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Full Length Research Paper

Bioconversion process of rice straw by thermotolerant cellulolytic *Streptomyces viridiochromogenes* under solid-state fermentation conditions for bioethanol production

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Enzymatic hydrolysis of the cellulose fraction of rice straw to glucose using solid-state fermentation for bioethanol production is a focus of current attention. A total of 10 actinomycetes isolates were isolated from soils and decayed rice straw. All these isolates were purified and screened for their cellulolytic activity; one strain was selected for further study and identified as *Streptomyces viridiochromogenes*. Optimization of fermentation conditions showed highest cellulolytic enzymes production on the 5th day at pH 6.5 and at 40°C. The production of enzymes reached its maximal value at 4.0 g of rice straw/250 ml flask. Avicelase and total cellulase productivity were highly increased by the addition of NH_4Cl as N-source, while maximum activity of CMCase was recorded by the addition of **peptone as N-source to the fermentation medium. The influence of various physico-chemical factors on enzyme activity was also investigated. The half life time of avicelase, total cellulase and CMCase at 60°C was 39.4, 50.0 and 78.58 min, respectively. A maximum of ethanol production 1.428±0.074% (v/v) by *Saccharomyces cereviseae* using dilute acid pretreated rice straw hydrolysate with initial soluble sugar 2.340±0.072% was recorded after 2 days of fermentation.**

Key words: Bioethanol, cellulolytic enzymes, rice straw, solid-state fermentation, *Streptomyces viridiochromogenes*.

INTRODUCTION

Bioethanol, a clean and renewable energy source, which can be produced through fermentation from renewable biomass, has drawn much attention from the government and researchers (Sun and Cheng, 2002). The demands for ethanol have the most significant market, where ethanol is either used as a chemical feedstock or as an octane enhancer or petrol additive.

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Abbreviations: CMC, Carboxymethyl cellulose; **CMCase,** carboxymethyl-cellulase; ***s*,** *Streptomyces.*

Large amounts of lignocellulosic wastes are generated through agricultural practices, paper-pulp industries, timber industries and many other agro-industries. The removal of lingocellulose waste is often disposed off by biomass burning, causing an environmental pollution problem and consequently affects public health, which is not restricted to developing countries, but is considered a global phenomenon (Levine, 1996). Furthermore, the chemical properties of lignocellulose components make them of enormous biotechnological values (Malherbe and Cloete, 2003). The production of ethanol from lignocellulosic material requires the release of monomeric sugar units constituting cellulose and hemicellulose. Based on the specific lignocellulosic structure, the common bioethanol process employs three steps including

substrate pretreatment (Grethlein and Converse, 1991), hydrolysis and sugars fermentation. Pretreatment reduces the size and breaks down the lignin and crystalline structure of lignocellosic biomass, thus facilitating the subsequent hydrolysis of cellulose and hemicellulose. The hydrolysis step employs enzymatic hydrolysis approaches to convert cellulose and hemicellulose into sugars (for example, hexose or pentose sugars). The sugars are finally fermented by yeasts or bacteria to ethanol. The overall reaction of hexose sugar fermentation by yeast has been expressed by Gay-Lussac which forms the basis of calculating fermentation efficiency.

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \xrightarrow{\textit{Saccharomyces cerevisiae}} \underset{\text{Ethanol}}{2C_2H_5OH} + \underset{\text{Carbon dioxide}}{2CO_2}$$

1kg 0.64 L 0.36 L

Rice straw is one of the abundant lignocellulosic waste materials in the world. In Egypt, rice straw is one of the most abundant agricultural wastes as compared to other straws. Currently, this straw is predominantly disposed of by direct burning in open field due to lack of effective utilization, it is a cheap disposal method. This also causes serious environmental pollution. It is an important issue to deal with the agricultural waste both for the comprehensive utilization of lignocellulosic resources and for the prevention of environmental pollution. One of the different approaches to overcome this problem is the biological conversion of rice straw to biofuels.

Actinomycetes, one of the known cellulose producers, has attracted considerable research interest due to its potential applications in the recovery of fermentable sugars from cellulose that can be of benefit for human consumption and to the ease of their growth (Jang and chen, 2003). For the degradation of cellulose, hemicellulose and lignin, different strains of the *Streptomyces* genus have been studied (Jang and chen, 2003; Schrempf and Walter, 1995). This study was to evaluate the best conditions for production of cellulolytic enzymes on rice straw as a cost-effective agricultural by-product in solid-state fermentation by a locally isolated *Streptomyces viridiochromogenes* NEAE-26, hydrolysis of acid-pretreated rice straw and the alcoholic fermentation of the enzymatic hydrolysate of rice straw by *Saccharomyces cerevisiae.*

MATERIALS AND METHODS

Substrate

Rice straw used in the experiments was obtained from local farmers at the end of the harvest season (2008). The straw was oven dried and milled to achieve the size of less than 1 cm. This straw was pretreated by dilute-acid. The pretreatment was carried out by soaking fifteen grams of ground rice straw into 300 ml of 1% H_2SO_4 in a 500 ml wide-mouth Pyrex bottle, the mixture was stirred with a glass rod to mix them well. The mixture was pretreated at 121°C in an autoclave for 1 h; it was then cooled to room temperature. The solid fraction was washed five times with distilled water until the supernatant pH reached 6.0 to 6.5, filtered and dried biomass in a 45°C oven for 3 days. Grinding of dried biomass was done to ensure more uniform particle size distribution.

Microorganisms and maintenance of cultures

The *Streptomyces* spp. used in this study were locally isolated from soil samples and decayed rice straw collected from rice fields in Dakahlia governorate. Actinomycetes from the soil had been isolated by pour plate technique. Dry colonies of actinomycetes were selected and isolated. Isolated colonies had been preserved in 20% glycerol and stored at -20°C. The medium used for isolation, cultivation and stock maintenance of isolated strains was starch nitrate agar medium (Waksman, 1959). It contained (g/L): Starch 20; KNO_3 2; K_2HPO_4 1; $MgSO_4.7H_2O$ 0.5; NaCl 0.5; $CaCO_3$ 3; $FeSO_4.7H_2O$ 0.01; agar 20 and distilled water up to 1 L. Plates were incubated for a period of 7 days at 30°C.

Inoculum preparation

The spore suspension was prepared from a 5 day old culture grown on starch-nitrate agar slant by adding 10 ml of sterile distilled water and suspending the spores with a sterile loop. This spore suspension was used as an inoculum.

