24**
4	Galactose	4.912	0.6787	109.45	41.67	4.58	Urea	5.564	0.5678	101.87	51.67	4.55
5	Cellobiose	3.803	0.5676	88.56	23.56	3.63	NH_4NO_3	4.346	0.4565	103.78	44.46	4.05
6	Glucose	2.812	0.3456	79.98	32.78	2.93	KNO_3	3.256	0.6581	99.77	36.89 ±	3.64
7	Control	2.701	0.3178	71.45	28.45	1.14	Control	2.89	0.4675	89.67	32.69	2.95

Significant production of biomass and proteins (Table 4) and component enzymes of cellulase were observed for *Aspergillus flavus*, when media supplemented by lactose. As majority of components of production medium were reported to be altering the rate of cellulase production therefore regulated to get a standardized optimal value for every isolate (Polyanna *et al.*, 2011). Our results were corroborated with the studies of Kathiresan and Manivannan (2006), Muthuvelayudham and Viruthagiri (2006) and Devanathan *et al.*, (2007) for use of lactose as carbon source. Even the addition of lactose and cellulose facilitated formation of FPase by *Penicillium echinulatum*on SmF (Ritter *et al.*, 2013). High levels of components of cellulase were reported by Naik *et al.*, (2020) also when supplemented by lactose.

Similarly effect of supplementation of external additives as nitrogen source on cellulase production in *Aspergillus flavus* was studied. Nitrogen source such as organic and inorganic nitrogen sources like peptone, beef extract, yeast extract, urea, NH_4NO_3, and KNO_3 in the study have shown different results. Biomass, proteins and cellulase secretion was observed on the 7th day of incubation (Table 6). Yields of cellulolytic enzymes were found to be superior with addition of yeast extract (1.5%) to the culture media. Therefore, lactose proved to serve as the best additional carbon source in enhancement of yields of cellulolytic enzymes; whereas others reduced the secretion of cellulolytic enzymes. Our results were corroborated with the studies of Prasanna *et al.*, (2016), Naik *et al.*, (2020) for yeast extracts, dos Reis *et al.*, (2015) for urea and Hayward *et al.*, (2000) for corn steep liquor. Utilization of nitrogen source by organisms depends on nutrients in medium provided. Urea supported growth and cellulolytic enzymes for *Aspergillus niger*on Czapek-Dox medium (Narasimha *et al.*, 2006). Naik *et al.*, (2020) reported high amounts of cellulase enzyme with *Fusarium*sp. when supplemented with yeast extract. In our study, yeast extract at 1.5% emerged as the optimal concentration for cellulase production for the potential culture.

Effect of surfactant, agitation and inoculum size

Similarly effect of surfactant on cellulase production in *Aspergillus flavus* was studied. Surfactants such as SDS, Triton X-100, Tween-20, Tween-80 and controlin the study have shown different results. Biomass, proteins and cellulase secretion was observed on the 7th day of incubation (Table 5). Yields of cellulolytic enzymes were found to be superior with addition of Triton X-100 to the culture media. Therefore, Triton X-100 was proved to serve as the best surfectant in enhancement of yields of cellulolytic enzymes; whereas others reduced the secretion of cellulolytic enzymes. Due to the structural diversity, hyphal heterogeneity, habitat diversity and nutritional specificity of fungus group; these are widely recommended and involved in extracellular synthesis of cellulase enzyme. To verify the effect of inoculum size on cellulase production in *Aspergillus flavus*, various sizes like 2 plugs, 3 plugs, 5 plugs, 7 plugs and 9 plugs were selected. Yields of cellulolytic enzymes were found to be superior with addition of 5 plugs inoculum size of fungal isolate to the culture media. Therefore, 5 plugs inoculum size was proved to serve as the best inoculum size in enhancement of yields of cellulolytic enzymes. Similarly to verify the effect of agitation rate on cellulase production in *Aspergillus flavus*, various agitation speeds like 100 rpm, 150 rpm, 200 rpm and 250 rpm were selected.

Table 5: Effect of surfactant, agitation and inoculum size on cellulase production by *Aspergillus flavus*.

