Int.J.Curr.Microbiol.App.Sci (2018) *7*(2): 429-445

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 02 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.702.055

Role of *Aspergillus flavus* on Biodegradation of Lignocellulosic Waste Millet Straw and Optimization Parameters for Enzyme Hydrolysis and Ethanol Production under Solid State Fermentation

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Keywords

Lignocellulosics waste, Millet straw, Cellulase, *Aspergillus flavus*, CMC agar medium, Solid state fermentation, Endoglucanase, Saccharification, *Saccharomyces cerevisiae*

Article Info

Accepted:
08 January 2018
Available Online:
10 February 2018

A B S T R A C T

Amongst six natural lignocellulosic waste/raw material screened, millet straw contained maximum cellulose (30 %) and physical and chemical pre-treatment yielded maximum cellulase activity. The composition of millet straw showed 30 % cellulose, 10 % hemicellulose, 8.73 % total organic carbon, 0.7 % total nitrogen and 0.8 ppm phosphorous. Amongst four fungi of genus *Aspergillus* isolated from local soil dumps of kitchen and garden waste, *Aspergillus flavus* showed maximum cellulase activity on CMC agar medium and used for degradation of millet straw. Amongst five media, Mandle and Sternberg fermentation medium showed maximum enzyme activity due to favorable pH and supplementing nitrogen and mineral requirements for growth and development of *Aspergillus flavus.* The optimum parameters for maximum cellulolytic enzyme activity in solid state fermentation were also studied which showed that 1 ml inoculum size, 4.5 pH of the medium, incubation at 28 °C and 70 % moisture content for 120 h, 3.0 % w/v substrate concentration, 0.1% supplementation of carbon source in form of Carboxy Methyl Cellulose and 0.5 % of nitrogen source in form of ammonium sulphate found effective for better endoglucanase activity. The hydrolysate was subjected to saccharification (fermentation through yeasts *Saccharomyces cerevisiae*) having potential of producing 0.15 g/hour/liter of ethanol at above optimized parameters.

Introduction

Besides growing demand of lignocellulosic materials for traditional applications (paper manufacture, biomass fuels, composting, animal feed, *etc*.), novel markets for conversion of lignocellulose to alternative energy carriers (*e.g.* bioethanol, acetone and butanol) are also well recognized (Kaylen *et al.,* 2000; Lee, 1997; Mitchell, 1998; Wheals *et al.,* 1999). Cellulose, hemicellulose and lignin are the main constituents of lignocellulosic materials (Deobald and Crawford, 1997). Agricultural resources of lignocellulosic waste are quite abundant as estimated by the Food and Agriculture Organization (FAO), USA. Around 2.9 £ 103 million tons from cereal crops and 1.6 £ 102 million tons from pulse crops, 1.4 £ 10 million tons from oil seed crops and5.4 £ 102 million tons from plantation crops are produced annually worldwide (Rajaram and Verma,

1990).The chemical composition of native agricultural waste obtained from plants differs considerably and is influenced by genetic and environmental factors. Amongst various types of natural lignocellulosic raw materials having varying amounts of cellulosic components; lignocellulose is a complex substrate and its biodegradation is not dependent on environmental conditions alone, but also the degradative capacity of the microbial population (Waldrop *et al.,* 2000). Most fungi are capable cellulose degraders. However, their ability to facilitate rapid lignocellulose degradation attracted attention from scientists and entrepreneurs alike. Therefore, the bioconversion of large amounts of lignocellulosic biomass into fermentable sugars has potential application in the area of bio-energy generation. Easy, cheap and abundant lignocellulosic raw materials having high cellulose content and resultant/ultimate high enzyme bio-synthesis has potential in bioconversion of cellulose into fermentable sugars and is abio-refining area that has invested enormous research efforts, as it is a prerequisite for the subsequent production of bio-energy. Sugars and starch comprise the feedstock for 90%of the produced ethanol today, but the most prevalent forms of sugar in nature are cellulose and hemicellulose. Lignocellulosic biomass can be converted to ethanol by hydrolysis and downstream fermentation processing. This processis much more complicated than just fermentation of C_6sugar (De Ruyck *et al.,* 1996) and is still far from being cost effective as compared to the production of bioethanol from starch or sugar crops. In hydrolysis, the cellulosic part of the biomass is converted into sugars, and fermentation converts these sugars to ethanol. Lignocellulosic biomass consists of 10–25%lignin, which contains no sugar and therefore impossible to convert into sugars. Lignin is therefore a residue in ethanol production and it represents a big challenge to convert it into a value-added product. However, attempt was made to screen lignocellulosic raw material such as wheat straw and leaf litter, millet straw, mustard straw, rice husk and cotton seed cake abundant in the region with high cellulose content with potential strain of local/ native cellulose degrading fungi for cellulolytic enzyme bio-synthesis in ideal fermenting medium for sugar and ethanol production in optimized condition.

