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Isolation of cellulase producing bacteria and optimizing production parameters for its potential application in industries

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ABSTRACT

Cellulases are a group of hydrolytic enzymes that are capable of degrading most abundant lignocellulosic material on earth. A high level of thermo stable cellulase has been produced from newly isolated strain *Flavobacterium bolustinum* (MTCC 10203) under submerged fermentation using basal medium supplemented with pineapple peel (1.5%) pH 9 at 37°C. Different culture conditions under submerged fermentation (SmF) were examined to assess their effect on enzyme production. Various production parameter included temperature, pH, inoculum age and volume, incubation time, carbon and nitrogen sources, salts and additives were optimized. After optimization there was increase of about 7.76 fold in cellulase production (265U/ml) which decreases the cost of enzyme production for its industrial application. Moreover, results showed pineapple peel as a excellent source of substrate for production of cellulase for commercial use.

KEYWORDS

Cellulases;
Flavobacterium bolustinum;
Lignocelluloses;
Pineapple peel;
Submerged fermentation.

INTRODUCTION

Cellulose is a fibrous, insoluble, crystalline polysaccharide. It is major polysaccharide constituent of plant cell wall, composed of repeating D-glucose units linked by 1,4-glucosidic bonds[1]. It being the most abundant carbohydrate polymer on the earth[2]. A promising strategy for efficient utilization of this renewable resource is the microbial hydrolysis of lignocellulosic waste. In the early 1970s, the oil crisis generated interest in using cellulose as a chemical and energy resource. One promising approach was to hydrolyze the cellulose to glucose with enzymes and then to ferment the glucose to ethanol which could be used as a liquid fuel[3] The growing concern about shortage of fossil fuel and air pollution has also resulted in increased focus on the production of Bioethanol(biofuel) from lignocellulosic material[4,5]. Among the potential alternative bioenergy resources lignocellulosic has been identified as prime source for biofuel. However in production of bioethanol, the cost of the enzyme to be used for hydrolysis of raw material needs to be reduced and their efficiency increased in order to make the process economically feasible[6]. Organism with cellulase system that are capable of converting biomass to alcohol directly are already reported[7,8] but none of these system described are effective alone to yield a commercially viable process. The use of pure enzymes in the conversion of biomass to ethanol is currently un-

economical due to the high cost of commercial cellulases. The effective strategies are yet to resolve which make the enzyme production cost effective.

Although fungi are good producer of cellulases but their slow growth rate and inability for cloning, there is need of new isolate that can be easily grown. This need is fulfilled by screening cellulase producing bacteria as they can grow easily, subjected to cloning and less inhibited to feedback inhibition. Cellulases hydrolyze cellulose (β-1, 4-D-glucan linkages) and produce as primary products glucose, cellobiose and cello-oligosaccharides[9]. There are three major type of cellulase enzymes endo 1,4,-β-D-glucanase[1,4-β-D-glucanases (CMCase, EC3.2.14)] and exo 1,4-β-D- glucanase (1,4-β-D-glucan cellobiohydrolase, FPA, EC 3.2.1.91) along with cellobiase (β-D-glucoside glucanohydrolase, EC 3.2.1.21)[10,11].

Cellulase yield depend on a complex relationship involving a varity factors like carbon source, inoculum size, pH, temperature, presence of inducer, medium additives, aeration, growth time, etc[12]. Therefore attention has been focused on studing cellulase enzyme production by several organisms on various agro products and in various environments. This work is focus on to complete the challenges in cellulase production by isolating bacterial species with cellulase activity and increasing its production by using cheap source of lignocellulosic waste as substrate and identification of better inducers.

MATERIAL AND METHODOLOGY

Chemical

All chemicals and reagents used in the study were of analytical grade.

Isolation and screening

To isolate the bacterial strain the soil sample was collected from sugar mill of district kaithal, haryana. The bacteria was isolated using media containing CMC (1%), Peptone (0.2%), yeast extract (0.2%), Nacl (0.5%), $MgSO_4$(0.01%) at pH 9. One gram of soil was inoculated in 100 ml flask containing 20 ml autoclaved media at 37C for 48 hrs in BOD incubator. Spread plate method was performed to isolate bacterial strain. The inoculated plates were incubated at 37°C for 24 hrs. After 24 hrs colonies so developed were screened for cellulase activity by growing isolates in a minimal agar plate consisting of soluble form of cellulose, Carboxymethylcellulose 0.5% (Na salt, Himedia). To visualize the zone of hydrolysis, plates were flooded with Congo red dye (0.5%) for 15 mins and washed with 1M Nacl[13]. on the basis of diameter of zone of hydrolysis best isolate was selected and maintained on agar slants at 4°C and sub cultured at interval of 1 month.

