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Characterization of newly isolated strain *Aspergillus fumigatus* JCF from spoiled jackfruit and optimization of cellulase for bioethanol production using fruit waste

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Abstract

Cellulase is a major enzyme which can be used for the production of many important products. A major constraint in cellulase production is the cost of production, so the objective of the present investigation was to increase the yield of cellulase enzyme by using different low cost substrate and subsequently optimize the conditions for production. Nine fungal strains were investigated for cellulase production, which were isolated from agricultural waste. A potential strain for cellulase was selected by Congo red assay. *Aspergillus fumigatus* JCF, isolated from spoiled jackfruit, was found to be the most potent cellulase producer from the set of fungal strains isolated. This strain was identified using microscopic examination, ITS gene sequencing and neighbourhood joining method. Cellulase production was carried out in various fruit and vegetable waste and on a detailed analysis it was found that optimum production was obtainted when apple waste was used as substrate. So apple waste was selected as a substrate in the current study. The conditions required for the production of cellulase were optimized statistically, by response surface methodology (RSM). The optimized conditions were found to be 15.79 % of apple waste, 0.27 g l^{-1} ammonium sulphate, 6.1 pH and 4.85 days as the incubation period, with optimum cellulase activity of 2.87 IU ml^{-1}. Enzyme activity of 2.87 IU ml^{-1} was further used for the production of bioethanol through simultaneous saccharification and fermentation, using apple waste as substrate. A bioethanol concentration of 13.59g l^{-1} was obtained in the current study.

Key words

Apple waste, *Aspergillus fumigatus* JCF, Cellulase, Fermentation, Gene sequencing, Saccharification

Introduction

Cellulase is a group of enzymes which have the capacity to hydrolyze ligno-cellulosic materials to glucose. Glucose produced from cellulosic substrate could be further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol, butanol, methane, amino acid and single-cell protein (Walsh, 2002). Cellulase is mainly composed of endoglucanase or carboxymethyl cellulase (CMCase) (endo β-1, 4-glucanase), exoglucanase or cellobiohydrolase (exo β-1, 4- glucanase) and β-glucosidase (β-D-glucoside glucohydrolase). Cellulases have broad application in food (Kanmani *et al*., 2011), paper pulp, detergent, fuel production (Sukumaran *et al*., 2009) as well as waste management and pollution treatment.

In today's context large quantity of agricultural cellulosic wastes are available in the environment. If these wastes are utilized in an effective way large amount of cellulase can be produced. In recent years, a lot of work has been carried out towards efficient utilization of agro-industrial residues to produce enzymes of commercial importance by microorganism (Botella *et al*., 2007; Sun *et al*., 2010). Over the years, a number of microorganisms, particularly fungi, possessing cellulose-degrading enzymes have been isolated and studied extensively. Production of cellulase by bacterial species of *Bacillus,*

Cellulomonas, Erwinia, Acetovibrio and by fungal species like *Aspergillus, Trichoderma* etc., have been reported (Gao *et al.*, 2008).

The cost of fermentation media is found to be one of the major factors which influence the production of cellulase. Therefore using agricultural waste as raw material cost of production can be reduced to an extent. Different types of waste materials like sugar cane bagasse, apple pomace (Sun *et al.*, 2010) and mango peel (Neelima *et al.*, 2012) have already been used as raw material. But studies on the use of fruit waste as raw material is meager.

Environmental and nutritional factors are known to have marked effects on enzyme production by microorganisms. Optimal conditions for the production of cellulase were established by response surface methodology (RSM). Response surface methodology (RSM), using central composite design (CCD), is a combination of mathematical and statistical techniques used to identify the effects of individual variables, evaluate relative significance and determine optimal conditions.

The objective of the present investigation was to produce cellulase enzyme using different low cost substrates and to increase the yield by optimizing the conditions for production. Conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase, derived from cellulolytic organisms, has been suggested as an important process and opens the way for the production of biofuels, which offers potential to reduce the use of fossil fuels and reduce environmental pollution (Lynd *et al.*, 1999). The present study also aims at producing bioethanol using low cost substrate.

Materials and Methods

Isolation and storage of cellulase producing micro-organisms : In the present study, fungal cultures were used for the production of cellulase. All the cultures were isolated from natural sources which included of fruits and vegetable wastes. Morphological features of all the isolated microorganisms were studied under microscope (Rengim Eltem *et al.*, 2004; Samson *et al.*, 1994). Then colonies were stored at 4°C in czapex dox agar medium.