Materials and Methods

Substrate selection and physico-chemical composition

Commonly available lignocellulosic waste/raw material *viz*., cotton seed cake, rice husk, millet straw, mustard straw, wheat straw and leaf litter were collected from the local farms near Sadra village; district Gandhinagar, Gujarat and each substrate were subjected to physical-chemical pretreatment for delignification and breaking down the macro and micro fibrils, increasing the surface area for better penetration of fungi. These lignocellulosic waste materials were subjected to mechanical grinding and sieving to finer particles of 3 mm size. Ten gram of substrate was soaked in 1 to 5N of NaOH for 24 h and washed repeatedly with distilled water till neutral pH and kept overnight at 60°C for drying.

Physico-chemical properties of substrate

The amount of cellulose was estimated by colorimetric method (Thimmaiah, 2006) by mixing dry sample with Acetic/nitric reagent, 67%H_2SO_4 and finally allowed to react with chilled Anthrone reagent. Amount of cellulose was estimated from standard curve using standard cellulose solution.

The amount of hemicellulose was determined colorimetric method (Thimmaiah, 2006). The

sample was refluxed using cold Neutral detergent fiber by adding 2.0 ml of decahydronapthalene and 0.5 g sodium sulphite in boiling water bath for 60 min. After drying at 100°C for 8 h crucible was cooled in desiccators and weighed. The amount of hemicelluloses was obtained by subtracting Acid detergent fiber from Neutral detergent fiber.

Total organic carbon was evaluated by titration method (Walkley and Black, 1934). The method is based on the oxidation of organic matter by potassium dichromate ($K_2Cr_2O_7$)-sulfuric acid mixture followed by back titration of the excessive dichromate by standard ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2.6H_2O$] in the presence of NaF or phosphoric acid and diphenylamine indicator.

For total nitrogen estimation, digestion method (Snell and Snell, 1954) was used. Plant sample was digested with concentrated H_2SO_4 and then with H_2O_2 till it became colorless. The intensity of colour developed by Nessler's reagent in the presence of NaOH and sodium silicate was measured on a spectrophotometer at 440 nm wavelength by using blue filter. The nitrogen content (ppm) was calculated with the help of standard curve.

Total phosphorus in plant sample was determined by vanadomolybdo phosphoric yellow colour method (Jackson, 1973). Orthophosphates obtained in triacid digest react with ammonium molybdate ammonium vanadate in HNO_3 medium and gives a yellow colour complex.

The colour developed in about 30 minutes and remained stable for 2 to 8 weeks. Intensity of colour was measured at 470 nm wavelength in a Spectrophotometer. The ppm content of phosphorus was obtained with the help of standard curve.

Microorganism selection and Inoculum Preparation

Amongst four fungi of genus *Aspergillus* isolated from local soil dumps of kitchen and garden waste was grown on Carboxy Methyl Cellulose (CMC) agar medium and the culture was selected on the basis of higher cellulase activity and pure culture was identified based on morphology and colony characteristics.

The selected culture was grown on PDA slants for further use as inoculum preparation.

Medium selection and composition

The growth medium used in solid state fermentation (SSF) process required supplementation of additional nutrients to stimulate growth and enhance enzyme synthesis.

Five different media *viz.*, Mineral Salt [Composition g/l: Peptone protease1.0g, $(NH_4)_2SO_4$ 1.4g, KH_2PO_4 2.0g, Urea 0.3g, $CaCl_2$ 0.3g, $MgCl_2.7H_2O$0.3g, $FeSO_4.7H_2O$ 0.005g, $MnSO_4.H_2$ 00.06g, $ZnSO_4.7H_2$ 0 0.4g at pH-5.0], Mandles and Sternberg [Composition g/l: Urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, $MgCl_2.7H_2O$ 0.3 g, Protease peptone 1.0 mg, $FeSO_4.7H_2O$ 5.0 g, $MnSO_4.7H_20$ 1.6 g, $ZnSO_4.7H_20$ 1.4 g, Tween-80 0.1%(v/v) at pH 5.2], Berg's medium [Composition g/l: $NaNO_3$ 0.5 g,$MgSO_4$0.05 g, $FeSO_4$ 0.01 g, $CaCl_2$ 0.02 g, $MgSO_4$0.02 g at pH 7.0], Czapek-dox medium [Composition g/l: Sucrose 30.0 g, yeast extract 7.0 g, KH_2PO_4 2.0 g, $NaNO_2$ 8.0 g, KCl 3.0 g, $MgCl_2.7H_2O$ 3.0 g, $FeSO_4.7H_2O$ 2.0 g, $ZnSO_4.7H_20$ 2.0 g, $CuSO_4$0.08g at pH 4.8] and Waber and Mandles [Composition g/l: KH_2PO_4 2.0 g, $MgCl_2.7H_2O$ 0.3 g, $CaCl_2.2H_2O$ 0.3 g, $CoCl_2$ 2.0 g, $MnSO_4.H_20$ 1.6 g, $ZnSO_4.H_20$ 5.0 g, Tween-80 2.0%(v/v) at pH 5.0] were evaluated for higher enzyme activity.

Optimization of parameters for enzymatic Hydrolysis

Various parameters such as inoculation size, pH of fermentation medium, incubation temperature, incubation time, moisture content of the substrate, substrate concentrations and supplementation of carbon and nitrogen source etc., were studied to find out the best hydrolyzing conditions for millet straw using cellulose produced by *Aspergillus flavus*.

The optimum condition obtained from each experiment was used in the next optimization study unless otherwise stated.