Plate screening of cellulase producer

A total of 10 strains of actinomycetes were isolated from soil samples and decayed rice straw. A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The actinomycetes strains were grown on CMC agar containing (g/L): CMC 10.0; $NaNO_3$ 1.2; KH_2PO_4 3.0; K_2HPO_4 6.0; $MgSO_4{\cdot}7H_2O$ 0.2; $CaCl_2$ 0.05; $MnSO_4{\cdot}7H_2O$ 0.01; $ZnSO_4{\cdot}7H_2O$ 0.001; agar 20; yeast extract 0.5 and distilled water up to 1 L. The pH was adjusted to 7.0. Agar plates were seeded with spores and incubated at 30°C for 7 days. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation. The largest clear zone diameter was assumed to contain the highest activity.

Saccharification of pre-treated rice straw by solid state fermentation

Dilute acid pretreated rice straw was used as the solid substrate for solid-state fermentation. Experiments were conducted in 250 ml Erlenmeyer flasks containing 3 g of pretreated rice straw moistened with 3 ml of basal medium containing the following (g/L): $NaNO_3$ 1.2; KH_2PO_4 3.0; K_2HPO_4 6.0; $MgSO_4{\cdot}7H_2O$ 0.2; $CaCl_2$ 0.05; $MnSO_4{\cdot}7H_2O$ 0.01; $ZnSO_4{\cdot}7H_2O$ 0.001; yeast extract 0.5 and distilled water up to 1 L. The pH was adjusted to 7.0. The flasks were autoclaved at 121°C for 30 min, inoculated with 3 ml of spore suspension of *Streptomyces* isolate and the contents of the flasks were thoroughly mixed. Flasks were incubated at 30°C, under static conditions for 5 days. After fermentation, the contents of the flasks were harvested for enzyme extraction and assay.

Enzyme extraction and assay

Cellulases were extracted by suspending the fermented rice straw

in 10 fold 0.1 M sodium acetate buffer (pH 5.5) and thoroughly mixed on a rotary shaker (200 rpm) for 30 min at 30°C and extract was kept for 6 h in the refrigerator. Following this, the residue of substrate and the biomass were separated by centrifugation (5000 rpm for 20 min) to remove all microbial cells and residue of substrate.

The clarified supernatant representing the crude enzyme preparation was used for assaying different enzyme activities. Total cellulase, avicelase and carboxymethylcellulase (CMCase) activities were assayed by measuring the release of reducing sugars in the reaction mixture of 0.1 ml of crude enzyme and 0.1 ml of 1.0% (w/v) cellulose (for total cellulase), carboxymethylcellulose (for CMCase) or Avicel pH 101 (for avicelase) dissolved in 0.1 M acetate buffers (pH 5.5) and incubated for 1 h (for total cellulase and avicelase) and 45 min (for CMCase) at 45°C. The amount of reducing sugars released by active enzymes was determined by Nelson (1944) and Somogyi (1952) method against boiled enzyme as control with D-glucose as standard. One unit of total cellulase, avicelase and CMCase activity is defined as the amount of enzyme required for liberating one µmole of glucose per minute under the standard assay conditions and was expressed as units per gram dry substrate (U/g ds). The specific activity was expressed in µmole $min^{-1}µg^{-1}$ protein (U/µg protein).

Determination of soluble protein

The soluble protein concentration was determined according to Bradford (1976), using crystalline bovine serum albumin as standard.

Optimization of fermentation conditions for cellulolytic enzymes production

Various process parameters influencing the enzyme production during solid-state fermentation were studied for maximal enzyme production as follows: The effect of incubation period on cellulolytic enzymes production was evaluated through 24 h interval by checking the enzyme activity. The effect of substrate concentration on the cellulolytic enzymes production was done by using different weights of rice straw (1.0 to 7.0 g/ 250 ml flask). The effect of cultivation temperature was examined at different temperatures (20 to 60°C). The effect of supplement of different nitrogen sources was examined on equivalent nitrogen basis. The effect of initial pH on the enzyme production was examined using different pH levels (4 to 8) of moistening agent (buffered basal medium) which adjusted with 0.1 M sodium acetate and 0.1 M phosphate buffers. The effect of initial total moisture content and inoculum's level were carried out.

Physicochemical properties of cellulolytic enzymes

The optimum incubation period of reaction mixture for the maximum activity of enzymes was determined at different time intervals. The optimum pH level for enzyme activity was determined by incubating each enzyme with its specific substrate at different pH levels (4 to 8) under optimal conditions. Effect of different substrate concentration on the enzyme activity was determined using 0.1 ml enzyme incubated with different concentrations of the substrate (0.50 to 3.0%) under optimal conditions. The effect of temperature on the enzyme activity was determined after incubation of the crude extract of enzyme with its specific substrate at different temperatures for 30 min for CMCase, total cellulase, and avicelase. The thermal and pH stabilities of different enzymes were examined. The calculated half life time ($T_{1/2}$) was detected through linear regression analysis of obtained data using Graph-Pad Prism 4 software.

Degradation of pretreated rice straw into fermentable sugars

Forty milliliter of crude enzyme produced by *Streptomyces* sp. was incubated with 2 g of dilute acid pretreated rice straw for 24 h at 50°C with constant stirring. The free reducing sugars produced were determined by anthrone method (Trevelyan and Harrison, 1952).

Yeast and inoculum preparation

Baker's yeast *S. cereviseae* was obtained from Biotech international R and D, Egypt. It was maintained on yeast peptone dextrose (YEPD) medium containing (g/L): Yeast extract 3; peptone 10; dextrose 20; agar 20 and distilled water up to 1 L. Plates were incubated for a period of 24 h at 30°C. It was stored under refrigerated conditions (4°C) and subcultured every month. For inoculum preparation, a loopful of twenty four hour old culture was inoculated to 50 ml of sterilized YEPD broth in 100 ml Erlenmeyer flask, the flask was incubated at 30°C for 24 h. This inoculum was used at 10% (v/v).

Fermentation process

Alcoholic fermentation was carried out with *S. cerevisiae* in 100 ml medium containing (% w/v): 2.34 initial soluble sugar; $(NH4)_2HPO_4$ 0.6; K_2SO_4 0.4; urea 1 at pH 5.5. Mineral salts and urea were autoclaved separately at 121°C for 30 min, and then added to the hydrolysate, aseptically. Ten percent (v/v) of the inoculum was used to inoculate the fermentation medium and allowed for fermentation for 4 days at 30°C (Sandhu et al., 1998). After 1 to 4 fermentation days; the ethanol content was measured by spectrophotometer. In addition to the remaining sugar during fermentation was also measured using the anthrone method (Hedge and Hofreiter, 1962). For calculation of fermentation efficiency the following equation was applied:

$$\text{Fermentation efficiency} = \frac{\text{Actual ethanol recovery}}{\text{Theoretical recovery}} \times 100$$

Where, actual ethanol recovery = actual ethanol obtained. Theoretical recovery = Amount of initial sugar content in fermentation solution × 0.64

Ethanol estimation

Determination of ethanol content was done by spectrophotomentric method (Caputi et al., 1968).