Screening of microorganisms for cellulase production : The media used for screening cellulase producing fungi included the following: 10 gm l^{-1} carboxy methyl cellulose; 0.2 gm l^{-1} $MgSO_4.7H_2O$; 1 gm l^{-1} K_2HPO_4; 1 gm l^{-1} KH_2PO_4; 1 gm l^{-1} NH_4NO_3; 0.05 gm l^{-1} $FeCl_3.6H_2O$; 0.02 gm l^{-1} $CaCl_2$ and 15 gm l^{-1} agar. Plates were inoculated with pure culture at the centre and incubated at 30ºC for three days. After three days, the plates were flooded with Congo red stain solution. The plates were kept as such for 15 min and thereafter washed with 1M NaCl. A clear hydrolysis zone was observed and diameter of inhibition zone was measured. Fungus which produced largest zone of clearance was then identified by using ITS gene sequencing and neighbourhood joining method.

Analysis of its (internal transcribed spacers) gene sequences of isolated microorganisms : From the sub-cultured fungal plate, a fungal mat was cut and ground into fine powder using liquid nitrogen with mortar and pestle. Genomic DNA of the fungi was isolated using CTAB method. Isolated DNA was then viewed by running in agarose gel electrophoresis.

A solution of nucleic acid strongly absorbs UV with an absorbance maximum of 260 nm and proteins at 280 nm which is linearly related with the concentration of DNA and RNA and the amount of contamination in the solution; the concentration of DNA in the fungal sample was calculated. Absorbance ratio should be 1.8 for pure DNA and between 1.7 and 1.9 for fairly pure DNA. If proteins are present, absorbance ratio will be less than 1.8 and ratio above 1.8 shows the presence of organic solvent.

PCR is an *in vitro* method of enzymatic synthesis of specific DNA sequence developed by Karymullis (1988). It is a simple and inexpensive technology for characterizing, analyzing, synthesizing, a specific DNA or RNA from virtually any living organism, plant, animal, virus or bacteria. It exploits the natural function of polymerase present in all living things to copy genetic material or to perform molecular photocopy.

Culture media for the production of cellulase : Apple waste was used as substrate for the production of cellulase. The sample was collected from a market in Chennai and the

Table 1 : Fungal cultures isolated from various sources

Isolate code	Source	Organism
P2	Rotten pomegranate	*Aspergillus sp.*
B1	Rotten banana	*Aspergillus niger*
O2	Putrid orange	*Aspergillus sp.*
J1	Decayed jackfruit	*Aspergillus sp.*
J2	Decayed jackfruit	*Aspergillus fumigatus*
C1	Putrid capsicum	*Aspergillus terreus*
On1	Onion	*Aspergillus niger*
G2	Garlic	*Aspergillus terreus*
Co	Corn	*Aspergillus sp.*

Table 2 : Screening of fungal cultures for cellulase production

Isolate code	Organism	Colony diameter (mm)	Hydrolytic zone (mm)
P2	*Aspergillus sp.*	30	10
B1	*Aspergillus niger*	55	24
O2	*Aspergillus sp.*	45	20
J1	*Aspergillus sp.*	35	Negligible
J2	*Aspergillus fumigatus*	35	30
C1	*Aspergillus terreus*	40	Negligible
On1	*Aspergillus niger*	35	Negligible
G2	*Aspergillus terreus*	30	Negligible
Co	*Aspergillus sp.*	47	18

Table 3 : Central composite design (CCD) and cellulase activity

Exp. No.	X_1 (%)	X_2 (%)	X_3	X_4 (DAYS)	Experimental cellulase activity (IU ml^{-1})	Predicted cellulase activity (IU ml^{-1})
1	15	0.2	6.0	4	2.29	2.42
2	25	0.2	6.0	4	3.73	3.28
3	15	0.4	6.0	4	2.19	2.21
4	25	0.4	6.0	4	3.12	3.27
5	15	0.2	6.4	4	2.35	2.41
6	25	0.2	6.4	4	2.89	3.07
7	15	0.4	6.4	4	2.24	2.00
8	25	0.4	6.4	4	3.00	2.96
9	15	0.2	6.0	6	2.02	1.95
10	25	0.2	6.0	6	1.94	2.27
11	15	0.4	6.0	6	2.05	1.97
12	25	0.4	6.0	6	2.78	2.60
13	15	0.2	6.4	6	2.62	2.47
14	25	0.2	6.4	6	2.73	2.59
15	15	0.4	6.4	6	1.97	2.30
16	25	0.4	6.4	6	2.76	2.73
17	20	0.3	6.2	5	3.54	3.53
18	20	0.3	6.2	5	3.57	3.53
19	20	0.3	6.2	5	3.54	3.53
20	20	0.3	6.2	5	3.54	3.53
21	15	0.3	6.2	5	2.73	2.67
22	25	0.3	6.2	5	3.27	3.31
23	20	0.2	6.2	5	3.27	3.33
24	20	0.4	6.2	5	3.38	3.29
25	20	0.3	6.0	5	3.46	3.46
26	20	0.3	6.4	5	3.54	3.51
27	20	0.3	6.2	4	3.49	3.53
28	20	0.3	6.2	6	3.24	3.17
29	20	0.3	6.2	5	3.43	3.53
30	20	0.3	6.2	5	3.46	3.53