Inoculation size

Fungi *Aspergillus flavus* was grown on PDA (Potato Dextrose Agar) slants and spores were harvested from 7 days old PDA slants. 10 ml of sterile distilled water was added to these slants. Spore count was measured and adjusted to10^7spores/ml by adjustment of optical density. Three gram of pretreated millet straw was wetted with Mandles and Stenberg medium was loaded with different concentrations of inoculum (0.5ml, 1.0ml, 1.5ml, 2.0ml, 2.5mland 3.0ml) and incubated at room temperature (28°C) for 7 days.

pH of the medium

The pH of the medium was adjusted to4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 by adding required amount of 1N HCl and 1N NaOH;1 ml of inoculum was added and finally incubated at room temperature (28°C)for 7 days.

Incubation temperature

Hydrolysis was performed at different temperatures (23°C, 28°C, 37°Cand 50°C) to determine the optimum temperature by adding 1.0 ml inoculum in the flask keeping pH of the medium as 4.5 and incubated for 7 days.

Incubation time

The enzymatic hydrolysis study was carried out for a period of 240 h by adding 1.0 ml inoculum in the flask keeping pH of the medium as 4.5 and incubating at the temperature of 28°C and every 24 h interval enzymatic activity was measured. Maximum hydrolysis occurred at 120h and was found to be decreased thereafter.

Moisture content of substrate

Moisture content was varied to 40%, 50%, 60%, 70% to 80% for enzyme biosynthesis; flasks inoculated with 1.0 ml inoculam, pH 4.5 followed by 120 h incubation period at 28°C.

Substrate concentration

The pretreated substrate at 70% moisture content was suspended in fixed volume (10 ml) of buffer (pH 4.5) with different weights (1.0, 3.0, 5.0, 7.0 and 10.0 g) and 1.0 ml inoculum was added and incubated for 120 hat 28°C.

Supplementation of carbon and nitrogen source

Mandles and Stenberg medium was contained 0.1% of different carbon sources like glucose, sucrose, CMC, xylose, wheat flour and cellobios; and 0.5% of different nitrogen sources like ammonium nitrate $(NH_4)_2NO_3$, ammonium sulfate $(NH_4)SO_4$, yeast extract, protease peptone and urea. The flasks inoculated with 1.0 ml inoculum, pH 4.5, moisture content 70%, were now incubated at 28°C for 120 h.

Extraction of enzyme

After incubation, 50.0 ml of citrate buffer (0.1M, pH 4.5) was added to each fermented flasks and homogenized at 10,000 rpm in a

rotator shaker for 30 min at room temperature to extract the product and extracted mixture was used to measure enzymatic activity and reducing sugars.

Cellulase activity

Carboxymethyl cellulase (CMCase) is mainly evaluated based on the procedure described by Mandels *et al.*, (1976).In this method, CMCase activity is measured by determining reducing sugars released after 5 min of enzyme reaction with 1.0 % Carboxy Methyl Cellulose at pH 4.8 and 50°C (Mandels *et al.*, 1976). Also, one unit (IU) of EG is defined as the amount of enzyme that liberates 1 µmol of glucose per minute under assay. The amount of reducing sugars released was measured by colorimetric method using dinitro salicylic acid according to Miller's method (Miller, 1959) with glucose as standard. The activity was expressed in international unit (IU), defined as the amount of enzyme required to produce 1 µmol glucose/min.

Enzymatic hydrolysis at optimized condition

Considering all above optimization parameters, enzymatic hydrolysis was performed and compared with the results of un-optimized conditions during experimentation.

Enzymatic saccharification and bioethanol production

Inoculum preparation

A culture suspension of *Saccharomyces cerevisiae* was prepared by adding 10 ml sterile distilled water in Glucose Yeast Extract Agar slants containing yeast culture. Optimal density was measured at 600 nm against distilled water as blank. The optical density of inoculum was adjusted to 1.0 O.D. (1×10^7 CFU/ml). 5% v/v of this inoculum taken for saccharification of fermented hydrolysate.

Fermentation process

The flasks containing 5g of millet straw was wetted with Mandles and Sternberg medium, at pH 4.5,with 70% moisture content; autoclaved at 121°C and 15 lbs; inoculated with 1.0 ml *Aspergillus flavus* (1×10^7 spores/ml) and incubated at 28°C for 120 h. After incubation 200ml of sterile distilled water was added to the fermentation flask; homogenized at 10,000 rpm for 10 min on environment shaker; filtered; centrifuged at 10,000 rpm for 10 min at 4°C; autoclaved at 10 psi for 10 min and transferred in sterilized anaerobic glass bottle with 250 ml capacity; inoculated with 5% v/v of *Saccharomyces cerevisiae* culture. Then, the anaerobic glass bottle was immediately stopped with the rubber stopper (sterilized by dipping it in boiled water for 10 min, just before use) having a hole through which a sterilized plastic tube (sterilized same as rubber stopper) was inserted in to the bottle whose another end was dipped in a 150 ml capacity conical flask containing 100 ml of 3N NaOH to collect CO_2 produced in bottle during fermentation process. The anaerobic glass bottle and NaOH containing flask was sealed by vaccum grease or parafilm tap and placed under stationary condition was done for 4 days. After 4 days, CO_2 estimation was done immediately from NaOH solution. The fermentation broth was centrifuged at 4000 rpm for 10 min and filtered through Whatman filter paper-1.For recovery of ethanol, 100ml fermentation broth was centrifuged; filtered and 100ml of distilled water was added and subjected for distillation process in condenser.