Determination of yeast from fermentation process

The concentration of yeast in the fermentation mash was determined gravimetrically as grams of dry weight in 1000 ml. The centrifuged and washed cells were dried to a constant weight at 103 to 105°C for 12 h (APHA, 2005). Duplicate determinations were made on all samples.

RESULTS AND DISCUSSION

Actinomycetes are well known for their ability to decompose complex molecules, particularly lignocellulose

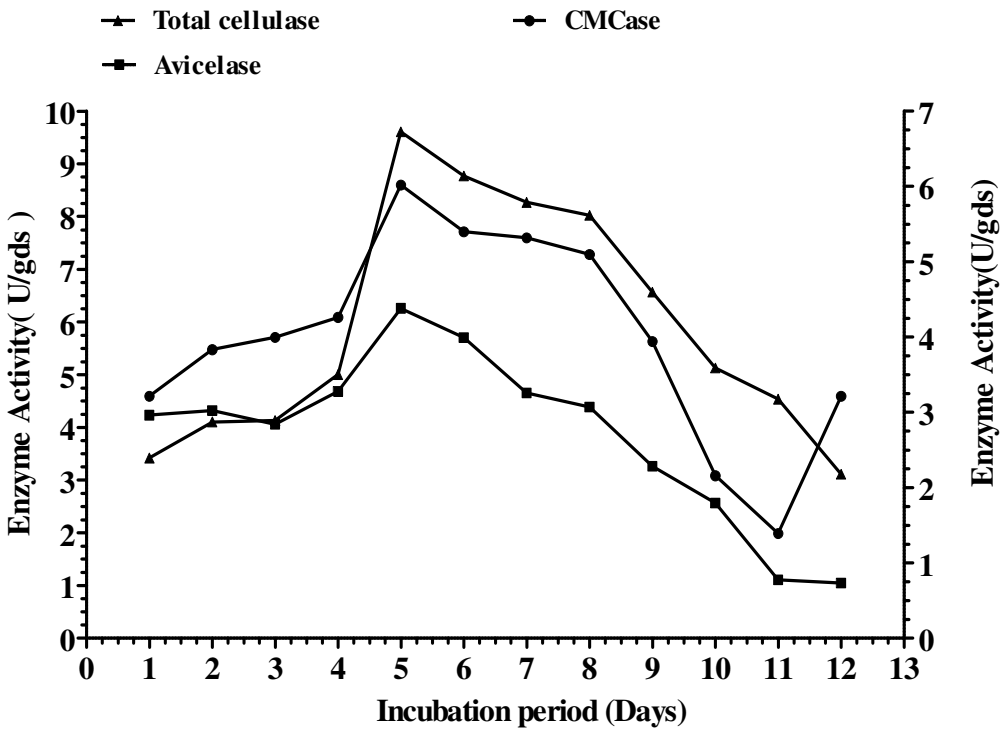

Figure 1. Effect of incubation period on cellulolytic enzymes production by *Streptomyces viridochromogenes*.

components. Additionally, the apparent widespread ability of actinomycetes to generate soluble lingo carbohydrate from straw has been confirmed (Ball et al., 1990). While screening a variety of actinomycetes for cellulolytic activities by a previously described method on CMC-containing agar plates, the strain NEAE-26 produced the largest clear zone diameter around its colonies indicating the highest cellulose degradation was selected for further study and identified as *S. viridiochromogenes*.

Effect of incubation period on cellulolytic enzymes production

Data illustrated graphically in Figure 1 showed that total cellulase, CMCase and avicelase displayed maximum activity after 5 days (9.604, 6.020 and 6.256 U/gds, respectively). The enzymes production increased gradually and reached its maximum production on the 5th day, then decreased gradually till the 12th day of incubation. This result is similar with that obtained by Alam et al. (2004) who reported that *Streptomyces omiyaensis* was found to show maximum activity after 5 days of incubation period and in addition, Jang and Chen (2003) reported that the transformed strain *Streptomyces* sp.T3-1 CMCase and avicelase recorded their maximum production after 3 and 5 days, respectively. In addition, Jaradat et al. (2008) observed that *Streptomyces* strain J2 showed the highest crude enzyme activity after 3 days of incubation. George et al. (2010) proved that *Streptomyces noboritoensis* SPKC1 was showing good CMCase activity on the 8th day. The decrease in cellulase activity shown by *S. viridiochromogenes* after attaining its maximum peak period of enzyme secretion could be attributed to catabolite repression by the products of cellulase action on rice straw (cellobiose and glucose …etc.) which inhibit enzyme secretion. Depletion of carbon and nitrogen sources causes starvation and hence, the organism may not grow and cellulase activity is growth related as reported by Dosoretz et al. (1990).

Effect of substrate concentration on cellulolytic enzymes production

The data shown in Figure 2 disclosed that lower or higher substrate concentration negatively affected the cellulolytic enzymes production by *S. viridochromogenes*. The moderate concentration (4 g/ 250 ml flask) led to high enzyme productivity. Activities of CMCase, total cellulase and avicelase recorded were 9.181, 16.337 and 13.154 U/gds, respectively. This result is in agreement with the result obtained by Fadel (2000). Substrate level is a vital factor in solid-state fermentation process especially under using tray or flasks during fermentation process (Satyanarayana, 1994). Substrate level has a lot of

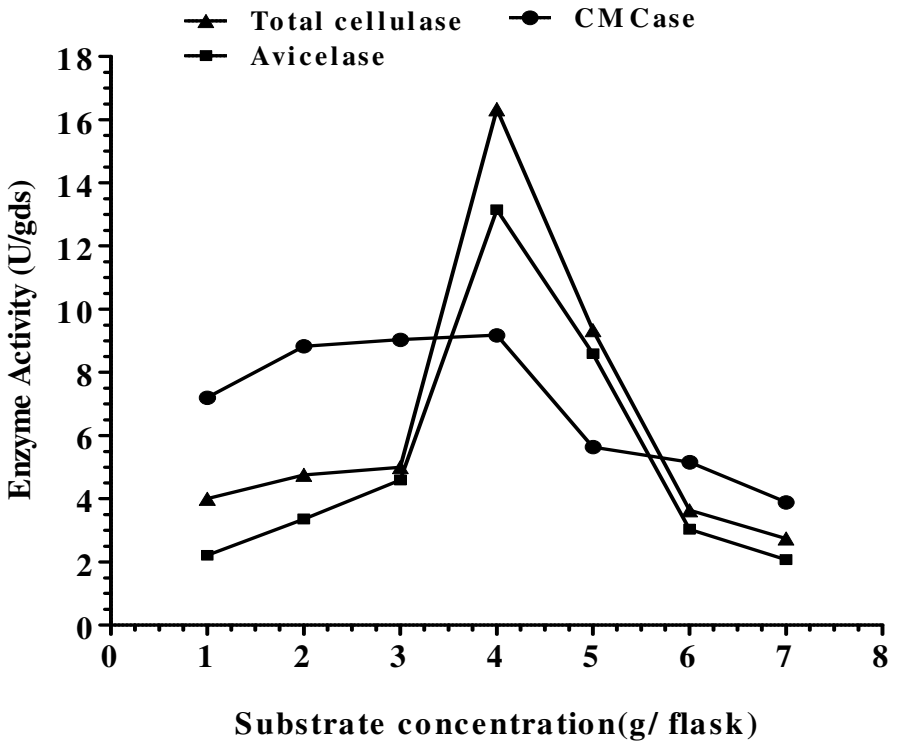

Figure 2. Effect of substrate concentration on cellulolytic enzymes production by *Streptomyces viridochromogenes*.