Enzyme production

The seed medium consisted of CMC(0.1%), Peptone (0.5%), Beef extract (0.15%), Yeast extract (0.15%), NaCl (0.5%), KH_2PO_4 (0.1%), wheat bran (1%) pH 9 was sterilized by autoclaving at 121°C for 30 minutes. After cooling the substrate was inoculated with 1% of inoculum of age 20 hrs. The flask was incubated at 37°C for 24 hrs at 200rpm.

Enzyme assay

The culture was harvested by centrifugation at 10,000 rpm for 20 min at 4°C using Refrigerated centrifuge (REMI). The supernatant was used as the crude extra cellular enzyme source. Cellulase activity was determined at 65°C by using carboxymethylcellulose (Sodium salts, Sigma, India) as substrate. A reactive mixture contained 450µl of 1 % (w/v) substrate in 0.1M Glycine-NaOH (pH 9) and 50µl of culture supernatant. The mixture was incubated at 65°C for 10 min. The reducing sugar released was measured using 3, 5-dintrosalicyclic acid (DNSA)[14]. One unit of enzyme activity was expressed as the amount of enzyme required to release 1 µg reducing sugars per ml under the above assay condition by using glucose as a standard curve.

OPTIMIZATION OF PARAMETERS INFLUENCING CELLULASE PRODUCTION

The protocol adopted for optimizing the process parameters influencing cellulase yield was to optimize one particular parameter and incorporating it at the optimized level in the next experiment[15]. The parameters analyzed included Incubation time (18 hrs to 48 hrs), inoculum age (14 hrs to 28 hrs), inoculum volume (1% to 5%), pH of the medium (pH 5 to 11), incubation temperature (25°C, 30°C, 37°C, 40°C, 45°C and 50°C), various carbon sources includes agro wastes and sugars and their concentration were tested for the

effect on cellulase production. Effect of selected nitrogen sources and their concentration on cellulase production were tested. Salts and additives were also analyzed to isolate best that affect the cellulase activity by conducting the fermentation under optimized conditions. All experiments were carried out in duplicate and mean values are reported.

RESULTS AND DISCUSSIONS

On the basis of physical and biochemical characteristics as shown in TABLE 1 revealed that isolate is *Flavobacterium bolustinum* which is identified by IMTEC, Chandigarh having MTCC no 10203.

TABLE 1 : Biochemical and growth characteristics of the *Flavobacterium bolustinum*

Characteristics/ biochemical tests	Observation
Gram's reaction	-
Configuration	Round
Surface	Smooth
Cell shape	Rod
Size (µm)	1.5-3µ
Arrangement	Scattered
Spore formation	-
Motility	-
Growth temperature	25°C-42°C
Growth pH	7-11.5
Growth on Nacl	2%-8%
Growth under anaerobic condition	-
Indole test	-
Methyl red test	-
Voges Proskauer test	-
Citrate utilization	-
Gas production from glucose	-
Casein hydrolysis	-
Starch hydrolysis	+
Urea hydrolysis	-
Nitrate reduction	+
Lysine decarboxylase	-
Catalase test	+
Oxidase test	-
Tween 20 hydrolysis	-
Acid production from Dextrose	+
Lactose	-
Maltose	-

+ Positive, - Negative

The extra cellular levels of cellulase were monitored from 18 to 48 hrs in agitated cultures of the bacterium grown in a basal medium. The enzyme production started after 18 h and production peaked at 24h (37.28U/ml) declined thereafter was shown in figure 1. Hydrolysis rates decline with time due to depletion of the more amorphous substrates, product inhibition and enzyme inactivation[16]. Cellulase production was found to increase when the age of inoculum's is 20 h (56.46U/ml) and increases further when inoculum's size was between 1 to 2 % (57.3U/ml). This decrease in cellulase production with further increase in inoculum's might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also, enzyme release[17]. The optimum incubation temperature for enzyme production was found to be 37°C (64.88U/ml) which is the optimum growth temperature of the bacterium while no activity was observed after 50°C. Effect of pH on cellulase production was shown figure 2. Optimal cellulase production was attained at pH 9 and it varies with slight change in pH. Maximum cellulase activity was obtained at pH 4.5, 7.5 and 6[18].