Table 4 : Analysis of Variance for cellulase activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	6.74498	6.74498	0.481784	19.36	0.000
Linear	4	2.43037	2.43037	0.607592	11.81	0.000
Square	4	3.51984	3.51984	0.879960	17.10	0.000
Interaction	6	0.79478	0.79478	0.132463	02.57	0.068
Residual Error	14	0.72048	0.72048	0.051463		
Lack-of-Fit	10	0.71936	0.71936	0.071936	255.77	0.000
Pure Error	4	0.00113	0.00113	0.000281		
Total	29	9.73528				

DF- Degrees of freedom, Seq SS -Sequential sum of squares, Adj SS -Adjusted sum of squares, Adj MS – Adjusted mean square

surrounding area in sterile polythene bags. The sample was then brought to laboratory for further study. Apple waste extract was prepared by following a modified version of Adoki (2007). Substrate was dried ground, weighed, dissolved in water and kept in autoclave for 25 min at 121ºC so that all the nutrients were extracted in the media. The solution was filtered and some more minerals were added to this filtrate according to modified method of Mandels and Sternberg (1976) with a minimal composition containing: 0.2% KH_2PO_4; 0.03% $CaCl_2$; 0.02% $MgSO_4.7H_2O$; 0.5% Peptone; 0.1% Tween 80; 5 mg l^{-1} $FeSO_4.7H_2O$; 1.6 mg l^{-1} $MnSO_4.H_2O$; 1.4 mg l^{-1} $ZnSO_4.7H_2O$ and 2 mg l^{-1} $CoCl_2$. The pH was adjusted and autoclaved. Fungal strain maintained in agar slants was inoculated in presterilized media by washing slant cultures by sterilized water with 0.1% Tween 80 (Smith *et al.*, 1996). Media was then kept in a shaker at 160 rpm and 30ºC for five days.

Optimization of cellulase using statistical method : Optimization of cellulase production was done statistically by Response surface methodology (RSM). Independent variables chosen included of waste apple (X_1), ammonium sulphate (X_2), pH (X_3) and incubation time (X_4) while cellulase activity was dependant output variable. Response surface methodology using 4 factors, 3 level central composite design with a total of 30 different combinations was employed for study by *Aspergillus fumigatus* JCF, constructed by MINITAB version14. A polynomial quadratic equation was fitted to evaluate the effect of each independent variable. Statistical analysis of the model was performed to evaluate analysis of variance (ANOVA). The quality of polynomial model equation was judged statistically by coefficient of determination (R^2) and its statistical significance was determined by F-test. The significance of regression coefficient was tested by t-test (Tandyildizi *et al*., 2005; Zhou *et al*., 2009; Wang *et al.*, 2012).

Simultaneous saccharification and alcohol fermentation : Four flasks were prepared for alcohol fermentation indicating I, II, III and IV. I and III contained 10 g of apple waste on wet basis in 100 ml citrate buffer. II and IV flasks were taken with 0.1% yeast extract along with 10 g of apple waste. Out of the four flasks I and II flasks was added with 2.5 ml of cellulase. Flask no.III and IV was added with 5 ml of cellulase. Cellulase that was used in the study was having an activity of 2.88 IU ml^{-1}. Fermentation was carried out at 120 rpm and 30ºC. Sample was collected at regular interval of 12 hr and ethanol concentration was determined for each sample.

