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Study of Cellulase by Isolated Fungal Culture from Natural Resources and Application in Bio-ethanol Production

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Abstract: ***Cellulase are a group of hydrolytic enzymes capable of hydrolysing the most abundant organic polymer i.e. cellulose to smaller sugar components including glucose subunits. In the current study the cellulolytic fungi were isolated from natural resources like cattle dung contaminated soil & cattle dung samples for cellulase production. The samples were collected from 3 different areas of valsad region. The primary screening of cellulolytic fungi were adopted by exposing Carboxyl Methyl Cellulose agar (CMC), which is a selective media for cellulolytic microorganisms, the 14 fungal isolates were screened. The Further screening was carried out on Czapek-Dox agar medium amended with 1% CMC; among 10 efficient Cellulolytic fungi were showing the Cellulase activity. Depending upon the maximum diameter of clear zone produced in CMC agar medium & Czapek-Dox agar medium 2 different fungi CPF-3 & CPF-8 were selected for higher production of one of the most highly demanded industrial enzyme cellulase using cheap and easily available agro wastes as sole source of carbon. The production of cellulase was substantially enhanced by optimizing the several parameters like pH, temperature, substrate concentration; nitrogen sources and agro based materials were evaluated under both fermentation conditions such as Solid State Fermentation (SSF) & Submerged Fermentation (SmF). The highest cellulase production was recorded in SSF medium than SmF by CPF-8 fungal isolate by optimizing the culture conditions. The optimum conditions found for cellulase production were achieved at 30°C temperature, pH 5 with urea as nitrogen source and the agro waste material saw dust to stimulate the production of cellulase. After the fermentation process the enzymes were partially purified by ammonium sulphate precipitation method. The untreated substrate saw dust was hydrolysed with the partially purified enzyme cellulases and the hydrolysed product was fermented by the yeast for the bioethanol production. The cellulase activity was assayed by Carboxyl Methyl Cellulase "CMCase" assay & the protein estimation by Folin-Lowry method. The maximum cellulase activity of enzyme achieved by CPF-8 fungal culture 903.7 IU/ml in SSF & where as in SmF cellulase activity was achieved 800 IU/ml. The higher enzyme activity producer fungal isolate CPF-8 were further used for bioethanol production. After partial purification of enzyme the maximum enzyme activity was achieved in both fermentation condition at the 80% saturation about 192.59 IU/ml in SSF & 133.33 IU/ml was achieved in SmF medium. The untreated substrate saw dust hydrolysed by cellulase of SSF medium yielded at 120 hour of incubation period 2.05 mg ml^{-1} sugar which produced 1.4 g% ethanol on fermentation. The ethanol yield of the hydrolysate of substrate saw dust by cellulase of SmF medium lower about 1.2 g%. The without pre-treated substrate saw dust, hydrolysed with cellulase of SSF medium is established as suitable for bioethanol production.***

Keywords: ***Cellulase, Cellulolytic fungi, Cattle dung, Fermentation, Bio-ethanol.***

I. INTRODUCTION

Biotechnological conversion of cellulosic biomass is potentially sustainable approach to develop novel bioprocesses & products [7]. As the consequence of industrialization and rapid growth in population hike globally, utilization of natural resources has been increased exponentially during the past few decades [18]. The plant based woody biomass, lignocellulosic biomass is considered as a potential resource for renewable energy. In which the cellulose is the most important part of the plant cell wall & as well as marine algae. Cellulose, a polymer of glucose residues connected by β 1-4 linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature [17]. The cellulose is the most important dietary fibers of the human body i.e. non digestible by some mammals. The dietary fiber cellulose which is water insoluble compound that hydrolyzes by most significant enzyme cellulase. Cellulase catalyzes the conversion of insoluble cellulose to simple water soluble products. [2].Cellulolytic enzyme that catalyzed by number of microorganisms such as species of Bacteria (*Clostridium, Cellulomonas, Bacillus,* etc.), Fungi (*Aspergillus,*

Rhizopus, Penicillium, etc.) & Actinomycetes (*Thermomonospora, Thermoactinomyces,* etc.). In this study, the fungal cellulases were used for the production of an enzyme because the fungi are the principle agent involved in the degradation of lignocelluloses. The fungal cellulases are simpler than the bacterial cellulase. The fungal degradation of lignocelluloses is mainly accomplished by producing two types of extracellular enzyme systems: hydrolytic and oxidative catalytic systems [20].