Table 1. Effect of temperature on the production of cellulolytic enzymes by *Streptomyces viridochromogenes*.

Temperature (°C)	Enzyme activity (U/gds)		
	Total cellulase	Avicelase	CMCase
20	5.351	3.365	4.055
30	16.492	16.537	12.081
40	24.310	20.143	13.748
50	22.651	16.628	3.470
60	10.770	8.635	1.571

influences on other culture fermentation factors such as substrate depth, aeration, heat transfer, moisture content, inoculum size, etc. (Hansen et al., 1993). Furthermore, it highly depends on substrate type and its particle size and used organism (Fadel, 2000).

Effect of incubation temperature on cellulolytic enzymes production

Results obtained in Table 1 showed the effect of temperature on cellulolytic enzymes production. The optimum incubating temperature for maximum total cellulase, avicelase and CMCase productivity by *S. viridochromogenes* (24.310, 20.143 and 13.748 U/gds, respectively) was found to be 40°C. This result is in agreement with the results reported by McCarthy (1987), who reported an optimal temperature for cellulase activity in the range of 40 to 55°C for several *Streptomyces* species including *Streptomyces lividans*, *Streptomyces flavogrisus*, and *Streptomyces nitrosporus.* Jang and Chen (2003) described a CMCase produced by a *Streptomyces* T3-1 with optimum temperature of 50°C, whereas Schrempf and Walter (1995) described CMCase optimum production by *Streptomyces reticuli* at 55°C.

Effect of nitrogen source on cellulolytic enzymes production

The results represented graphically in Figure 3 indicated

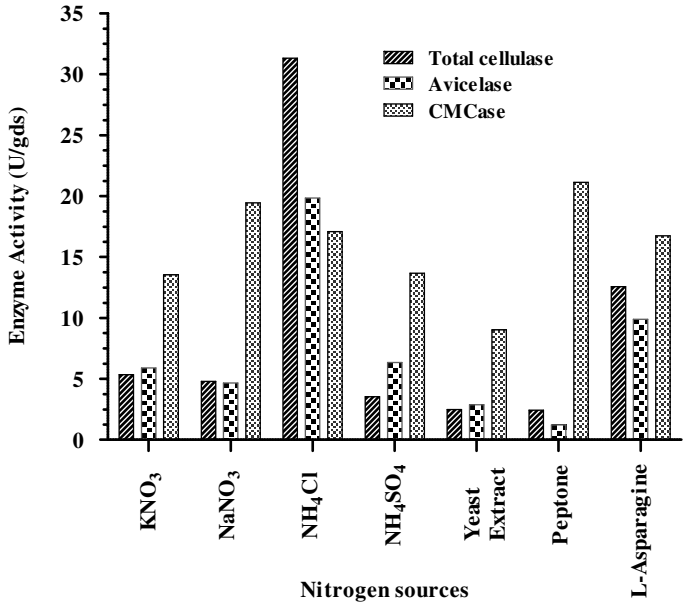

Figure 3. Effect of nitrogen source on cellulolytic enzymes production by *Streptomyces viridochromogenes*.

that *S. viridochromogenes* avicelase and total cellulase productivity highly increased with the addition of NH_4Cl and reached 31.325 and 19.860 U/gds, respectively. On the other hand, the optimum CMCase productivity (21.131 U/gds) was achieved by the addition of peptone to the fermented culture medium. This result is similar to that reported by Jaradat et al. (2008) who showed that the highest level of enzyme production was achieved when NH_4Cl was added to the CMC medium as sole source of nitrogen. In addition, Alam et al. (2004) recorded *S. omiyaensis* can produce maximum cellulase with beef extract as a nitrogen source. Chellapand and Himamhy, (2008) found that maximum cellulase productivity by *Streptomyces* sp. BRC2 was obtained when ammonium phosphate was added to the production media.

Effect of initial pH on cellulolytic enzymes production

The results in Figure 4 illustrated the maximum productivity of different cellulolytic enzymes by *S. viridochromogenes* at pH 6.5. However, activities decreased at highly acidic pH or pH level above 6.5. Highest productivity of total cellulase, avicelase and CMCase amounted to 40.022, 27.455 and 14.821 U/gds, respectively. The production of *S. viridochromogenes* cellulolytic enzymes under solid-state fermentation is dependant upon the level of initial pH in the fermented medium. The higher liquefaction of cellulose due to enzyme activity at pH 6.5 to 7.5 was reported by many workers (Hachiro and Kazuhiko, 1991; Hossain et al., 1998). *S. omiyaensis* showed heavy growth and high cellulase activity at pH 6.5 (Alam et al., 2004). The role of pH factor attributed to affect the permeability of cells as well as stability of produced enzyme (Mase et al., 1996).

Effect of moisture level on cellulolytic enzymes production

The results in Table 2 showed that all determined activities were maximal at 50% moisture, above or below it, these activities decreased. The maximal activities of total cellulase, avicelase and CMCase by *S. viridochromogenes* were 55.87, 47.50 and 17.41 U/gds, respectively. These results are generally in agreement with the results obtained by Lu et al. (2003); they reported that high enzyme production was obtained at 40 to 50% moisture using dry koji. Increase in moisture level is believed to decrease enzyme productivity due to decreasing substrate porosity, alteration in particle structure, lowering oxygen transfer and higher soluble protein. On the other hand, low moisture content causes reduction in the solubility of nutrients as well as low degree of substrate swelling (Gervais and Molin, 2003).