Analytical methods : Concentration of reducing sugars was determined by DNS method (Miller, 1959). Endoglucanase activity (CMCase) in reaction mixture containing 1.5 ml of 1% carboxymethyl cellulose in 50 mM sodium citrate acetate buffer (pH 4.8) and 1.5 ml of filtrate. The reaction mixture was incubated at 50 ± 2ºC for 10 min and the reducing sugar produced was determined by DNS method. To the reaction mixture, 3ml of DNS reagent was added and incubated for 15 min at 100ºC. The reaction mixture was cooled and 1 ml of Rochelle salt was added and OD was taken at 575 nm. One unit (IU) of cellulase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min. The amount of ethanol produced in fermentation media was estimated by using the dichromate method. One milliliter of cell free extract was diluted four times and 1 ml of potassium dichromate was added. After keeping all the tubes containing the above mixture in ice water, and 5 ml of concentrated H_2SO_4 was added gently through the walls and optical density was measured at 660 nm (Bennette., 1971, Balasubramanian *et al.*, 2011).

Results and Discussion

Isolated colonies were subjected to screening using Congo red assay and diameter of the zone of clearance was measured. Zone of clearance clearly indicates cellulase activity produced by the fungi. The fungal strain which produced largest zone was selected for cellulase production. Out of nine fungal isolates, five were screened for maximum production of cellulase enzyme. From five fungal isolates, (Fig.1 A), isolate from spoiled jackfruit, showed maximum production (Fig.1 B). This was selected as better cellulase producer (Krootdilaganandh, 2000).

Fungal DNA was isolated from fungal species present in spoiled jackfruit by CTAB method. The absorbance ratio of the sample was found to be 1.75, 1.82 and 1.78 which showed that the sample was pure. Isolated DNA was then subjected to

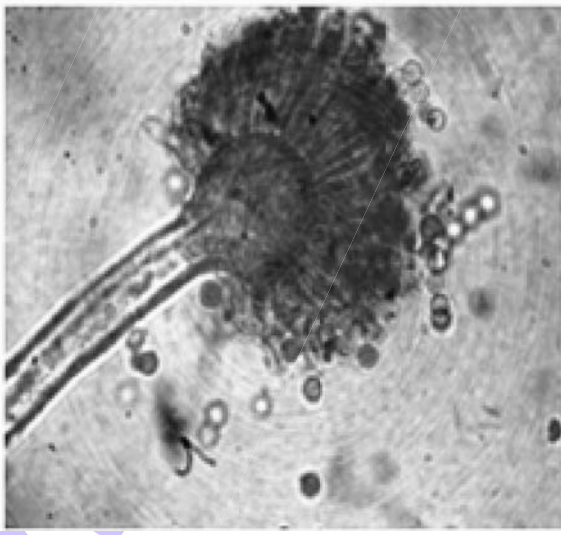

Fig. 1(A) : Microscopic features of *Aspergillus fumigatus* stained with lactophenol cotton blue

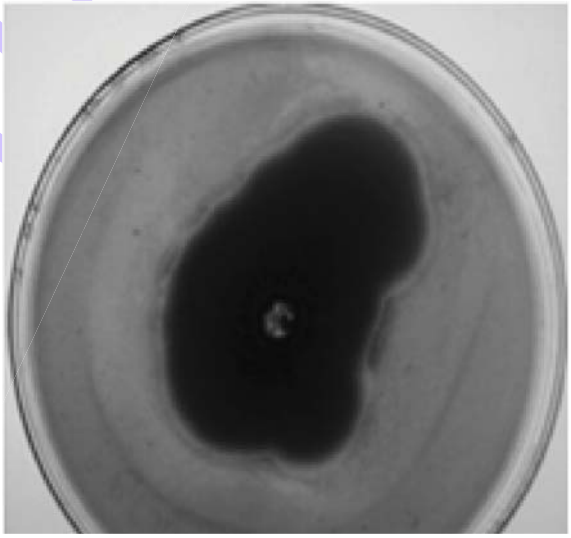

Fig. 1(B) : Cellulase production by *A.fumigatus* isolated from spoiled jackfruit

agarose gel electrophoresis and compared with 1kb DNA ladder. The results showed that the DNA isolated was pure and results visualized is shown in Fig. 2. Isolated gene was amplified and was run in agarose gel along with a DNA marker (Fig. 3). PCR reaction was performed for ITS gene. This amplified gene was then sequenced. The result obtained was compared with the already existing gene sequences in the National Centre for Biotechnology Information (NCBI). From the neighbourhood joining method analysis (Fig. 4) it was confirmed that isolated sequence was *Aspergillus fumigatus* JCF. The sequence was submitted to NCBI with sequence number KF541346.