Cellulase is a family of 3 groups of enzymes: endo-(1, 4)-β-D-glucanase (EC 3.2.1.4), exo-(1, 4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Cellulases have been commercially available for more than 30 years and these enzymes have represented a target for both academic as well as industrial research [24]. Cellulases have various industrial applications. Glucose produced from cellulosic substrate by hydrolysis could be further used as substrate for subsequent fermentation or other processes which could yield valuable end products such as ethanol [8], butanol, methane, amino acid, single cell protein, organic acids etc. Cellulase also used in pulp and paper industry, e.g., in deinking of fiber surfaces and improving pulp drainage [12]. Additional potential applications include the production of wine, beer and fruit juice. In the current study, by enzymatic hydrolysis of lignocellulosic biomass produce bioethanol. For the production of bioethanol one major problem facing the development of lignocellulosic biofuel is the cellulose hydrolysis [16]. So through the development of enzymatic hydrolysis decrease the ethanol cost. So this study examines the potential of lignocellulosic substrate i.e. saw dust hydrolyzed by fungal cellulases in the bio energy industry particularly in the production of bioethanol.

II. METHODOLOGY

A. Isolation for Cellulase Producing Fungi

Cattle dung & Cattle dung contaminated soil samples were collected from 3 different areas of valsad region. The strain isolation was done by serial dilution method & dilutions were spread on Carboxyl Methyl Cellulose Agar (CMC) plate. The following composition of CMC (g/L): $NH_4H_2PO_4$ – 1, KCl –0.2, $MgSO_4$.$7H_2O$– 1, Yeast extract – 1, CMC – 1%, Agar – 24. The pH of the medium was adjusted to 5 [16]. Then the plates were incubated at 28°C for 7 days and observed the clear zone around the colony. To visualize the hydrolytic zone around the colonies, the plates were flooded with about 10 ml of an aqueous solution of 1% Congo red staining solution & plates were shaken for 15 min. The staining solution was then discarded & washed with about 10 ml of 1M NaCl [21]. This is indicating the cellulase activity of the organisms. The fungal culture grown on the medium were sub cultured on sabraud dextrose medium for repeated times. Pure culture was transferred on to the sabraud dextrose agar slants and maintained at 4°C for further studies.

B. Screening of Cellulolytic Fungi

The isolated fungal cultures were further screened for their ability to produce cellulase enzyme. For the further screening Czapeak – Dox medium were used. The following composition(g/L): Sucrose – 30, $NaNO_3$– 2, K_2HPO_4– 1, $MgSO_4$– 0.05, KCl- 0.5, $FeSO_4$ – 0.01, CMC – 1%, Agar – 24. The pH of the medium was adjusted to 5. After autoclave and solidification of the plate the cavities of 8 mm size were made in the solidified medium and inoculated with 0.1 ml of fungal suspension prepared from 7 day old slants. The plates were incubated at room temperature for 3-5 days to allow fungal growth, then again incubated for 18 hour at 50°C which is the optimum temperature for cellulase activity. After incubation, 10 ml of 1% Congo red staining solution was added & plates were shaken for 15 min. The staining solution was then discarded & washed with about 10 ml of 1 N NaOH and shaken the plates for 15 min. After discarded the 1 N NaOH the plates were analysed by noticing the formation of clear or yellowish zones around the fungal spore inoculated wells [14].

C. Morphological Identification of Isolates

Fungal isolates were identified on the basis of primary morphological characteristic, done by mounting fluid lacto phenol cotton blue [14]. The higher zone producer fungi was identified by molecular characterization technique based on 18s rRNA sequencing method.

