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AVICELASE ENZYME FROM SAWDUST: ISOLATION, PRODUCTION AND OPTIMIZATION

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Abstract

Economical disposal of sawdust is a problem of growing concern to the wood industries as waste. On an industry-wide basis most of sawdust is green and has many uses that able to be converted to useful products such as fuel, chemical feedstock or textiles. Cellulose is a potential substrate to the enzyme that can be produced from the activation of cellulose conversion into monomer. Using microorganisms such as bacteria and fungi as a converter, cellulose can be degraded and converted into reducing sugar with the help of various enzymes including Avicelase enzyme. The utilization of sawdust as a potential substrate for producing enzymes may serve a dual purpose of reducing the environmental pollution along with producing a high value commercial product. Effect of nutritional parameters such as initial pH, carbon and nitrogen sources for Avicelase production from *Bacillus sp*. was investigated. From three different sources of isolates, only one of the most potent isolate was selected from a food source CL5 which has been partially identified and suggested to be *Bacillus* species. Optimum pH-value, carbon and nitrogen sources for Avicelase production found to be 7.0, lactose and KNO_3 were (2.450±0.009 IU/ml) increased up to 3556.7% with sp. act. 1.528 IU/mg; (1.286±0.074 IU/ml) increased up to 1548.7% with sp. act. 0.631 IU/mg and (2.287±0.109 IU/ml) increased to 3313.4% with sp. act. 0.979 IU/mg, respectively.

Key words: Avicelase production, Waste management, Bacterial isolates, Optimization.

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INTRODUCTION

The cellulolytic activity is described as extracellular in the present work since it was assayed in cell-fee culture supernatants. It is not possible to say if such enzymes are products of cell lysis or have been actively secreted by the bacteria, which is known to occur even during exponential growth of bacterial cultures [1]. Cellulase(s) are important enzymes that are sold in large scale for use in different industrial applications, for example in animal feed production, starch processing, malting and brewing, grain alcohol fermentation, extraction of fruit and vegetables juices, textile industry and pulp and paper industry. Submerged fermentation (SmF) is used for industrial production of cellulases. The cost of production is high and low production of these enzymes cause big problems for industrial applications. It has been reported that solid state fermentation (SSF) as an potential technique to produce cellulase(s) which is economical as it only needs lower capital investment and lower operating financing [2]. Cellulases are employed in the removing of inks, coating and toners from paper [3]. Also been used for the enhancement of the soil quality. Typically straw development is considered an important way to enhance soil quality and decrease the need of mineral fertilizers. Numerous researches have attempted to speed up straw breaking down using bacterial degradation. Cellulolytic fungi programs such as *Aspergillus, Chaetomium*, and *Trichoderma*, and actinomycetes have proven appealing results [4]. A specific endo-β-1,4-D-glucanase (Avicelase EC 3.2.1.91) was partially purified from a thermophilic bacterial strain was isolated from different soil samples when grown on cellulose enrichment sugarcane bagasse-SCB substrate as the sole carbon source [5].

The objective of this study is to find an optimum method of Avicelase enzyme production and extraction by using biomass-degrading microorganisms. Hence, ability of the microorganism in exploring waste cellulosic material as an alternative energy can be developed. In general, this study is based on a few steps which are isolation of microorganism from different sources such as soil, water,

food and crop waste containing cellulosic material; screening to obtain active organisms that can convert cellulose to form reducing sugar and finally enzyme assay.

MATERIALS AND METHODS

Isolation of Bacterial Isolates

Nutrient agar (NA) was prepared for bacterial isolation from different sources (soil, water and food samples) collected from different areas of Kuantan, Pahang, Malaysia. The plates were incubated at 37 and 50 °C for 24 to 48 h.

Sawdust Pretreatment

In this experiment, sawdust waste collected from any timber will was used as a substrate for microbial degradation power. Dry sawdust that has been ground into fine pieces was prepared in 1 L beaker. A sufficient amount of 4N NaOH was poured into the beaker until the sawdust submerged and kept at room temperature overnight. Then the sawdust washed using tap water several times until the color of the water become clear or less dense. The pH was checked using pH meter to neutralize the solution then the sawdust was filtered using Muslin cloth to drain the water then left to dry in oven overnight at 40-50 °C.

Inoculum Preparation

About 50 ml nutrient broth media prepared into 100 ml conical flask and autoclaved for 20 min at 121 °C, purified isolates were inoculated and incubated at 37 or 50 °C for 24 to 48 h according to the isolate.

Production media Preparation

Modified M9 (MM9) Minimal Medium (glucose free) was prepared by adding 500 ml MM9 Minimal Salts (2X) into 1L beaker. Two ml of 1.0 M $MgSO_4$ solution and 0.1 ml of 1.0 M $CaCl_2$ solution were added to the beaker contents. The total volume was adjusted to 1000 ml with dH_2O and mixed until homogenous. Fifty ml of MM9 media was poured into 100 ml conical flask. Pretreated sawdust which served as substrate (the sole carbon source) was weighed to 1.5 g and added to the media then autoclaved at 121 °C for 20 min. After that, media was inoculated with 5 ml of bacterial suspension isolate and incubated at 37 or 50 °C for 24 to 48 h.