The results of RSM experiments with different combinations of apple waste, ammonium chloride and pH on the production of cellulase by *Aspergillus fumigatus* JCF is shown in Table 3. By applying multiple regression analysis on the experimental data, the following second-order polynomial equations were obtained:

$$Y = 3.53+0.32X_1-0.02X_2+0.03X_3-0.18X_4-0.55X_2^2-0.05X_3^2-0.18X_4^2+ 0.08X_1X_2-0.05X_1X_3-0.13X_1X_4-0.05X_2X_3+0.06X_2X_4+0.13X_3X_4 \ldots\ldots (2)$$

Statistical significance of Eq. 2 was checked by F test. Each coefficient was checked for its significance by its P and F values. Model F value was calculated as ratio of mean square regression and mean square residual. F value of 9.36 and P value of 0 from ANOVA model showed that the model was significant (Table 4). P values in Table 4, shows that all linear and quadratic terms had significant effect ($p<0.01$) on cellulase production. Larger value of t test and smaller p-value shows high significance of the corresponding coefficient (Karthikeyan *et al.*, 1996, Tandyildizi *et al.*, 2005). Coefficient of regression (R^2) was found to be 92.6% for cellulase activity. Determination of coefficient of 92.6 % is in reasonable agreement with adjusted R^2 value of 84.7% (Md. Zahangir Alam *et al.*, 2008). These results indicated that the model was statistically sound.

The 2D contour plots are graphical representations of the regression equation with large number of combinations of two variables keeping the other two constant. The contour plots present a way for visualizing the relation between the

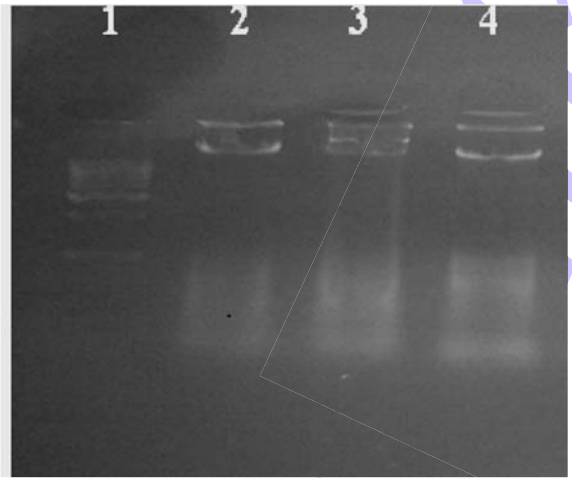

Fig. 2 : Isolated DNA in agarose gel (Lane 1: 1Kb Ladder (10,000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp; Lane 2: Genomic DNA of Fungi; Lane 3: Genomic DNA of Fungi; Lane 4: Genomic DNA of Fungi)