D. Production of Enzyme

1) *Inoculum Development:* The inoculum was prepared from 7 days old slant by adding 10 ml of sterilized 1% Tween 80 solution into it. The spores were scratched with the help of sterilized wire loop to make a homogeneous suspension of spores. The suspension was used as inoculums (10^7/ml) spores count by Haemocytometer [13].
2) *Submerged Fermentation (SmF):* Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of fermentation medium. The composition of the medium contained the following per litre of distilled water. Urea, 0.3 g; $(NH_4)_2SO_4$, 1.4 g; KH_2PO_4, 2.0 g; $CaCl_2$, 0.3 g; $MgSO_4$, 0.3 g; Peptone, 1 g; $FeSO_4$, 5.0 mg; $MnSO_4$, 1.6 mg; $ZnSO_4$, 1.4 mg;

$CoCl_2$, 2.0 mg and carboxymethyl cellulose (CMC) 10 g. pH of the medium were adjusted to 5. The medium was sterilized by autoclaving at 121ºC for 15 min. Each flask was inoculated with 1ml of the above said inoculum. The cultures were incubated on a rotary shaker (120 rpm) at 30ºC for 120 hour [1].

3) *Solid State Fermentation (SSF):* Solid state fermentation was carried out in 250 ml Erlenmeyer flasks that contained 5 g of lignocellulosic substrate- saw dust and 15 ml of mineral salt solution (moistening agent). The following composition of mineral salt solution (g/L): Urea, 0.3 g; $(NH_4)_2SO_4$, 1.4 g; KH_2PO_4, 0.4 g; $MgSO_4$, 0.3 g; Peptone, 0.75 g; $FeSO_4$, 0.05 g; $MnSO_4$, 0.01 g; $ZnSO_4$, 0.01 g; $CoCl_2$, 0.01 g, pH 5.0 [10]. The flasks were sterilized at 121ºC for 15 min and cooled to room temperature. About 1ml of inoculum was added, mixed well and incubated at 30ºC in static condition for 120 hour. The flasks were periodically mixed by gentle shaking.
4) *Enzyme Extraction:* At the end of the fermentation the culture broth from submerged fermentation was centrifuged at 6000 rpm for 15 min and the supernatant was used as a source of extracellular enzyme. In solid state fermentation (SSF) the enzyme was extracted from the saw dust by mixing homogenously the entire waste with (1:10 w/v) distilled water and agitated on a rotary shaker (120 rpm) at 30 ºC with a contact time of 1hour. Dampened cheese cloth was used to filter the extract and pooled extracts were centrifuged at 6000 rpm for 15min and the clear supernatant was used as a source of extracellular enzyme [28].

E. Assay for Cellulase Activity

1) *Endoglucanase activity- Carboxyl Methyl Cellulase (CMCase):* Carboxyl methyl cellulase activity was determined following the method of the International Union of Pure and Applied Chemistry [25]. An aliquot of 0.5 ml of cell free supernatant was transferred to a clean test tube and 0.5 ml of CMC (1% w/v) in 0.05 M Sodium citrate buffer (pH 4.8) was added. The tubes were incubated in water bath at 55º C for 15 min. After incubation 3 ml of DNS reagent was added to stop the reaction. The reactants in test tubes were incubated in boiling water bath for 10 min. After incubation librated sugars were determined by measuring absorbance at 540 nm in colorimeter. Enzyme activity was determined in terms of International Unit (IU) which is defined as an amount of enzyme that produces 1µ mole of glucose per minute.
2) *Protein determination:* Protein Concentrations in crude sample were determined by using Lowry method [27] with bovine serum albumin (BSA) as a standard.

F. Optimization of Process Parameters in SmF and SSF

The optimization for cellulase production was performed based on the modification of environmental and nutritional parameters. The influence of all factors on enzyme activity was determined by measuring cellulase activity at varying pH values from 4 to 9 and temperature varying from 25 to 50°C. The effects of substrate concentrations CMC including 1% to 5%. Various nitrogen sources such as urea, papaic digest of soybean meal, yeast extract & ammonium sulphate. To find out the suitability of agro based waste as substrate for enzyme production, different substrates, i.e. coconut coir pith, saw dust & wheat bran were taken.

G. Partial Purification of Enzyme with Ammonium Sulphate

Solid $(NH_4)_2SO_4$ was added to supernatant of both the medium to achieved 80% of saturation. The mixture was centrifuged and the precipitates of both the medium was dissolved in 0.05 M phosphate buffer at pH 7.0 [26]. Endoglucanase activity (CMCase) was determined by DNSA method.