Effect of inoculum amount on cellulolytic enzymes production

The data recorded in Table 3 showed the effect of inoculum amount on cellulolytic enzymes production. The

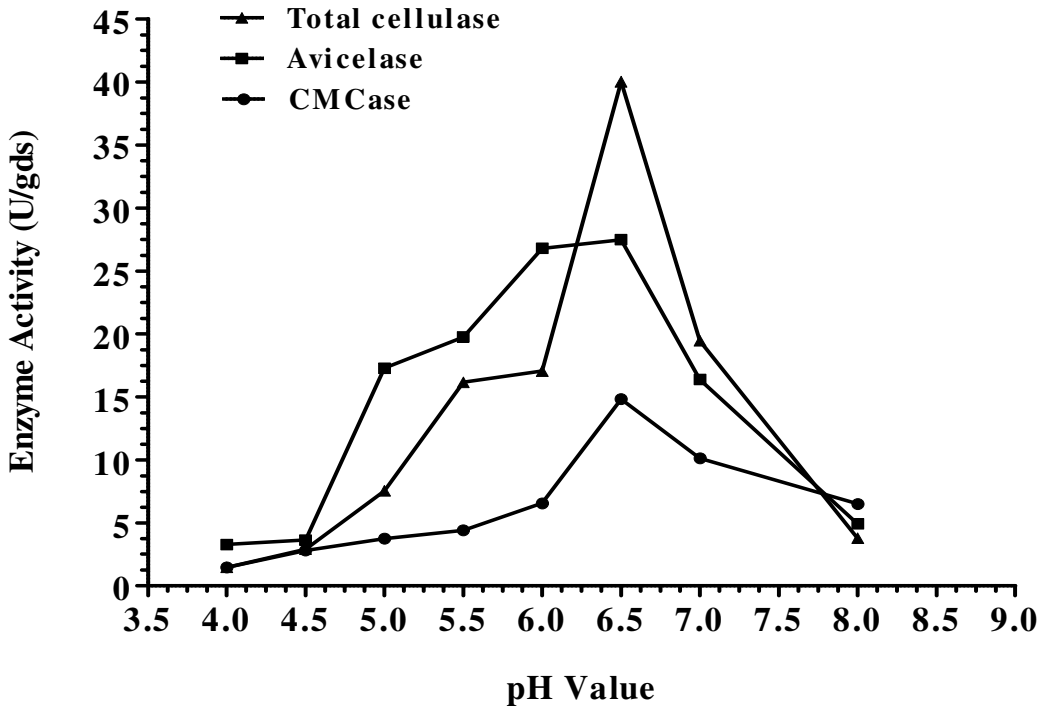

Figure 4. Effect of initial pH values on cellulolytic enzymes production by *Streptomyces viridochromogenes*.

Table 2. Effect of moisture level on cellulolytic enzymes production by *Streptomyces viridochromogenes*.

Moisture content (%)	Enzyme activity (U/gds)		
	Total cellulase	Avicelase	CMCase
33.0	45.90	39.60	14.54
50.0	55.87	47.50	17.41
67.0	15.66	9.75	5.47
75.0	5.10	6.61	4.62
80.0	2.51	5.36	2.52

Table 3. Effect of inoculum amount on cellulolytic enzymes production by *Streptomyces viridochromogenes.*

Inoculum size (ml)	Enzyme activity (U/gds)		
	Total cellulase	Avicelase	CMCase
0.5	2.343	1.722	2.482
1.0	46.754	24.642	6.632
1.5	51.539	36.575	18.085
2.0	59.323	51.893	22.462
2.5	50.012	44.432	11.161

optimum inoculum amount for maximum total cellulase, avicelase and CMCase productivity by *S. viridochromogenes* (59.323, 51.893 and 22.462 U/gds, respectively) was found to be 2 ml. A decline in enzymes production was observed at inoculum amount of 2.5 ml. Our results are agreement with Kunamneni et al. (2005) who showed that further increase in inoculum amount resulted in decreasing enzyme yield due to limitation of nutrients. Low inoculum may require longer time for microbial multiplication and substrate utilization to produce

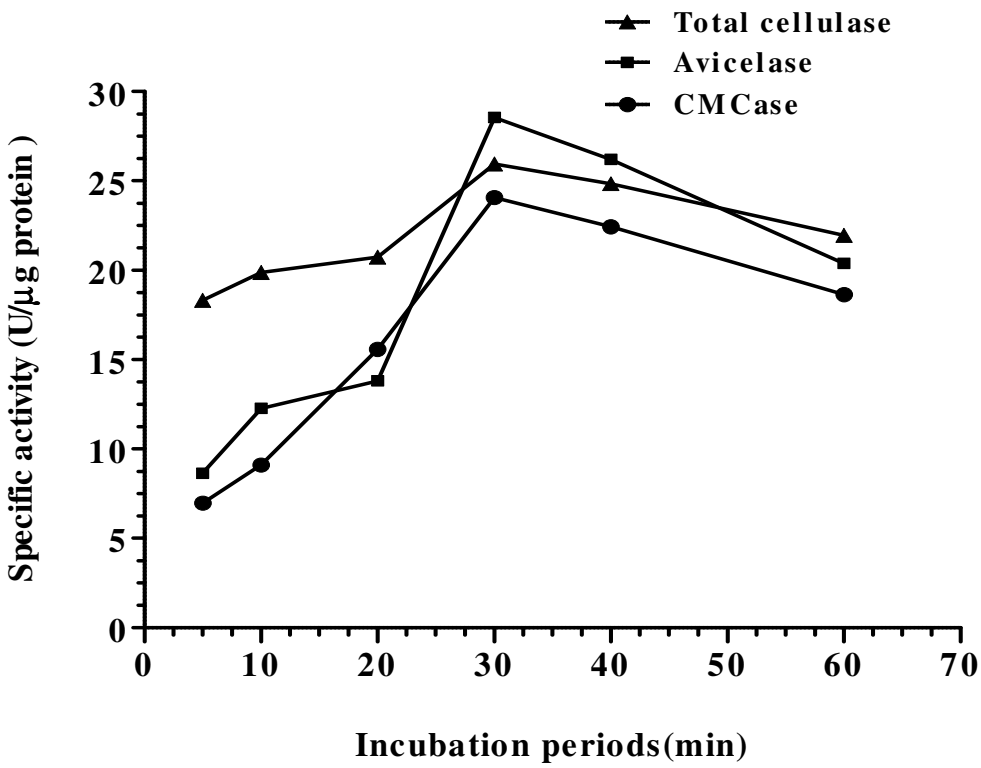

Figure 5. Effect of incubation period on cellulolytic enzymes activities by *Streptomyces viridochromogenes*.

desired enzyme. On the other hand, high inoculum would ensure rapid proliferation of microbial biomass. So, a balance between the proliferating biomass and substrate utilization would yield maximum enzyme activity as recorded by Ramachandran et al. (2004).

Effect of incubation period on cellulolytic enzymes activity

The effect of incubation periods on cellulolytic enzymes activities of *S. viridochromogenes* are depicted in Figure 5. It was observed that total cellulase; avicelase and CMCase activities gradually increased and reached its maximum activities (25.94, 28.55 and 24.07 U/µg protein, respectively) at 30 min, after which enzyme activity slowly decreased. This result is in agreement with Muthezhilan et al. (2007) who reported that 60 min incubation was the optimum for endoglucanase and exoglucanase activities.