Protein Determination and Enzyme Assay

Protein determination was carried out according to [6] Lowry method by Folin Reaction and all samples were in replicates. Dinitrosalicylic colorimetric (DNS) method [7] was used to detect the presence of free carbonyl group (C=O) or reducing sugars and the equivalent enzyme used in the reaction can also be detected in one ml of the cell-free filtrate obtained from the production media was incubated with 1 ml of 2 % (W/V) Avicel in 0.1 M phosphate citrate buffer (pH 6.6) at 40 °C for 2 h distilled water was used instead of cell-free filtrate as blank. The reducing sugar was determined using 1.5 ml of DNS reagent was added to 1.5 ml sample in a capped tube to avoid the liquid from evaporating. The mixture was heated at 90 °C for 5-15 min to develop the red-brown color. One ml of 40 % potassium sodium tartrate solution was added to stabilize the color. After cooling to room temperature, the absorbance was measured using spectrophotometer at 575 nm.

Parameters Controlling Enzyme Productivity

Different four carbon sources were used (lactose, glucose, sucrose and starch) and five different nitrogen sources used ($NaNO_3$, $(NH_4)_2$ SO_4, yeast, KNO_3 and peptone) to optimize the enzyme productivity. About 40 ml of MM9 media added to 1.5 g of pretreated sawdust substrate into100 ml conical flask, 0.5 ml of each source was added into the flasks and then inoculated with about 5 ml bacterial suspension of the selected most potent (CL5) isolate and incubated at 37 °C for 24-48 h. On the other hand, different pH-values within the range of 4.0 to pH 9.0 determined. At the end of incubation period, the cell-free filtrate prepared for proteins and enzyme assay. Gram reaction was done for partial identification of the most potent isolate under study.

RESULTS AND DISCUSSION

Protein and enzyme assay

Data recorded in table (1) exhibited about 12 most potent isolates out of 55 were selected for more screening against enzyme activity and protein content. These isolates were classified as only 9 isolates that were successfully grown on sawdust at 37°C and only 3 isolates grown at 50°C were selected. All results were expressed as IU/ml for enzyme activity and mg.protein/ml in case of protein content. An enzyme which degrades crystalline cellulose was detected in cultures of cellulose by measuring the formation of soluble carbohydrate but was not detected by determining the reducing sugar released

[8]. The difficulty in comparison between cellulase(s) activities depends on several factors including the- assay determination, difference between strains used in production and condition of production SmF or SSF. According to Mukherjee and others [9] states that crystalline regions are considered to be more difficult to be degraded than the amorphous regions. The key element in bioconversion process of lignocellulosics is the hydrolytic enzymes mainly cellulases. The use of media with high cellulose content appears to be desirable when greater yields of cellulase are required. This means the cellulase production is directly proportional to the quantity of enzyme being consumed by the microorganism. The higher cellulose, the higher cellulase enzyme can be produced.

Table 1: Screening of most potent isolates for avicelase activity and protein content from sawdust

Isolate code	Production TemP. (°C)	Avicelase activity (IU/ml)	Protein content (mg/ml)
		Mean ± SD	Mean ± SD
Control	37	0.102±0.018	1.416±0.001
CL8A	37	0.077±0.018	1.532±0.069
A5	37	0.102±0.016	1.812±0.001
CL5	37	0.108±0.006	1.771±0.018
PS1	37	0.104±0.003	1.766±0.011
CL4B	37	0.088±0.006	1.795±0.000
RSS3	37	0.102±0.003	1.752±0.025
CL2 (Branch)	37	0.062±0.006	1.820±0.007
KK2S5	37	0.094±0.212	1.804±0.001
JF outer 2B (PDA)	37	*NG	
Control	50	0.144±0.010	1.456±0.014
A1	50	0.148±0.006	1.890±0.008
A9	50	0.140±0.009	1.975±0.000
GL1	50	0.116±0.003	1.823±0.006

*NG = No Growth

Parameter controlling the enzyme activity

Only one isolate, CL5 was selected out of 12 most potent isolates to continue for the effect of parameters controlling the production of Avicelase enzyme at 37°C which are effect of carbon source, nitrogen source and pH value as well. As shown in figure 1 the effect of different carbon sources on Avicelase productivity revealed that in the presence of lactose sugar exhibited maximum enzyme activity than others to be (1.286±0.074 IU/ml) when CL5 isolate grown on sawdust substrate at 37°C for 48 h incubation. The most weak carbon source of Avicelase productivity was starch with only (0.212±0.001 IU/ml). Induction of avicelase enzyme was reported in presence of lactose as the carbon source when produced by *Cellulomonas fimi* and *Cellulomonas cellusea* strains [10].