H. Hydrolysis of Waste Biomass with Partially Purified Cellulase of the Isolated Fungi

About 0.05 M Citrate buffer (pH 4.8) (100 ml) containing untreated substrate saw dust, (10 % w/v) in 250 ml Erlenmeyer flask was inoculated with 10 ml of the partially purified enzyme filtrates of both the medium SSF & SmF and incubated at 50°C for 4 h. Samples (1 ml) were withdrawn aseptically from the flask at 1hr interval and analysed for reducing sugar by the DNSA method to determine the optimum time in hours for cellulase digestion of substrate. The hydrolysate was then used for fermentation to produce ethanol [29].

I. Fermentation of the Products of Waste Biomass Digestion to Alcohol

1) *Organism:* Baker's yeast, *Saccharomyces cerevisae* was purchased from a local area and cultured on yeast extract agar. Dried yeast sample (1 g) was measured into 10 ml sterile distilled water in Erlenmeyer flask. The flask was shaken rigorously for even distribution of the cells. An aliquot (0.1 ml) of the cell suspension was then used to inoculate yeast peptone dextrose agar medium. Plates were incubated at room temperature for 48 h. Colonies were purified by sub culturing on YPDA (Yeast Peptone Dextrose Agar) and pure cultures maintained on agar slants at 4°C [19].

2) *Inoculum Preparation:* Yeast peptone dextrose broth (100 ml) in 250 ml Erlenmeyer flask was inoculated with pure colonies of yeast from agar slant with the aid of an inoculating loop. This was incubated at 28-30°C on a gyratory shaker at 150 rpm for 48 h [19].
3) *Fermentation:* The fermentation broth (100 ml) comprised of (%w/v), peptone, 2; yeast extract, 2 and the product of hydrolysis of substrate saw dust as the fermenting sugar. The broth (80 ml) was filled into a 100 ml sealable bottle, sterilized in an autoclave and inoculated with 10 ml of the inoculums culture of yeast at 3.8 x 10^6 cells ml^{-1}. The bottles were sealed with the aid of an adhesive tape and incubated at 28-32°C for a period of 8 to 48 h. Bottles were removed at every 24 h interval to determine the amount of ethanol produced and the residual sugar in the medium. Alcohol was produced by distillation process [19]. The fermented broth was assayed for ethanol using Dipotassium chromate method [30].
4) *Determination of Residual Sugar in the Fermentation Medium:* The amount of sugar in the fermentation medium after each period of fermentation was determined following the DNSA method of Miller (1959). Dinitro-salicylic acid (DNSA) reagent (1 ml) was added to an aliquot (1 ml) of the fermentation medium in a test tube and properly mixed. The mixture was boiled for 5 min and cooled under running tap water. 5 ml of 40 % Rochelle salt solution was added to the mixture and absorbance was read in colorimeter at 540 nm. Amount of reducing sugar was read off a curve of glucose standard and expressed as mg ml^{-1} [31].

III.RESULTS AND DISCUSSION

A. *Isolation And Screening Of Cellulase Producing Fungi*

Total 14 fungal isolates were isolated & identified. Out of 14 fungal isolates total 6 fungal isolates were isolated from cattle dung and 2 were isolated from cattle dung contaminated soil. Other 6 were previous year student's isolates. The fungal isolates were grown on the selective media CMC. Out of 14 fungal isolates, only 2 fungal strains were observed to have highly positive zone activity based on their growth and the diameter of clear zone which is determined by hydrolytic zone using Congo red.
The results were showing in the following Figures and Table.