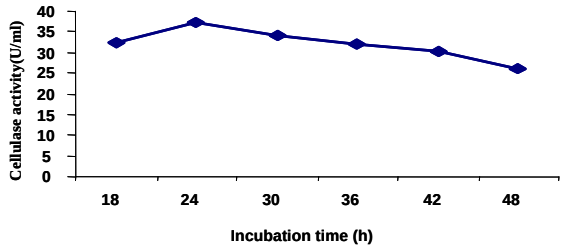

Figure 1 : Effect of incubation period on cellulose production

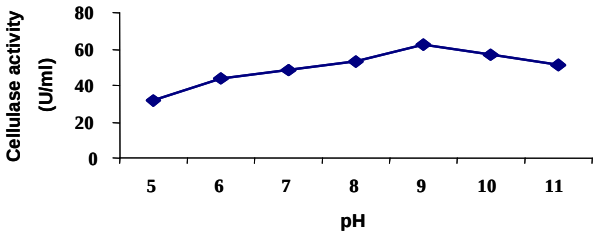

Figure 2 : Effect of pH on celluase production

Different agriculture waste byproduct such as Saw dust, Coconut waste, Banana waste, pineapple peel, pineapple pulp, wheat bran, wheat straw, rice husk, rice straw, bagasses were used as sole carbon sources for enzyme production[19-23] and results shown in figure 3 and the effect of substrate concentration was shown in figure 4. A decrease in production beyond optimum concentration is explained to be as a result of an inhibi-

FULL PAPER

tory effect of accumulated byproducts and formation of thick suspension of substrate which hinder the proper mixing of medium in shake flasks[24,25]. Low cellulase production after optimum very probably highlights sugar depletion from the substrates into the medium. Pineapple peel was identified as best carbon source as it is rich in cellulose, hemi cellulose and other saccharides[26]. The effect of additional carbon sources on enzyme production was tested by addition of sugars such as glucose, CMC, fructose, lactose, sucrose, cellulose etc was shown in figure 5. Results suggest that fructose can enhance cellulase production up to significant level. For cellulase synthesis cellulose, lactose and glucose act as inducer[11]. Glucose, lactose and fructose induces the cellulase production by *Cellulomonas cellusea*[27]. Effect of various organic and inorganic nitrogen sources listed peptone, beef extract, yeast extract, NH_4cl, Ammonium sulphate, Ammonium nitrate etc, are shown in figure 6. NH_4Cl was found best nitrogen source for cellulase production by *Cellulomonas cellusea*[28]. When medium is supplemented with 1.8% ammonium sulphate shows increase in cellulase production[28,21]. Effect of NH_4Cl concentration was shown in figure 7. Different salts were studied for their effect on cellulase production was shown in figure 8. $MgSO_4.7H_2O$ was suggested as best to induce cellulase production and its concentration effect was shown in figure 9.