Biochemical parameters		**Surfactants**					**Agitation (rpm)**				**Inoculum size (plugs)**				
		SDS	**Triton X-100**	**Tween-20**	**Tween-80**	**control**	**100**	**150**	**200**	**250**	**2**	**3**	**5**	**7**	**9**
Protein (mg/ml)		4.056	**6.7**	5.919	4.677	3.076	4.989	**6.688**	4.788	3.065	3.098	4.97	**6.519**	4.477	3.096
Biomass in (mg/flask)		0.4566	**0.9772**	0.8862	0.8993	0.4472	0.8707	**0.9795**	0.8884	0.4655	0.4565	0.8906	**0.9864**	0.8996	0.4478
Cellulolytic enzymes (IU/mL)	CMCase	87.45	**116.48**	98.99	88.9	77.23	98.45	**113.34**	99.56	89.34	87.34	98.56	**115.34**	99.34	89.89
	FPase	39.45	**55.67**	50.67	47.87	48.89	41.56	**56.65**	49.34	38.34	39.56	41.34	**55.45**	49.45	38.23
	BGL	3.96	**6.76**	5.24	4.67	3.99	5.43	**6.55**	4.67	3.48	3.94	5.11	**6.31**	4.52	3.92

Yields of cellulolytic enzymes were found to be superior with use of 150rpm agitation speed to the culture media. Therefore, 150rpm agitation speed was proved to serve as the best agitation speed in enhancement of yields of cellulolytic enzymes. These increased yields of CMcase, FPase, and BGL in culture broth of *Aspergillus flavus* inoculum of five plugs (Table 5) indicate the optimum sib of inoculum. Our results were corroborated with the studies of Techapu and Prosesor, (2003), Sanghi *et al.*, (2009), Kumar *et al.*, (2013) and Nailk *et al.*, (2020). Naik *et al.*, (2020) reported highest enzyme production with *Fusarium*sp at 3 plugs levels. However, the present study indicates that for *Aspergillus flavus*, five plug of inoculum size is essential. Patterns of growth of *Aspergillus flavus* cultivated with different inoculum sizes in the present study are parallel to the pattern of cellulolytic enzymes production of the culture of *Fusarium* sp cultivated (Naik *et al.*, 2020). Growth of *Aspergillus flavus* at agitation of 150 rpm also resulted in maximum production of cellulolytic enzymes. Biomass of the potential culture reached peak at agitation speed of 150 rpm, as also extracellular protein content (Table 7). In case of Sanghi *et al.*, (2009) and Kumar *et al.*, (2013) the highest rate of cellulase production by *B. pumilus* VLK-1 was achieved at an agitation rate of 200 rpm after 48 h of incubation. Similarly in case of *Streptomyces* species AB106, xylanase was produced at 150 rpm agitation (Techapu and Prosesor, 2003).

Conclusion

The fascinating structural diversity of fungi makes it an eligible candidate for biosynthesis of many natural compounds. The study depicted the method for isolation, screening and identification of most potent fungal sources for cellulase production. In the results, out of 120 fungal isolate samples, through primary and secondary screening four potent fungal isolates were selected and these were identified through microscopic and molecular characterization to be *Aspergillus flavus* (CSK), *Aspergillus niger* (K9), *Aspergillus nomius* (SK1) and *Ganodermalucidum* (K99). Amongst all of these finally *Aspergillus flavus* was selected for cellulase production with optimizing the cultural conditions as pH to 5 providing highest proteins (0.8028 mg/ml), biomass (5.781 mg/flask), carboxymethylcellulase enzyme (114.56 IU/mL), Filter paperase enzyme (52.56 IU/mL), agitation speed to 150 rpm and temperature to 30°C providing highest proteins (5.302 mg/ml), biomass (0.8965 mg/flask), carboxymethylcellulase enzyme (111.92 IU/mL), Filter paperase enzyme (51.05 IU/mL). Similarly the pH ranges was also optimized to pH 5, with addition of lactose (1.5%) and yeast extract (1.5%) to the culture media. The optimization methods would facilitate maximized quantity of cellulase production thereby validating the proposed method.

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