Estimation of ethanol

Ethanol obtained by distillation was oxidized by acid potassium dichromate and measured

calorimetrically at 600 nm using working and standard ethanol solution (5%). From standard ethanol solutions, working standard solution of 1%, 2%, 3%, 4% and 5% were prepared. In order to estimate ethanol content, eight volumetric flasks of 50 ml capacity were taken and labeled from 1 to 8. 1.0 ml working standard solutions of 1%, 2%, 3%, 4% and 5% were added into flask no. 2 to 6,respectively; whereas, flask no. 7 and 8 were added with 0.5 and 1.0 ml of distilled ethanol sample respectively. Then 25 ml of acid potassium dichromate solution (Dissolve 3.4 g $k_2Cr_2O_7$ in 50 ml distilled water in a 100 ml capacity volumetric flask. Put it in ice bath and add 30 ml of concentrated H_2SO_4 in it, mix well and make up the volume up to the mark with distilled water) was added in all the flasks and incubated in water bath at 80°C for 15 min followed by cooling at room temperature under running tap water and distilled water added to make final volume to 50 ml. Optical density (O.D.) at 600 nm wavelength was measured; standard curve was prepared and ethanol concentration was calculated. Ethanol (g/l) is equivalent to the yield of 100g of dried substrate.

In the present study all experiments were performed with the above mentioned treatments repeated thrice to ensure accuracy and means of all treatments were taken into consideration of each parameter for interpreting the results.

Results and Discussion

Selection of substrate

India being an agriculture country, large amounts of lignocellulosic wastes is generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro industries. These wastes have been insufficiently disposed off leading to environmental pollution (Fabiyi *et al.,* 2011). Such renewable resources are becoming increasingly important, plentifully available in nature and have a potential for bioconversion into fuels and chemicals, both natural and man-made (Thompson *et al.,* 1999), to change the world economically, socially, and environmentally (Sanchez, 2009).Amongst six natural lignocellulosic waste/raw materials *viz*., cotton seed cake, rice husk, millet straw, mustard straw, wheat straw and leaf litter screened, millet straw contained maximum cellulose of 30 %followed by mustard straw (28 %), wheat straw (24%), leaf litter (23%), rice husk (20%) and cotton seed cake (19%) as estimated by colorimetric method. Owing to maximum cellulose content, millet straw was selected as substrate. Cotton seed cake, wheat straw and rice husk are widely used as food for animals in India because they provide enough energy and protein to meet an animal's requirement. Besides this, there is limited availability of mustard straw in local region; furthermore, biochemical composition of leaf litter is variable and diseases and insecticides application interfering with the final results; hence, leaf litter is also not ideal choice for cellulase biosynthesis. In India, every year 64.4 million tones millet is produced with processing of millet grains. Besides, millet waste as animal feed is limited. Considering these, millet straw seems ideal choice on account of higher cellulose content and negligible lignin, plentiful and easy availability in local region. Bioethanol production from sugarcane and maize were well known in South Gujarat. Considering ample availability in rain fed area and high cellulose content as well as little work on enzymatic hydrolysis and potential of bioethanol production, millet straw was selected as substrate for further experimentation. Wu *et al.,* (2006) reported that the pearl millet could be a potential feedstock for ethanol production in bioreactor using fermenting yeast, *Saccharomyces cerevisiae* in rain fed region where wheat and

other grain crops could not be raised owing to shortage of moisture.

Pretreatment of the substrate

Pretreatment of lignocelluloses is intended to disorganize the crystalline structure of macro and micro fibrils in order to increase the polymer chains of cellulose and hemicelluloses and modify the pores in the material to allow the enzyme to penetrate into the fibers to render them amenable to enzymatic hydrolysis. Physical pretreatment is intended to increase accessible surface area and size of pores, partial hydrolysis of hemicelluloses and partial depolymerization of lignin (Sun and Cheng, 2002). When substrates were grinded mechanically, substrate of 3mm particle size showed 3.703 U/gds enzymatic activities which were higher than that obtained through 1 cm particle size (0.462 U/gds).Cellulose degradation was found to be decrease with increase alkali concentration (Table 1). Basically, pretreatment is a delignification process, and the underlying mechanism is the saponification of intermolecular ester bonds cross linking xylan hemicelluloses and lignin (Sun and Cheng, 2002). In addition, alkaline pretreatment also removes acetyl and other acidic substitutions on hemicelluloses that protect cellulose from attack by cellulase (Chang and Holtzapple, 2000). Various alkalis including sodium hydroxide, lime and aqueous ammonia have been studied (Gupta and Lee, 2010; Kim *et al.,* 2003). Considering these, millet straw was treated with five different concentrations of alkaline treatments *viz*., 1N, 2N, 3N, 4N and 5N NaOH solutions. The maximum enzyme activity (3.549U/gds) was observed with 1N NaOH. As concentration of NaOH was increased, there was decrease in cellulase degradation (Table 1). This was due to higher concentration of alkali which causes rapid accumulation of volatile fatty acids which inhibits the cellulosic activity while lower concentration may prolong the time of degradation (Gu *et al.,* 2014; Chandra *et al.,* 2012; Zhou *et al.,* 2011). Increased cellulase activity at 1N NaOH concentration might be due to increased solubilization of lignin and increase in swelling of cellulose I to II. Koullas *et al.,* (1992), Aguiar *et al.,* (2001) and Vyas *et al.,* (2005) also reported 1N NaOH pretreatment of lignocellulosic substrates best for cellulase production.