Effect of pH value on cellulolytic enzymes activity

It is clear from the recorded results in Table 4 that the three tested cellulolytic enzymes showed their optimal activities at pH range of 5 to 5.5 as follows: 46.756 U/µg protein for total cellulase at pH 5.5 and 48.581 U/µg protein for avicelase and 28.208 U/µg protein for CMCase at pH 5.0. This result is similar to that reported by Theberge et al. (1992) who showed that the optimum pH for endoglucanase from a strain of *S. lividans* was 5.5 and considerably similar to that reported with Jaradat et al. (2008) who showed that CMCase enzyme from the active *Streptomyces* isolate J2 was found to be active over a pH range of 4 to 7 with maximum activity at pH 6. Kluepfel et al. (1986) reported that the maximal values of *S. lividans* 1826 CM-cellulase activity were obtained at pH7.

Effect of substrate concentration on cellulolytic enzymes activity

Total cellulase, avicelase and CMCase of *S. viridochromogenes* were evaluated under optimal conditions with varied substrate concentrations ranging from 0.5 to 3.0% (w/v). The results obtained in Figure 6 indicated that CMCase and avicelase enzymes reached their highest activity rate (45.290 and 57.500 U/µg protein) at 2.5% (w/v) substrate concentration. Total cellulase offered its highest activity rate (80.202 U/µg protein) at the substrate concentration of 2.0% (w/v). An increase in substrate concentration made more binding sites available for the enzymes to adhere and the rate at

Table 4. Effect of pH value on cellulolytic enzymes activities by *Streptomyces viridochromogenes*.

pH value	Specific activity (U/µg protein)		
	Total cellulase	Avicelase	CMCase
4	35.337	39.797	10.754
4.5	36.689	46.824	16.810
5	37.635	48.581	28.208
5.5	46.756	45.675	26.740
6	44.797	45.202	22.286
6.5	39.797	43.378	18.400
7	33.783	42.567	13.229
8	33.513	41.824	12.435

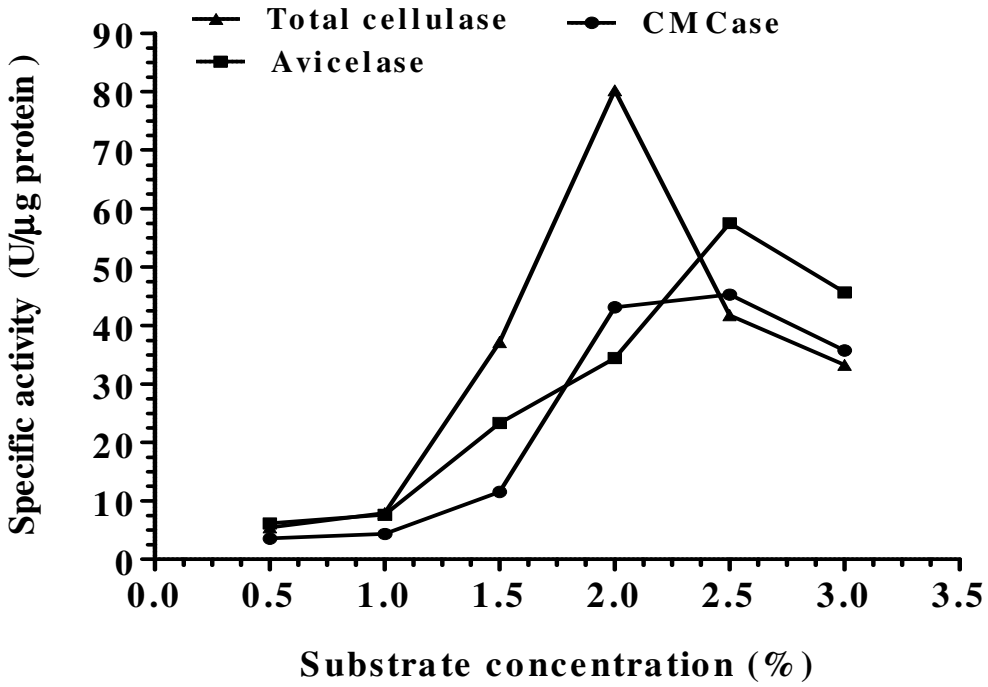

Figure 6. Effect of substrate concentration on cellulolytic enzymes activities by *Streptomyces viridochromogenes.*

which product formation would be achieved therefore would be faster (Dixon and Webb, 1971). El-Azab (2007) reported that the cellulases reached their maximum activity at 1.5% substrate concentration.

Effect of temperature on cellulolytic enzymes activity

The data recorded in Figure 7 showed that the optimum cellulolytic enzymes activities of *S. viridochromogenes* were reached at 50°C (86.58, 68.72 and 78.75 U/µg protein for total cellulase, avicelase and CMCase, respectively). This result is in agreement with Aboul-Enein et al. (2010) who recorded that the optimum temperature of cellulase enzyme produced by actinomycete isolate was found to be around 50 to 55°C and agreed with Jang and Chen (2003) who reported that maximum activity for cellulase were obtained at 50°C for *Streptomyces transformant* T3-1 and Kluepfel et al. (1986) who reported that the highest values for CM-cellulase activities were obtained at 50°C for *S. flavogriseus* enzymes and the highest values for CM-cellulase activities were obtained at 55°C for *S. lividans.*

pH stability of the enzymes

The results represented in Table 5 showed that the

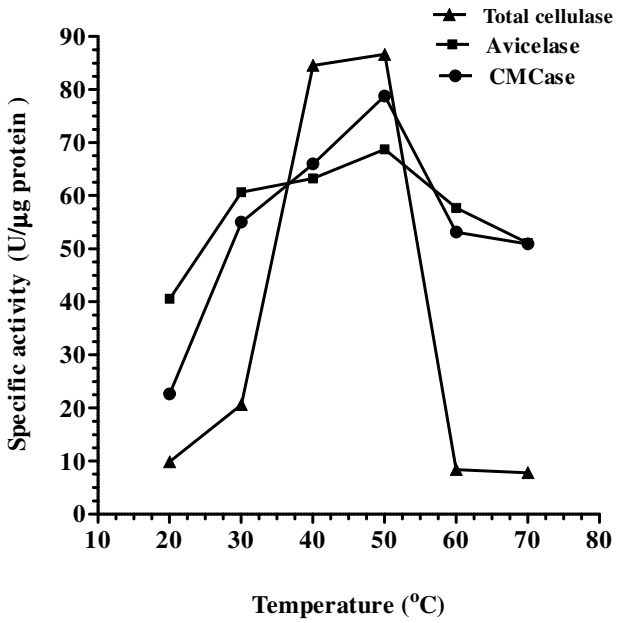

Figure 7. Effect of temperature on cellulolytic enzymes activities by *Streptomyces viridochromogenes*.