TABLE I
Showing fungal isolates on to the medium

Sr. No.	Nature of Sample	No. of Fungal Isolates
1	Cattle dung contaminated soil	2
2	Cattle dung	12

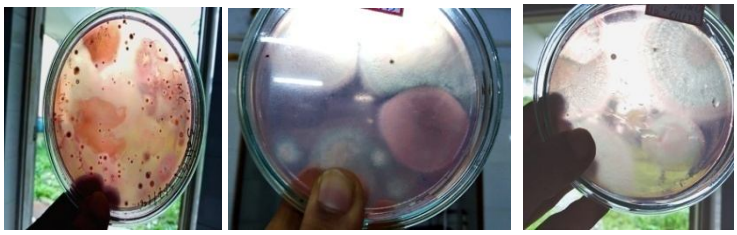
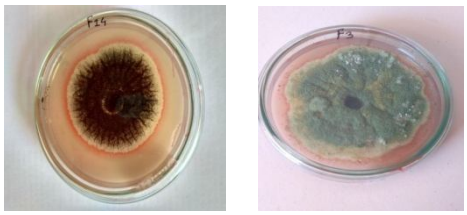


Fig. 1 Fungal isolates

On the basis of their zone size the fungal isolates were selected for further studies. The fungal strain CPF-8 showed highest zone of clearance as 7 mm of colony diameter. The highest zone producing fungi were further used for the enzyme production & other analysis. The results were as shown in Fig. 2 & Table 2.



CPF- 8 CPF- 3

Fig. 2 Fungal isolates showing cellulase activity

TABLE II
Zone of clearance around the fungal strain

Sr. No.	Fungal Isolates	Zone of Diameter (mm)
1	CPF 3	5
2	CPF 8	7

B. Optimization of Fermentation Parameters in both SSF & SmF

1) *Effect of pH on Enzyme Production:* The CPF-8 higher zone producer fungal isolates were allow to grow in both SSF & SmF medium at different pH ranging from 4 to 9. The maximum enzyme activity was observed in SSF medium than the SmF medium at the pH range 5. The pH of the medium plays important role for the production of the enzymes. The results were showing in the following Fig. 3.

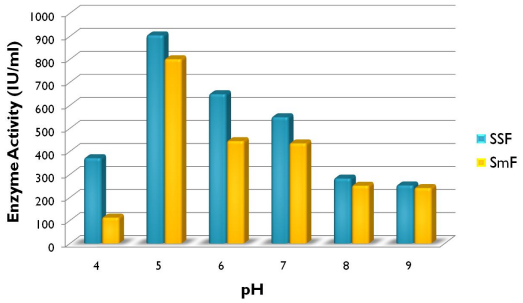


Fig. 3 Effect of pH on cellulase activity

2) *Effect of temperature on enzyme production*: The maximum enzyme activity was recorded at different temperature revealed that fungal isolate yielded maximum cellulase production in both the culture condition in SmF & SSF at 30℃ (Fig. 4). The temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane [22].

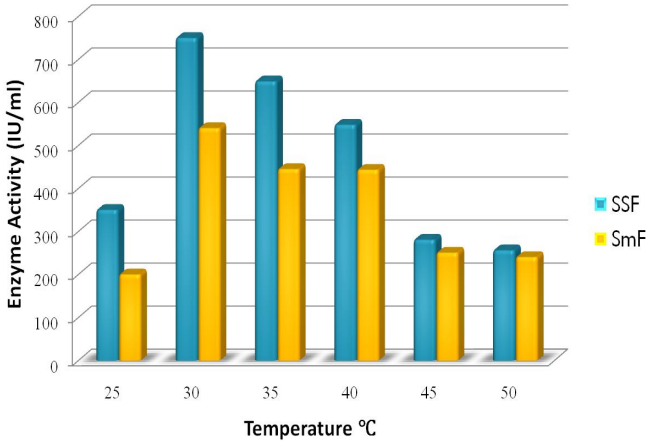


Fig. 4 Effect of temperature on cellulase activity

3) *Effect of Nitrogen Source on Enzyme Production:* Production of extracellular enzyme has been shown to be sensitive to repression by different carbohydrates and nitrogen sources [22]. The various nitrogen sources which are tested such as urea, papaic digest of soybean meal, yeast extract & ammonium sulphate. Among the various nitrogen source tested in both the fermentation medium urea was found to be the best nitrogen source for the production of cellulase enzyme. Nitrogen is one of the major cell proteins and urea is the good source of nitrogen which is the best source for the production of higher yield of enzyme.