Physico-chemical parameters of the millet straw

The composition of selected substrate (millet straw) showed 30 % cellulose, 10 % hemicellulose, 8.73 % total organic carbon, 0.7 % total nitrogen and 0.8 ppm phosphorous (Table 2). In solid state fermentation, enzyme production was carried out using lignocellulosic biomass. Cellulose and hemicelluloses are used as carbon source for the production of cellulolytic and hemicellulolytic enzymes. Furthermore, cellulose and hemicelluloses are the effective inducer for cellulolytic and hemicellulolytic enzymes respectively and millet straw is rich in cellulose and hemicellulose.

Microorganism selection and Inoculum Preparation

The ability to secrete large amounts of extracellular cellulase is characteristics of certain fungi. Moreover, fungi have ability to produce higher amount of enzymes than bacteria (Amouri and Gargouri, 2006). Cellulase characteristics and production by *Aspergillus* spp. have been well documented in the literature (Lockington *et al.,* 2002; Ong *et al.,* 2004 and Wang *et al.,* 2006). Therefore fungi *viz*., *Aspergillus oryzae, Aspergillus flavus, Aspergillus niger* and *Aspergillus fumigatus* isolated from local soil dumps of kitchen and garden waste were grown on Carboxy Methyl Cellulose (CMC) agar

medium and examined for cellulolytic enzyme activity. Fungus, *Aspergillus flavus* showed higher cellulose activity (4.012 U/gds) than other three species (Table 3). The culture of *Aspergillus flavus* showing high cellulase activity was selected and grown on PDA slants and the spores were harvested aseptically from 5 day old PDA slants. The slants were freshly made twice in a month and stored at 4 °C.

Sterile distilled water (10 ml) was added to each fungal slant and vortexed. Spore count was measured in Neubers Chamber and adjusted to 10^7spores/ml by adjusting absorbance maxima. The potentiality of *Aspergillus flavus*as one of the potent lignocellulolytic organisms for cellulose production was also reported by Solomon *et al.*, (1999). Ojumu *et al.*, (2003) also found that *Aspergillus flavus* grown on sawdust gave the highest cellulase activity of 0.0743 IU/ml than bagasse and corncob.

Selection of fermentation medium

In order to obtained good biosynthesis of cellulose, five different media were used for enzyme activity and Mandels and Sternberg medium showed maximum enzyme activity (4.629 $U.gds^{-1}$) (Table 4). The composition of medium (g/l) includes Urea 0.3 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2.0 g, $CaCl_2$ 0.3 g, $MgCl_2.7H_2O$ 0.3 g, Protease peptone 1.0 mg, $FeSO_4.7H_2O$ 5.0 g, $MnSO_4.7H_20$ 1.6 g, $ZnSO_4.7H_20$ 1.4 g, Tween-80 0.1%(v/v) at pH 5.2.

The medium showed maximum enzyme activity due to favorable pH and supplementing nitrogen and mineral requirements for growth and development of *Aspergillus flavus*. This medium was selected for solid state fermentation using *Aspergillus flavus* for enzymatic hydrolysis. The cellulase hydrolyzing activity is further improved by some metal ions $Mg^{2+,}$ Mn^{2+}, K^+ and Ca^{2+} (Zhang *et al.*, 2012).Urea provides nitrogen which is essentially important for cell growth and building block of enzymes. Stimulation of endoglucanase activity by ammonium salt may be due to their direct entry into protein synthesis. In the current study Tween 80 was used as a surfactant. Wu and Ju, (1998) reported enhanced enzymatic saccharification of waste newsprint by addition of surfactant like Tween 20 or Tween 80. The rate of enzymatic hydrolysis was improved by 33% using Tween 80 as a surfactant in the hydrolysis of newspaper (Castanon and Wilke, 1981).

Optimization for enzymatic hydrolysis

Optimization is important aspect in order to find out suitable growth condition for maximum biosynthesis of cellulolytic enzyme. Various parameters such as inoculum size, pH, temperature, incubation time, moisture content, substrate concentration, carbon source and nitrogen source were studied for optimization for maximum CMCase biosynthesis (Table 5).