Table 5. pH stability of cellulolytic enzymes activities produced by *Streptomyces viridochromogenes*.

pH value	Specific activity (U/µg protein)		
	Total cellulase	Avicelase	CMCase
4	3.445	3.783	5.616
4.5	33.783	19.527	11.406
5	33.918	21.621	26.962
5.5	58.986	41.283	52.611
6	50.655	34.864	39.581
6.5	44.256	26.081	25.729
7	43.918	24.324	18.049
8	43.378	21.418	16.686

highest stability of the enzymes was at pH 5.5. Decrease in the *S. viridochromogenes* enzymes stability was at lower pH levels (pH 4 to 5) for total cellulase, avicelase and CMCase enzymes. Marginal decrease in enzymes stability was also detected at higher pH levels (pH 6.0 to 8.0). Optimum activity for CMCase from *Streptomyces drozdowiczii* occurred at pH 5.0, but another peak at pH 10.0 was also observed (Lima et al., 2005). According to George et al. (2001), CMCase from culture supernatant obtained from a species of *Thermomonospora* presented optimum activity at pH 5.0, whereas Jang and Chen (2003) obtained a CMCase produced by *Streptomyces* T3-1 with optimum activity at pH 7.0. The effect of pH on enzyme stability may be due to denaturation of the enzyme proteins which occur at pH differ or changing from the optimum of such enzyme (Kalra and Sandhu, 1986).

Thermostability of the enzymes

Thermal stability results of *S. viridochromogenes* avicelase (Figure 8) indicated that 88.9, 85.3, 25.03 and 19.49% of its activity was retained at 40, 50, 60 and 70°C, respectively after 30 min incubation. The half life period of avicelase activity was 50.41, 47.89, 39.43 and 27.99 min at 40, 50 60 and 70°C, respectively. The results in Figure 9 showed that *S. viridochromogenes* total cellulase half life period was 102.42, 100.12, 50.0 and 49.6 min at 40, 50, 60, and 70°C, respectively. The result in Figure 10 indicated that CMCase of *S. viridochromogenes* was highly stable comparing with the

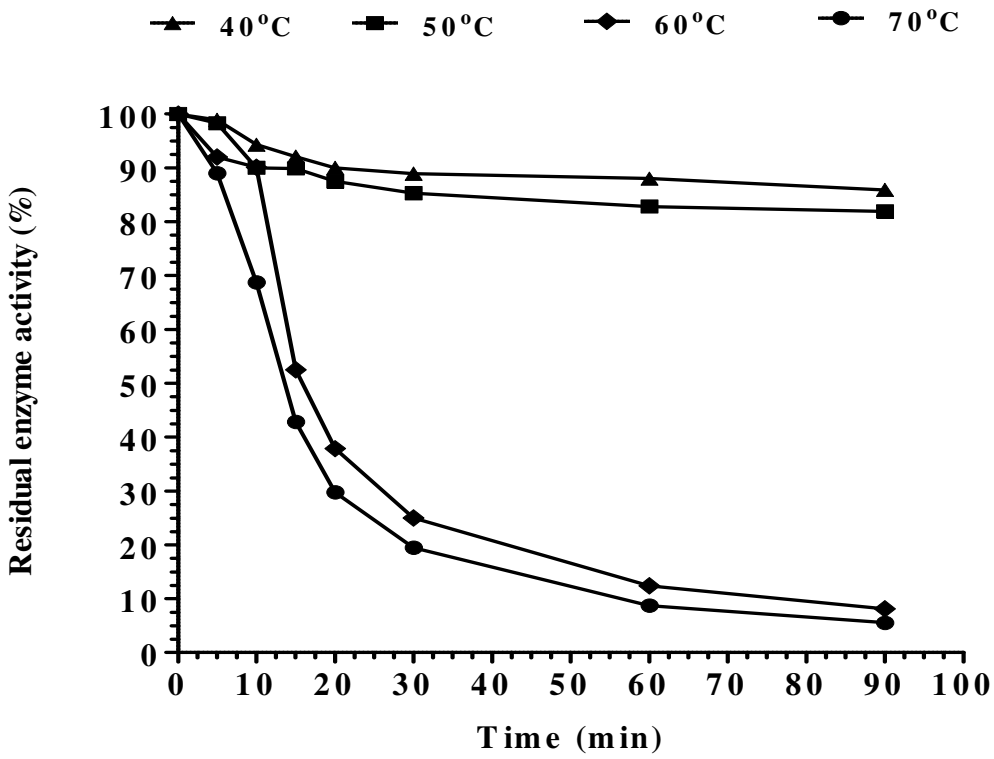

Figure 8. Thermostability of avicelase produced by *Streptomyces viridochromogenes*.