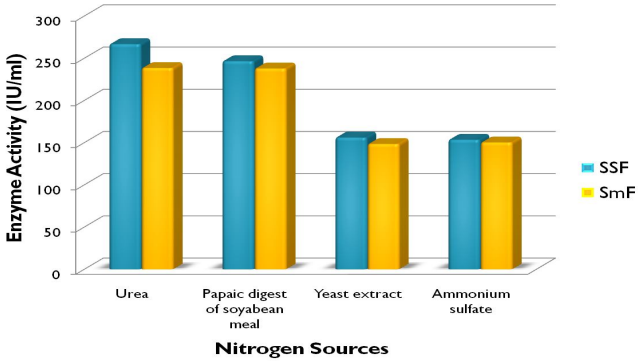


Fig. 5 Effect of nitrogen source on cellulase activity

4) *Effect of Substrate Concentration on Enzyme Production:* The estimation of enzyme activity was studied by varying the substrate concentration (Fig. 6). Different concentrations of CMC (Carboxyl Methyl Cellulose) were used for the enzyme production ranging from 1 to 5% in the both fermentations medium. At 1% substrate concentration were optimized maximum productions of cellulase.

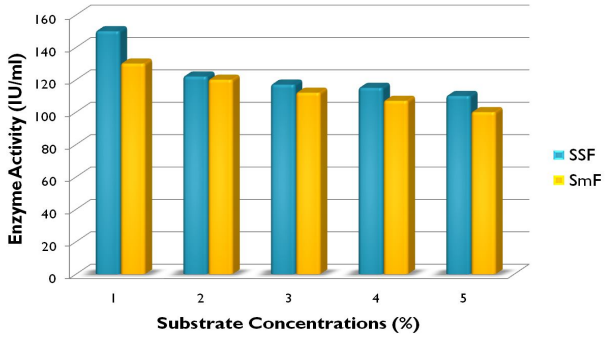


Fig. 6 Effect of substrate concentration on cellulase activity

5) *Effect of Agro Based Waste Materials on Enzyme Production*: The effect of agro based by products as alternative substrate on cellulase production was studied under both the fermentation conditions. In this study, the saw dust was found to be the best inducer of cellulase product

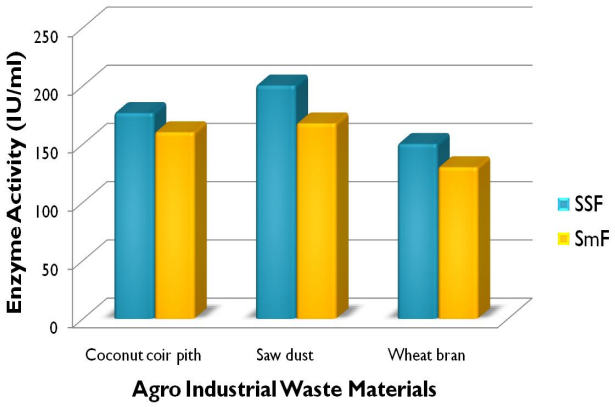


Fig. 7 Effect of agro based waste materials on cellulase activity

C. Enzyme Activity Assay

The crude enzyme filtrate was utilized for the determination of enzyme activity. The cellulase activity was measured by DNSA method [31]. The maximum enzyme activity achieved 903.7 IU/ml in SSF & where as in SmF the cellulase activity was achieved 800 IU/ml. of crude was achieved at 120 hour incubation period in both the fermentation conditions. The results were shown in fig. 8.

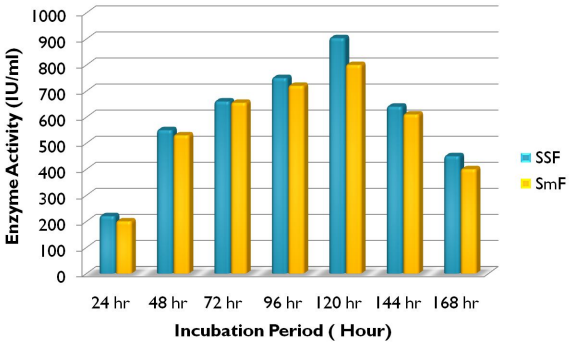


Fig. 8 Endoglucanase activity

D. Partial Purification Of Cellulase

Partial purification of cellulase enzyme was carried out by ammonium sulphate precipitation method. The maximum enzyme activity was achieved at 80 % saturation in SSF medium 192.59 IU/ml & where as in SmF cellulase activity was achieved 133.33 IU/ml. The partially purified enzymes were used for bioethanol production.