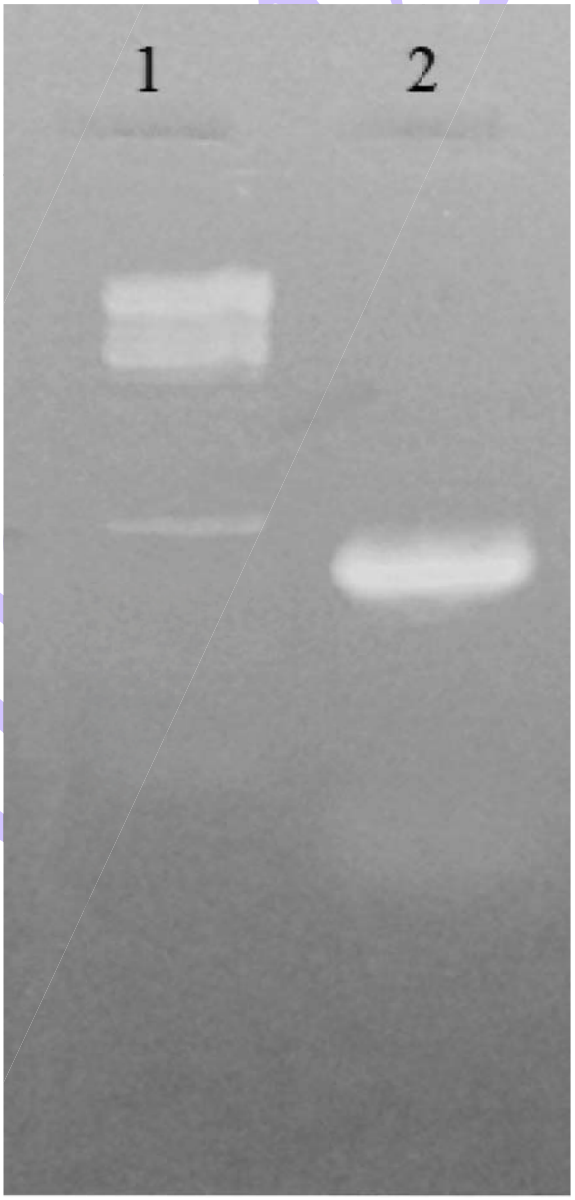

Fig. 3 : Isolated gene in agarose gel (Lane 1:1Kb Ladder (10,000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp; Lane 2: PCR amplified ITS region)

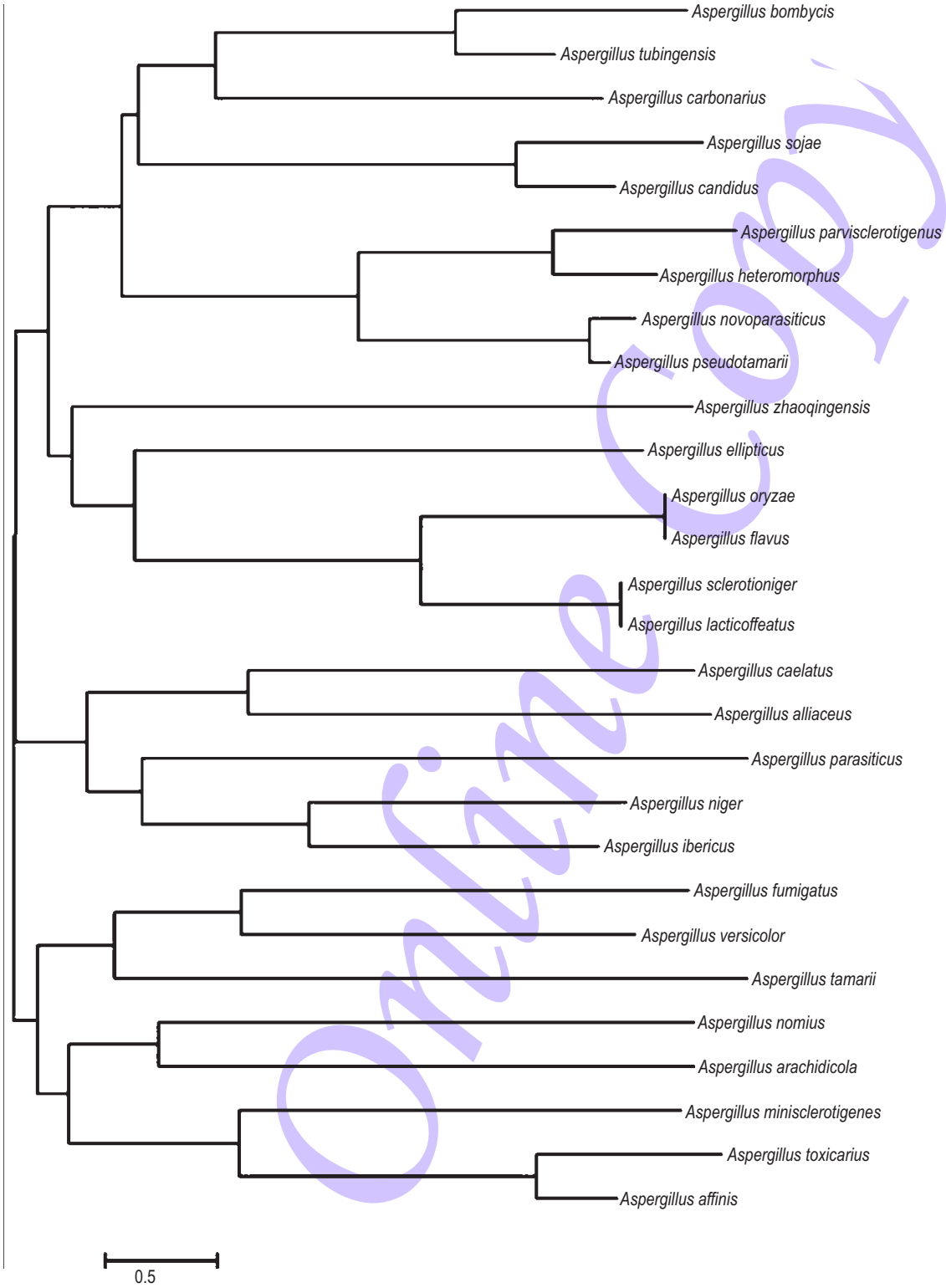

Fig. 4 : Neighbourhood joining tree based on ITS sequence of *Aspergillus fumigatus* JCF