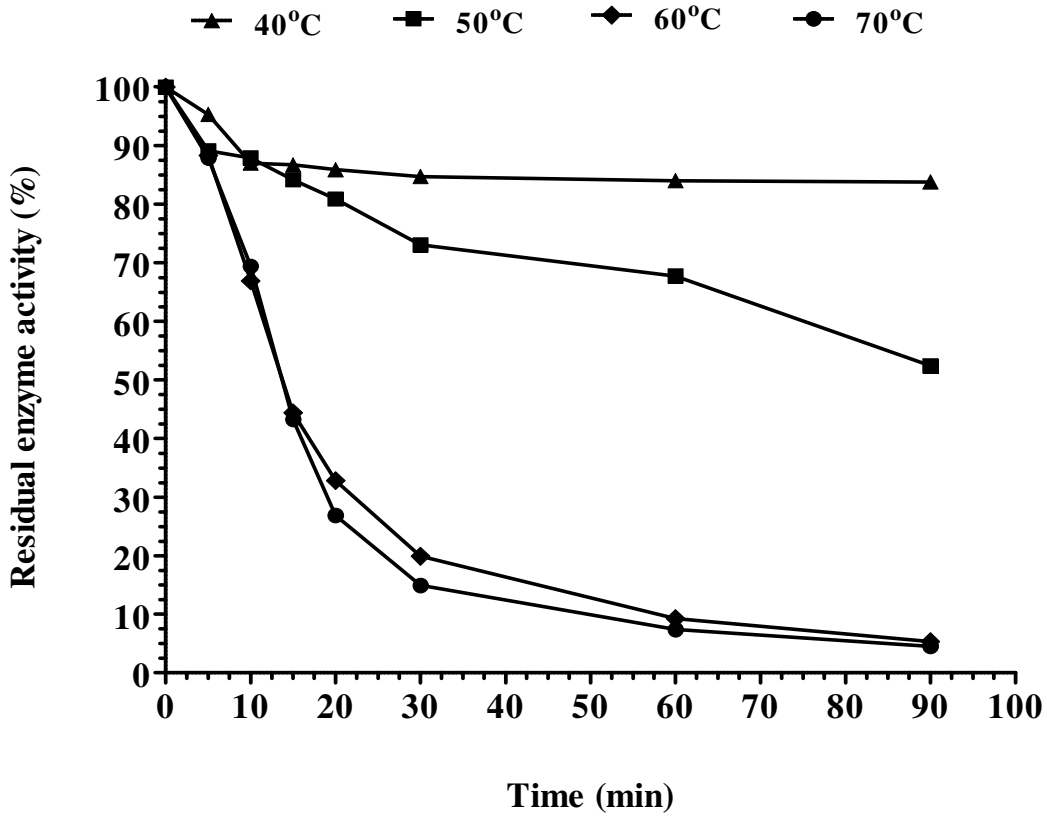

Figure 9. Thermostability of total cellulose produced by *Streptomyces viridochromogenes*.

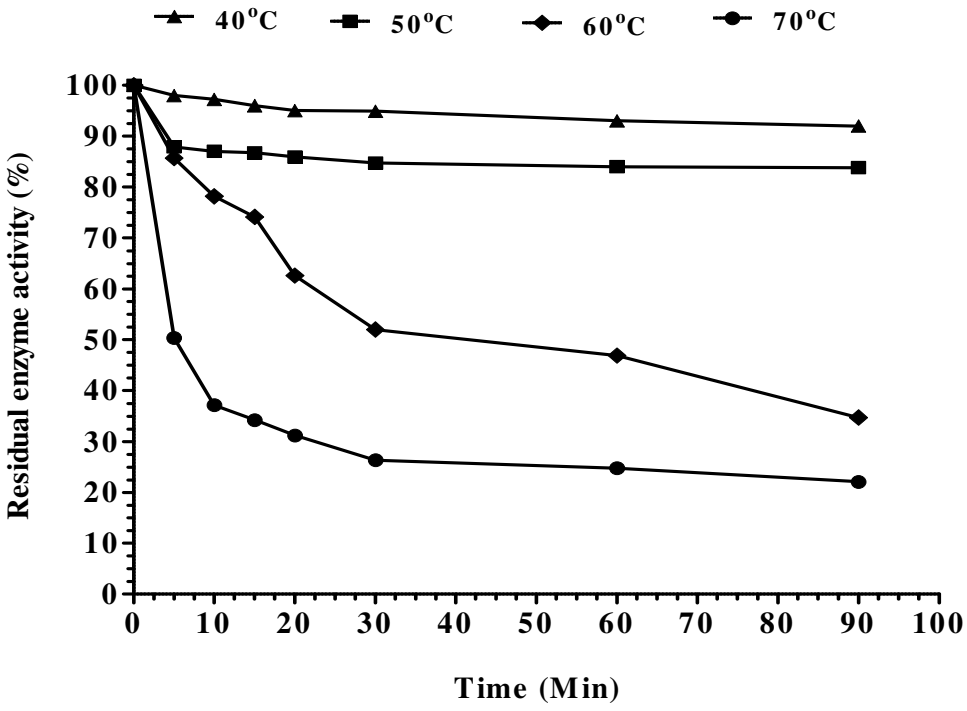

Figure 10. Thermostability of CMCase produced by *Streptomyces viridochromogenes*.

other tested enzymes. This was due to slightly high values of half life times; 652.40, 357.14, 78.58 and 71.73 min at 40, 50, 60 and 70°C respectively. Thermostable cellulases are considered ideal for biotechnological applications. The thermal stability of the CM-cellulase of *S. viridochromogenes* is more than twice to that of *S. lividans* (Kluepfel et al., 1986) which showing a half-life of about 30 min at 60°C, but considerably less than that of the thermophile *Thermomonospora sp.* (Hagerdahl et al., 1980) which proved to be stable at 60°C for at least 24 h. Eighty percent residual activity after 30 min incubation at 45°C has also been cited for a *Streptomyces* endoglucanase (Hoshino et al., 1999). Crude enzyme from *S. drozdowiczii* was able to retain 40% residual CMCase activity at 50°C after 2 h, but only 20% after 8 h incubation (Lima et al., 2005).

Fermentation process

The data in Tables 6 and 7 showed that the amount of produced ethanol, fermentation efficiency (%), remaining sugars and yeast productivity after fermentation process for free sugars in rice straw hydrolysate was obtained through enzymatic hydrolysis of rice straw by *S. viridochromogenes.* Fermentations of dilute acid pretreated rice straw hydrolysate (with initial soluble sugar of 2.340±0.072%) showed maximum ethanol production after 48 h (1.428±0.074%, v/v) with fermentation efficiency of 95.348%. The ethanol production was decreased after 48 h of fermentation, the decrease in ethanol level is probably a result of ethanol's volatility (Michilka, 2007). Little sugar consumption was observed, this is more likely to be due to the inability of *S. cerevisiae* to ferment pentose sugars as the native ethanologen *S. cerevisiae* is capable of fermenting only hexoses, and cannot utilize pentoses like xylose, which is the main component of the hemicellulosic fraction of lignocellulose, and can contribute to as much as 30% of the total biomass. Undesirable toxic compounds might also originate due to the pretreatment; thus, besides sugars, compounds such as lignin residues, acids, and aldehydes can be released. The concentrations of inhibitors in the hydrolysate have been shown to inhibit not only the yeast, but also the enzymes (Tengborg, 2001). Gaur (2006) reported that with initial 5% soluble sugar, after 48 h of fermentation, ethanol production was 2.56% and Sherief et al. (2010) reported that with initial 4.1% soluble sugar, after 48 h of fermentation, ethanol production was 2.26% with fermentation efficiency of 86.17%.

Conclusion

Bioconversion offers potentially low cost and safe method of not only disposing the agricultural residues, but also it has the potential to convert lignocellulosic wastes into usable forms such as reducing sugars that could be used for ethanol production. It is an economical and cleaner

Table 6. Ethanol yield during fermentation process.

Time of fermentation (h)	Alcohol (%,v/v)	Fermentation efficiency (%)
24	0.889±0.056	59.361
48	1.428±0.074	95.352
72	1.378±0.074	92.014
96	1.306±0.061	87.206

Table 7. Soluble sugars concentration and yeast productivity.

Parameter (s)	Value
Initial soluble sugar (%)	2.340±0.072
Remaining sugars (%) after fermentation	
24 h	1.313±0.0600
48 h	1.122±0.1077
72 h	1.109±0.0819
96 h	1.103±0.0215
Yeast production after 144 h fermentation	9.86 gL^{-1}

substitute for direct combustion of the lignocellulosic wastes.

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