E. Sugar Yield From Hydrolysis Of Substrate Saw Dust With Partially Purified Enzyme Cellulase

The maximum yield of 2.05 mg ml^{-1} of sugar was produced on hydrolysis of untreated substrate saw dust with cellulase within 4 hr in SSF medium compared to SmF medium. In the SmF medium yield of 1.86 mg ml^{-1} sugar was produced by the hydrolysis of the substrate. The results were as shown in Fig. 9.

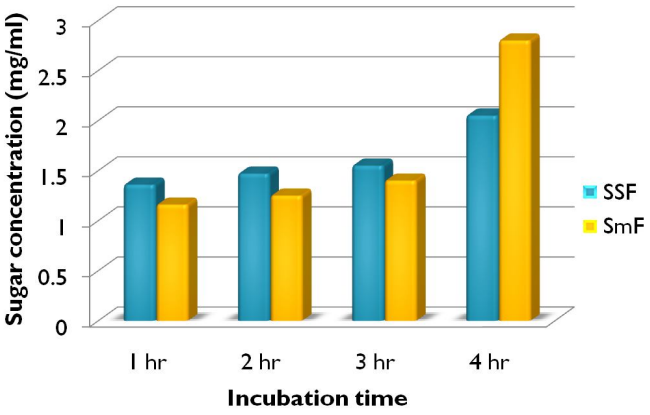


Fig. 9 Time period for the hydrolysis of substrate with partially purified cellulase

F. Ethanol Production Yield

Fermentation of the sugar (product of enzymatic hydrolysis of substrate saw dust) by the yeast, yielded ethanol in accordance with the sugar concentration obtained. Substrate saw dust was hydrolysed by cellulase of the SSF fermentation medium yielded, maximum ethanol production 1.4 g%. By utilizing the hydrolysate of SmF medium yielded, 1.2 g% ethanol was produced. As were shown in the following Fig.10. Fermenting yeast, however, consumes sugar for growth and production of other metabolic products. Moreover since growth commences during the aerobic phase, some amount of sugar gets used up before the anaerobic stage which is characterized by ethanol production [19].

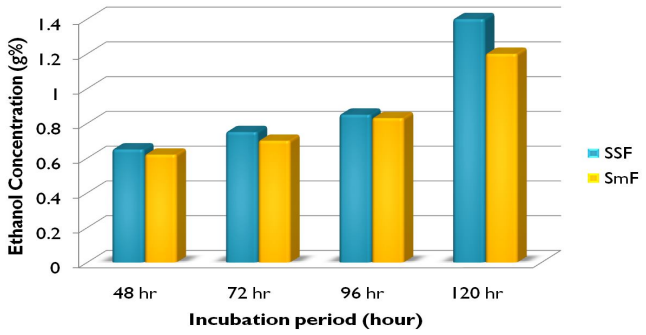


Fig. 10 Time period for ethanol production by hydrolysis of substrate saw dust with cellulase

IV. CONCLUSIONS

The present work was carried out to optimize the nutritional and environmental parameters for improving cellulase production by cellulase producing isolated fungi. Because the fungi are the principle agent involved in the degradation of lignocellulose. From this present study, the result showed that cellulase producing fungi CPF-3 & CPF-8 can grow at optimized condition and characterized by morphological identification & partial purification. In this present study the enzyme were produced by both the culture conditions such as Solid State Fermentation (SSF) & Submerged Fermentation (SmF). Among this condition maximum enzyme production was achieved by cost effective technology solid state fermentation. That is a suitable technology for economical production of cellulase by using lignocellulosic residues saw dust as substrate. The saw dust was affirmed as a good substrate for energy production particularly in the production of biofuel [19]. The partially purified enzyme converts cellulose into fermented reducing sugar by yeast to produce ethanol. Overall it concluded that saw dust was hydrolysed by cellulase of cellulase producing fungi was most productive in terms of ethanol yield and can therefore be increased in the production of bioethanol. The future aspects of this study are by using waste lignocellulosic materials for enzyme production may reduce the production cost and easily applicable for industrial perspective. Biofuel may include many benefits that reduction of greenhouse gas emission & reduce environmental pollution.