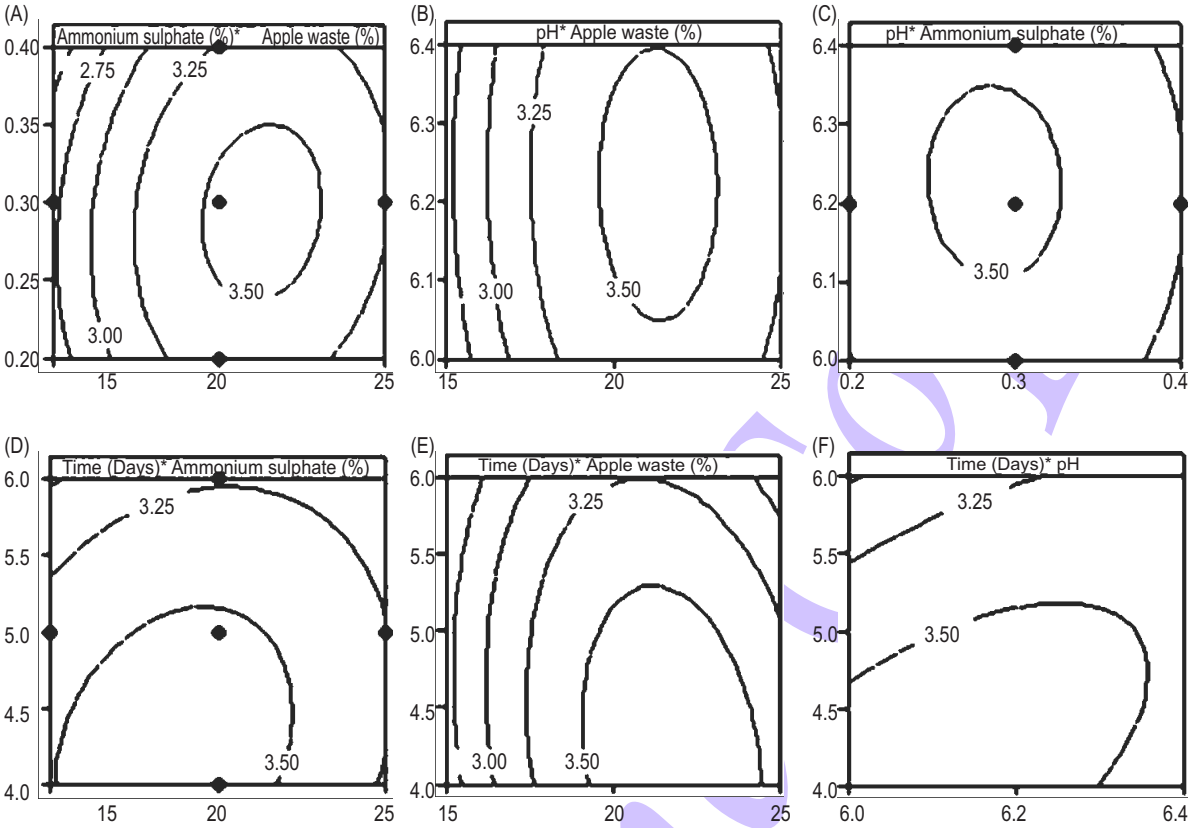

Fig. 5 : (A) Contour plots showing the interaction between Ammonium sulphate and apple waste; (B) Contour plots showing the interaction between pH and apple waste; (C) Contour plots showing the interaction between pH and Ammonium sulphate; (D) Contour plots showing the interaction between time and Ammonium sulphate; (E) Contour plots showing the interaction between time and apple waste; (F) Contour plots showing the interaction between time and pH