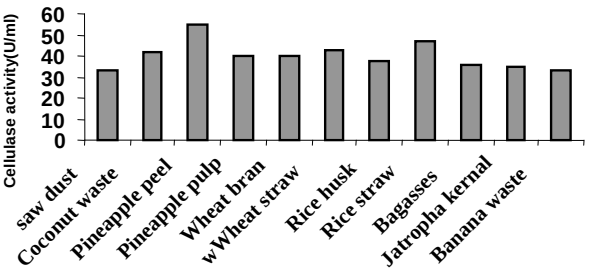

Figure 3 : Effect of various agrowastes on cellulase production

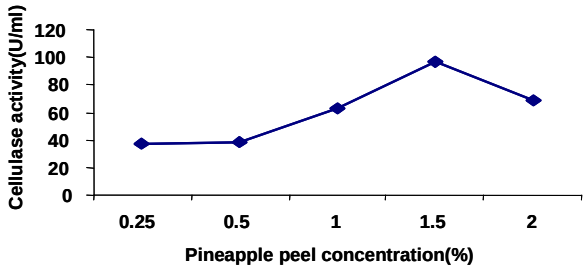

Figure 4 : Effect of concentration of Pineapple peel on cellulase production

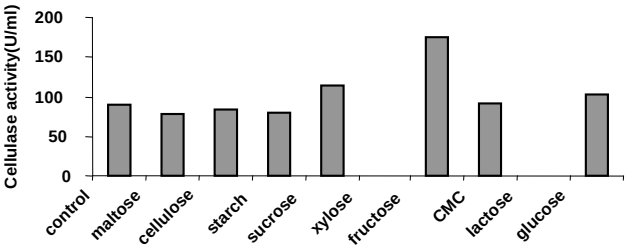

Figure 5 : Effect of various carbon sources on cellulase production

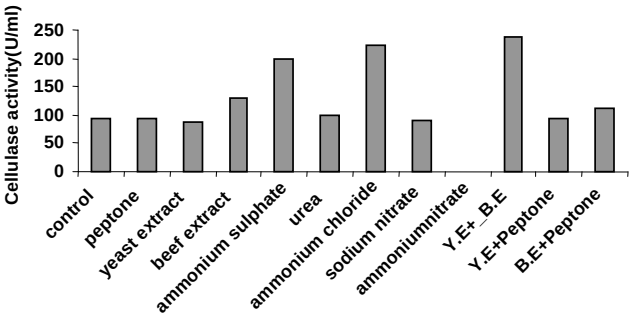

Figure 6 : Effect of various nitrogen sources on cellulase production

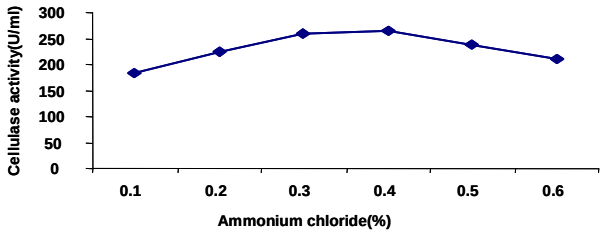

Figure 7 : Effect of ammonium chloride concentration on cellulase production

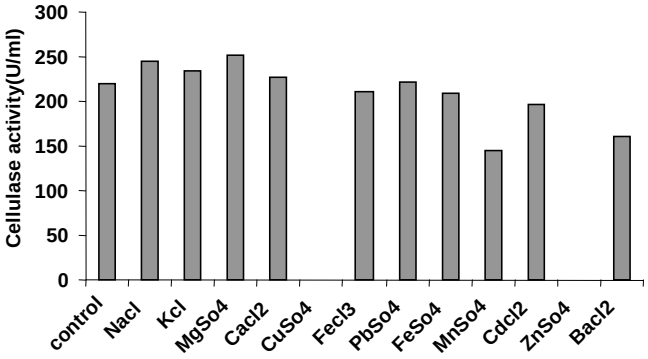

Figure 8 : Effect of various salts on cellulase production

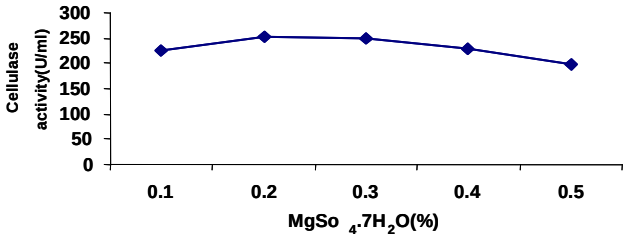

Figure 9 : Effect of $MgSo_4.7H_2O$ concentration on cellulase production

The production can be improved by standardizing the culture conditions. After optimizing parameters in this investigation it was observed that cellulase yield was increased to 7.76 fold and showed very simple nutrients requirement.

CONCLUSION

This study revealed that pineapple peel, which are examples of domestic and industrial agro-wastes was screened as best carbon source to produce large amounts of cellulase enzyme when hydrolyzed by cellulolytic microorganisms and instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce cellulase. Fructose a disaccharide as a best inducer to enhanced the cellulase production. By optimizing fermentation parameter for cellulase production there is 7.36 fold increases in cellulase activity thus reducing the cost for the production the enzyme which is one of the obstacle in the path of bioethanol production to overcome the increasing energy crisis. Moreover, pineapple waste could be directly used for production of ethanol, thus making the ethanol production process more economical and less time consuming.

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