V. ACKNOWLEDGMENT

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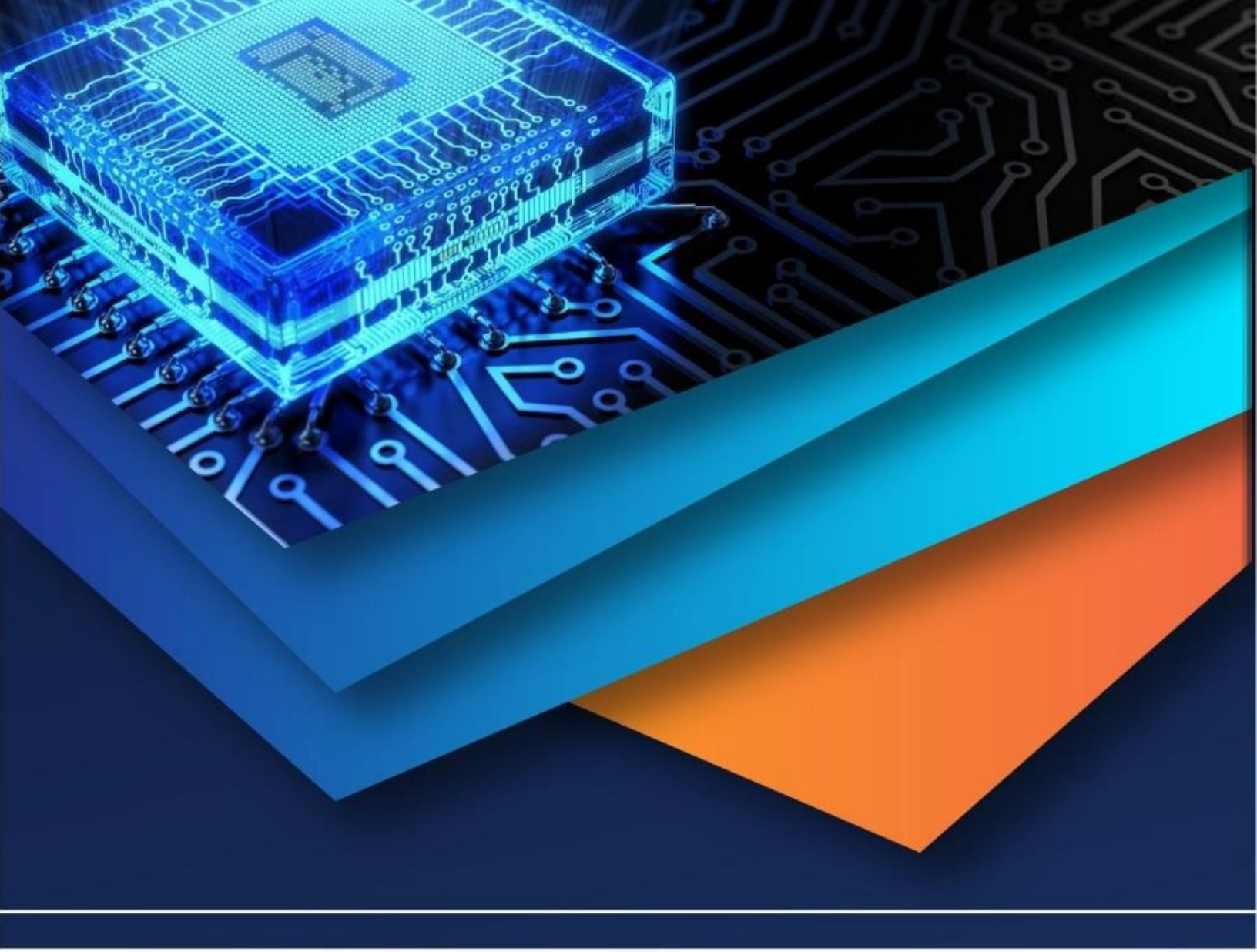

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