Effect of inoculum size on CMCase biosynthesis

Pretreated millet straw @3g wetted with Mandles and Stenberg medium was loaded with different concentrations of inoculum (0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml and 3.0ml) and incubated at room temperature (28°C) for 7 days. The results revealed that optimum inoculum size for CMCase synthesis by *Aspergillus flavus* was 1.0 ml showing maximum CMCase activity of 4.7830U/gds (Table 5). It was observed that the increase in the inoculum size yielded gradual reduction in the enzyme activity. This decrease in enzyme activity with further increase in inoculum might be due to clumping of cells which could have reduced sugar and oxygen uptake rate

and in turn reduced release of enzyme (Srivastava *et al.,* 1987). Nadagouda *et al.,* (2016) reported that lower inoculum size resulted in longer time for substrate utilization and cellulase release. While too high inoculum size may lead to rapid fungal biomass synthesis up to certain limit, after that the completion and interaction may lead to affect enzyme synthesis. This might be due to the nutrient depletion in biomass enhancement owing to completion, which may affect rate of metabolic activity. Hence, the inoculam size of 1.0 ml showing high cellulase activity seems optimum for conducting further studies in rotary shaker experiment.

Effect of pH on CMCase biosynthesis

The pH of the medium was adjusted to4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 by adding required amount of 1N HCl and 1N NaOH and 1 ml of inoculam was added and finally incubated at room temperature (28°C) for 7 days. The biosynthesis of enzyme was found to be maximum (5.092 U/gds) when pH of the medium was maintained at 4.5, more over when the pH level increased, the enzyme production was decreased (Table 5).

The initial pH of the medium has a great effect on the growth of the organism, permeability in membrane, as well as on the biosynthesis and stability of the enzymes (Shoichi *et al.,* 1985; Poorna and Prema, 2007). It was reported that optimum pH for CMCase from *Aspergillus aculetus* was found to be 4.5 to 5.0 (Murao *et al.,* 1988). Akiba *et al.,* (1995) reported that the production was high at pH 4.0 and 4.5 by *A. niger*. Coral *et al.,* (2002) observed that the enzyme activity has a broad pH range between 3 and 9. These might be due to the fact that the enzyme system within the same species may vary, depending on the strain under study. Based on the results obtained, the pH of the medium was adjusted to 4.5 for *A. flavus* in subsequent experiments.

Effect of temperature on CMCase biosynthesis

Amongst different incubating temperatures (23°C, 28°C, 37°C, and 50°C), the maximum CMCase biosynthesis was observed in flasks incubated at 28°C (6.018 U/gds) (Table 5). As the temperature increased, there was gradual decrease in the enzyme biosynthesis which might be due to fact that too high temperature could change membrane composition and could cause the protein catabolism and inhibition of fungal growth. Shahriarinour *et al.,* (2011) found the optimum temperature of 28°Cforbetter growth and cellulase production by *A. terreus*. Jadhav *et al.,* (2013) reported 30°C as best temperature for cellulase production by *Aspergillus niger* from the rice husks, millet husks, wheat bran and banana peels. The slight difference in the incubating temperature condition might be due to different favourable range for different species and substrate composition.

Effect of incubation time on CMCase biosynthesis

Incubation time is required for appropriate growth and adaptation in the environment. The enzymatic hydrolysis study was carried out for different periods from 48 to 240 h by adding 1.0 ml inoculum of *Aspergillus flavus* in the flask keeping pH of the medium 4.5 and incubating at the temperature of 28°C.At every 24 h interval enzymatic activity was measured. The biosynthesis of enzyme increased with the incubation period and reached upto maximum activity (6.790 U/gds) at 120 hours of incubation (Table 5).

Further increase in incubation period resulted in the decreased production of cellulase. The incubation time of 120 h was also reported ideal for *Aspergillus niger* kk_2 (Ojumu *et al.,* 2003) as well as for *Aspergillus phoenix* (Dedavid *et al.,* 2008).

Table.1 Comparison of chemical pretreatment for delignification

Alkaline (NaOH) treatment normality (N)	Enzyme activity (U/gds)
1	3.549
2	3.395
3	2.932
4	2.623
5	1.540

Table.2 Physico-chemical parameters of the selected substrate (millet straw)

No.	Parameter	Estimation Method	Reference	Content
1	Cellulose	Colorimetric	S. K. Thimmaiah	30 %
2	Hemicellulose	Gravimetric	S. K. Thimmaiah	10 %
3	Total Organic Carbon	Titrometric	Weakly and Black, 1934	8.73 %
4	Total Nitrogen	Nessler	Snell and Snell, 1995	0.7 %
5	Phosphorus	Colorimetric	Jackson, 1967	0.8 ppm

Table.3 Selection of organism for cellulase activity

Fungi	CMCase activity (U/gds)
Aspergillus oryzae	2.932
Aspergillus flavus	4.012
Aspergillus niger	2.777
Aspergillus fumigates	1.234

Table.4 Effect of growth medium on cellulase activity

Medium	CMCase activity ($U.gds^{-1}$)
Mineral Salt	2.930
Mandles and Sternberg	4.629
Berg	3.549
Waber and Mandles	3.240
Czapek-Dox	2.160

Table.6 Enzyme biosynthesis at optimized parameters under solid state fermentation

Substrate	CMCase activity (U/gds)	
	Unoptimized condition*	Optimized condition
Millet Straw	4.012	10.648

Table.7 Bioethanol production from millet straw through yeast fermentation

Incubation Period Hours	Ethanol Concentration from graph (%)	Ethanol Concentration (g/l)	Productivity (g/hr/lit)
120	1.15	18.46	0.15