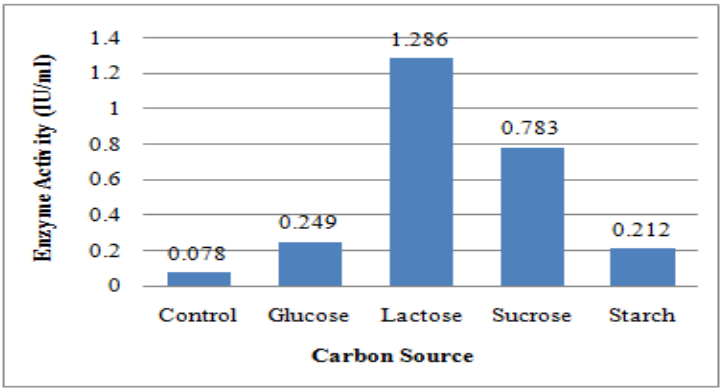

Fig. 1. Graph of different carbon sources used.

As recorded in fig. 2 the effect of different nitrogen sources on Avicelase activity exhibited that the maximum activity obtained in presence of KNO_3 as the best source as compared with others to be (2.287±0.109 IU/ml) for enzyme activity when CL5 isolate grown on sawdust at 37°C for 48 h. In this study, both organic and inorganic nitrogen sources were used to detect the best nitrogen sources for Avicelase enzyme production. This result show a contrast with Mukherjee and others [9] which show a higher productivity of Avicelase using peptone as nitrogen source. Type of substrate, enzyme assay technique or reagent used may also contributed to the contradict results. According to Rosma and Cheong [11] have found that for the fermentation medium with 0.01% (w/v) nitrogen content, potassium nitrate, KNO_3 showed the highest yield among the other inorganic nitrogen sources which are $NH_4H_2PO_4$ and $(NH_4)_2SO_4$. According to Kubisi and others [12], potassium nitrate is a naturally occurring mineral source of nitrogen, but only several yeasts are able to assimilate nitrate as a nitrogen source.

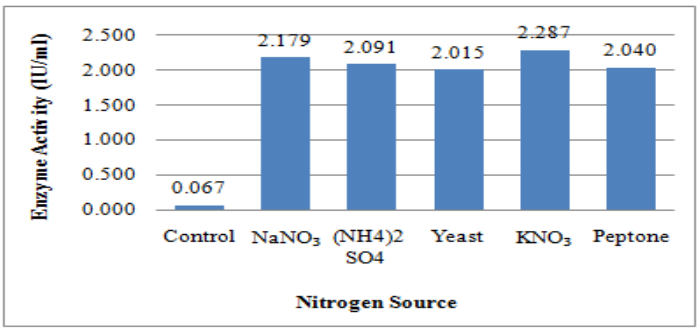

Fig. 2. Graph of different nitrogen sources used.

Data recorded in fig. 3 the effect of different pH value on Avicelase activity exhibited that the maximum activity obtained at pH 7 as compared with others to be (2.450±0.009 IU/ml) for enzyme activity when CL5 isolate grown on sawdust at 37 °C for 48 h. Other research also expressed that Avicelase enzyme exhibited high pH stability when media production is in range 7.0-7.2 [8]. The consistency between both results showed that Avicelase has higher tendency to be produced in natural condition. Other reports indicated that pH 7.0 was more suitable for Avicelase (0.30 and 1.57 IU/ml) for both untreated sugarcane bagasse (UntSCB) and treated sugarcane bagasse (TSCB), respectively. Either increase or decrease in pH beyond the optimum value showed decline in enzyme activities. Avicelase enzyme showed good pH stability between (5-8) and (4-9) for UntSCB and TSCB, respectively [5].

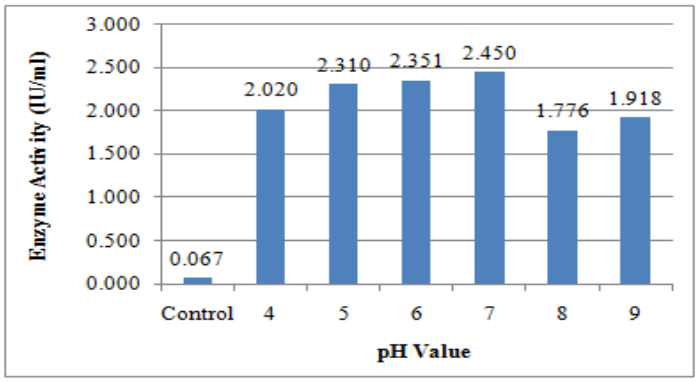

Fig. 3. Graph of different pH values

Partial Identification of Bacterial Isolate

It was revealed that CL5 isolate is the most potent selected out of 55 isolates. CL5 is a type of common bacteria that grow at optimum temperature of 37°C. It has been confirmed that the most potent isolates CL5 is a Gram-positive bacteria as it shows general characteristics and morphological structure of *Bacillus* species. *Bacillus* is a genus of Gram positive and rod shaped bacteria. However,

the exact species of the *Bacillus* is not being tested. CL5 is a mesophile bacterium as it grows best in moderate temperature, neither too hot nor too cold, typically between 20 and 45°C.

CONCLUSION

In summary, a simple microbial production method has been developed for production of Avicelase enzyme from sawdust by the most potent *Bacillus sp.* isolate when characterized in different parameters controlling the enzyme production when grown at 37°C for 48h incubation period.

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