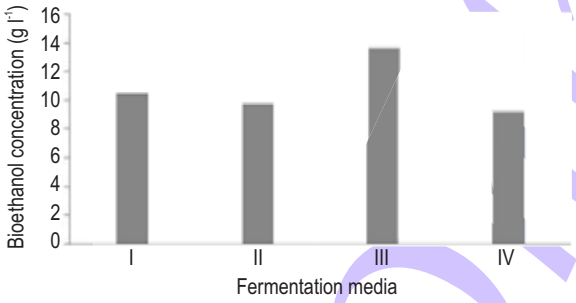

Fig. 6 : Bioethanol production (I-Apple waste + 2.5ml Cellulase, II-Apple waste + 5ml Cellulase, III- Apple waste + Yeast extract + 2.5 ml cellulase, IV- Apple waste + Yeast extract + 5 ml cellulase)

experimental and predicted values of different variables and type of interaction between the variables (Narra *et al.*, 2012). Fig. 5 shows 2D contour plots of cellulase for each pair of parameters by keeping the other two parameters constant. In graphical representation, circular contour plots indicated interaction between the corresponding variables as negligible, whereas elliptical shaped contour plots indicated that interaction between the corresponding variables as significant. Fig. 5 A shows relation between apple waste and ammonium sulphate, with maximum cellulase activity of 3.58 IU ml^{-1} at 21.52% apple waste and 0.29 % ammonium sulphate. Interaction between pH and apple waste is shown in Fig.5 B. Maximum CMCase activity was found to be 3.58 at a pH of 6.2 and 21.22 % of apple waste. Similarly cellulase activity of 3.53 IU ml^{-1} was found at 6.19 pH and 0.299 % ammonium sulphate (Fig.5 C). 3.53 IU ml^{-1} at 0.302 % ammonium sulphate and incubation time of 5.01 days (Fig.5 D), 3.65 IU ml^{-1} at 21.94 % of apple waste at 4.34 days (Fig.5 E) and 3.56 IU ml^{-1} at 6.20 pH and incubation time of 4.79 (Fig.5 F). Contour plots indicated interaction between apple waste and time and between pH and time. Maximum cellulase activity was seen at pH 6.2 according to Fig. 5B, C and F. The maximum cellulase production occurred near pH 6.1-6.2, when wheat bran-rice straw mixture was used as substrate (Das *et al.*, 2013). Optimization of incubation time is of prime importance for cellulase production by fungi. Reczey *et al.* (1996) observed maximum cellulase

synthesis was between 3 and 5 days, which is similar to the results obtained in the present study.

Thus, the optimum media composition required for the production of cellulase was 15.79% of apple waste, 0.27 g l^{-1} ammonium sulphate, 6.1 pH and 4.85 days. Validation of the experimental model was checked by carrying out the experiment in shake flasks under optimum combination of the variables obtained from response surface method. Three parallel experiments were carried out in optimized media and the results were compared. Cellulase activity obtained from the validation experimental study of 2.90 IU/ml was very close to the predicted value of 2.87 IU ml^{-1}. This proved the validity of the model.

In simultaneous saccharification and fermentation, enzymatic cellulose hydrolysis and glucose fermentation to ethanol by yeast proceed simultaneously within one vessel. Yeast *Saccharomyces* sp. is usually preferred due to its high bioethanol production efficiency than others. In the present study bioethanol production was tried on apple waste. The results of the ferementation study with enzymatically saccharified sample is shown in Fig 6. Maximum bioethanol concentration of 13.59 g l^{-1} was obtained with apple waste and peptone along with 2.5ml of crude cellulase enzyme (Fig.6). Crude cellulase enzyme extract produced by *Trichoderma reesei* CBS439.92 produced a final ethanol concentration of 5.0 g l^{-1} after 96 h of simultaneous fermentation and saccharification) (Lever *et al*., 2010). Thus simultaneous saccharification and fermentation is an efficient method for bioethanol production.

In the current study, out of the set of fungal cultures isolated, one isolated from spoiled jackfruit displayed maximum zone of clearance. This fungus was characterized by ITS gene sequencing and amplification. Identification was done by microscopic analysis and neighbourhood joining method and was confirmed to be *Aspergillus fumigatus* JCF. It was concluded that apple waste, which generally goes with the municipal waste can be effectively used as substrate for the production of cellulase enzyme. Ammonium sulphate was selected as nitrogen source for the study as it is cheap and effective. Optimum pH and incubation time was found to be 6.2 and 5 days respectively for cellulase production. Results of the current study were statistically good for the production of cellulase enzyme as experimental and predicted run showed close concordance. A bioethanol concentration of 13.59g l^{-1} was obtained during fermentation with apple waste. Thus from the current study, natural wastes can be successfully converted to useful products like cellulase and thereby, bioethanol.

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