Table.5 Optimization parameters for CMCase biosynthesis using millet straw as substrate under solid state fermentation

Inoculam size		pH		Temperature		Incubation time	
Inoculam (ml)	**CMCase activity (U/gds)**	**pH**	**CMCase activity (U/gds)**	**Temperature (°C)**	**CMCase activity (U/gds)**	**Incubation time (hours)**	**CMCase activity (U/gds)**
0.5	0.617	4.0	4.012	23	0.771	48	3.308
1	4.783	4.5	5.092	28	6.018	72	5.401
1.5	4.012	5.0	4.783	37	3.086	96	5.555
2	2.777	5.5	1.697	50	2.314	120	6.790
2.5	1.543	6.0	1.388			144	6.018
3	1.080	6.5	1.080			168	4.938
		7.0	0.617			192	4.783
						216	4.629
						240	4.475
Moisture content		**Substrate concentration**		**Carbon source**		**Nitrogen source**	
Moisture content (%)	**CMCase activity (U/gds)**	**Substrate concentration (g)**	**CMCase activity (U/gds)**	**Carbon source (g)**	**CMCase activity (U/gds)**	**Nitrogen source (g)**	**CMCase activity (U/gds)**
40	6.635	1	7.870	Glucose	6.635	Ammonium nitrate	8.029
50	7.253	3	9.104	Sucrose	7.870	Ammonium sulphate	9.722
60	7.870	5	6.851	CMC	9.259	Yeast extract	7.561
70	8.333	7	3.502	Xylose	8.179	Protease peptone	7.253
80	6.018	10	1.712	Wheat flour	6.790	Urea	6.327
				Cellobiose	6.635		

U/gds = unit per gram dry substrate

Datt and Kumar (2012) found that the CMCase activities of *Aspergillus flavus* AT-2 increased steadilywith increasing incubation period and attained maximum (9.20 IU/ml) on the 5th day (120 h) of incubation. Okonkwo *et al.,* (2014) also reported that *Aspergillus flavus* culture may be effectively harvested in their highest cellulase production capacity in 5 days (120 h). The possible reason for decreased enzyme activity is associated with the fact that the enzyme production in various microorganisms reached the maximum level in stationary phase and declined during the death phase. During the death phase, depletion of nutrient concentration and cellular fragmentation is very common, resulting in release of intracellular material and proteases in fermentation broth (Dhillon *et al.,* 2011; Bansal *et al.,* 2012). By considering these, incubation time was selected as 120 h for subsequent studies.

Effect of moisture content on CMCase biosynthesis

The moisture content of substrate is the key factor in SSF because it affects growth, biosynthesis and secretion of enzymes. The maximum CMCase activity (8.333 U/gds) was found at 70% moisture content (Table 5). Lower moisture content caused the solubility of substrate, low degree of swelling and high water tension (Moo-young *et al.,* 1983). Inter particle mass transfer of oxygen; nutrients and enzyme are dependent on substrate characteristics and moisture content of fermenting media. Contrary, at higher moisture content, void space within solid phase is filled with water and air is driven out, affecting growth of fungus and ultimate enzyme synthesis. Xia *et al.,* (1999) found maximum cellulase production under SSF at water content of 70per cent. Liang *et al.,* (2012) also observed highest cellulase activity at 70% moisture content by incubating with *Aspergillus* sp. (SEMCC-3.248) in solid state fermentation. An increase or decrease in moisture content from the optimum value resulted in lower cellulase activities. A higher than optimum moisture content led to a decrease in porosity, gummy texture, and lower oxygen transfer whereas a lower moisture content led to decreased swelling of substrate and solubility of nutrients (Kaur *et al.*,2011). This inhibits effective uptake of nutrients by fungi. If moisture is too high during SSF, risk of contamination by unfavorable microorganisms is greater (Yoon *et al.,* 2014).

Effect of substrate concentration on CMCase biosynthesis

The pretreated substrate at 70 % moisture content was suspended in fixed volume (10 ml) of buffer (pH 4.5) with different weights (1.0, 3.0, 5.0, 7.0 and 10.0 g) and 1.0 ml inoculum was added and incubated for 120 h at 28°C. 3 g pretreated substrate showed higher CMCase activity (9.104 U/gds) (Table 5). Increased in quantity of substrate and concentration, there was reduction in enzyme activity. Increase in substrate concentration limits the saccharification yields because of stirring difficulties, reduction of aqueous movable phase and end product inhibition (Szczodrak 1999). The present results was in accordance with Vyas *et al.,* (2005) who also reported 2 % substrate concentration was optimum for maximum endoglucanase activity (2.889 IU/ml).

Effect of carbon source on CMCase biosynthesis

The carbon source is one of the most important nutrients for the biosynthesis of enzyme. The substrates not only serve as a carbon source but also produce the necessary inducing compounds for the organisms (Haltrich *et al.,* 1996). In our study, influence of supplementation of different carbon source

such as glucose, sucrose, carboxy methyl cellulose (CMC), xylose, wheat flour and cellobiose in 0.1% concentration to Mandles and Sternberg medium on cellulase production by *Aspergillus flavus* was tested. Amongst carbon sources tested, carboxy methyl cellulose showed higher CMCase activity (9.259 U/gds) (Table 5). Earlier it has been reported that endoglucanase was induced by CMC but repressed by glucose (Ahmed *et al.,* 2005). It was also found that CMC was preferred substrate for endoglucanase production (Lucas *et al.,* 2001). Similarly, Malik *et al.,* (1986) reported that negligible cellulases were produced with glucose as carbon source from *T. harzianum*. Niranjane *et al.,* (2007) observed highest yields of cellulase on CMC. In the present study also similar results was noticed with less cellulase activities in the presence of glucose, while CMC proved to be a strong inducer of cellulase enzymes.

Effect of nitrogen source on CMCase biosynthesis

Nitrogen requirement of microbes used in SSF process are obtained from the substrate itself in same instance, whereas supplementation of additional nitrogen in organic or inorganic form is often reduced. In this study, effects of nitrogen source *viz*., ammonium nitrate, ammonium sulphate, yeast extract, protease peptone and urea were tested at 0.5% concentration individually. Amongst them, ammonium sulphate showed highest enzyme activity (9.722 U/gds) (Table 5). Nitrogen is the major constituent of protoplasm building block of enzyme (proteins). Stimulation of endoglucanase activity by ammonium sulphate may be due to their direct entry in protein synthesis (Mandels, 1975). The present finding in accordance with the result of Linko *et al.,* (1978) who reported ammonium salt to be excellent source of nitrogen for cellulose production by *Trichoderma sp*. Similarly, Sasi *et al.,* (2012) reported that *A. flavus* showed the highest production of cellulase enzyme utilizing ammonium sulphate as nitrogen source than yeast extract. Sethi and Gupta, (2014) observed ammonium sulphate as the best nitrogen source for *Aspergillus niger*. The enzyme production was remarkably decreased in presence of urea (Acharya *et al.,* 2008).

Enzymatic hydrolysis under solid state fermentation at optimized condition

Based on the above results on optimization parameters, the flasks containing 3 gof millet straw was wetted with 10 ml of Mandles and Sternberg medium, at pH 4.5,with 70% moisture content; autoclaved at 121°C at 151 lbs; inoculated with 1.0 ml *Aspergillus flavus* (1×10^7 spores/ml) and incubated at 28°C for 120 h. Simultaneously, enzymatic hydrolysis also carried out with unoptimized condition using 5 ml of inoculum, pH 7.0, temperature 50°C, 40% moisture content, 10 g of substrate providing cellobiose and urea as carbon and nitrogen source respectively. The CMCase activity was enhanced considerably from 4.012 observed before optimization to 10.648 U/gds after optimization (Table 6).

Ethanol production

Ethanol fermentation was performed in flasks on a rotary shaker using yeast, *Saccharomyces cerevisiae.* The ethanol production was 18.46 g/l from 100 g dried millet straw when incubated for 120 h at optimized parameters and fermented through yeast *Saccharomyces cerevisiae* (Table 7).

Millet straw seems ideal choice on account of ample availability in rain fed region, higher cellulose content and negligible lignin in local region and amenable to high cellulase activity after pre-treatment. *Aspergillus flavus* was the

potent culture for synthesis of cellulose under solid state fermentation with 1.0 ml inoculam size. The organism gave good activity at pH 4.5, 28°C temperature and 3 g substrate concentration for 120 h incubation period. In addition, 70% (w/w) moisture content, 0.1% CMC and 0.5% ammonium sulphate enhanced cellulase activity. The CMCase activity was 4.012 U/gds before optimization which was found to be increased upto 10.648 U/gds after optimization. The biodegradation of cellulose further applied for the production of ethanol through yeast fermentation. The hydrolysate was undergone to saccharification and it was observed that 0.15g/h/lit ethanol can be produced.

This study also indicates that millet straw is most suitable raw material for cellulase production as it gave maximum biosynthesis of enzyme. The bioethanol obtained by hydrolysis and saccharification was highly satisfactory and eco-friendly energy source produced from lignocellulosic waste millet straw. Hence, it is promising lignocellulosic feedstock for bioethanol production through fermentation by *Saccharomyces cerevisiae*. This work will not only benefit the fuel ethanol industry in semi-arid or arid rural areas by finding alternative raw materials but will also provide valuable information for breeders to modify existing millet genotypes amenable for improving genotypes for maximum cellulase activity and bioethanol production.

Acknowledgments

Authors are highly thankful to Dr. Pratik Shilpakar, Lecturer; Dr. Nikhil Bhatt, Research Scientist and Dr. Srinivas Murthy, Research Scientist from Biogas Research Center, Gujarat Vidyapeeth, Sadra, Gandhinagar, Gujarat for providing valuable feedback and guidance for conducting the work.

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How to cite this article:

Prathvi V. Narsale, Sejal R. Patel and Pradeep Acharya. 2018. Role of *Aspergillus flavus* on Biodegradation of Lignocellulosic Waste Millet Straw and Optimization Parameters for Enzyme Hydrolysis and Ethanol Production under Solid State Fermentation. *Int.J.Curr.Microbiol.App.Sci.* 7(02): 429-445. doi: https://doi.org/10.20546/ijcmas.2018.702.055