Biofuel Production from Cellulosic Wastes by Local Isolates of *Streptomyces sp*.

Nadem H. Hayder*, Banan Mahmoode
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.

Abstract

The current study aims to produce cellulase enzyme from *Streptomyces spp*. isolates and study the effect of some cultural conditions on cellulase production; biofuel production from cellulotic waste through enzymatic and acids hydrolysis. Out of 74 isolates of *Streptomyces sp*. were screened for cellulse production in solid and liquid media. Results showed higher capability of isolate *Streptomyces* sp. B 167 for cellulase production and bioconversion of cellulose, therefore selected for further studies. The results of optimization revealed that the cellulase enzyme productivity by the selected isolate reached 2.1 and 2.28 U/ml after 48 h of incubation time and pH 7 respectively. Cellulase productions in tested isolate improved (2.57 U/ml) by supplementation of cellulose liquid medium with 1 % of yeast extract as nitrogen source. Additives of carbon sources like (manitol, glucose, maltose, sucrose and starch) to the process of saccharification not improve the cellulose productivity. The bioconversion of cellulosic waste to reducing sugar was maximum with Banana peels (77.78 %) followed by the rice husk (75.56 %), orange peels (71.11 %), corn steep peels (60.0 %) and lowest bioconversions (53.33 %) recorded with sawdust. The degradation of cellulosic waste increases with increasing substrate concentration. Maximum cellulase productivity (3.18 U/ml) and bioconversion (86.1 %) was obtained at 3 % (w/v) of cellulosic waste (Banana peels). Saccharification of cellulosic waste with different treatment methods was studied. The pretreatment of cellulosic waste with 1 % HCl and H_2SO_4 produces 21 and 15.8 g of reducing sugar / 100 g of cellulosic waste. In comparison, hydrolysis with *Streptomyces sp*. B 167 enzymes, resulting significantly higher amount of reducing sugar yield (25 g / 100 g cellulotic waste). Further fermentation of cellulosic hydrolysates were preformed using *Saccharomyces cerevisiae* using stationary fermentation condition, maximum yield of ethanol were (0.30, 0.19 and 0.10 g ethanol / g glucose) observed with *Streptomyces sp.* B 167 enzymes, HCl and H_2SO_4 hydrolysates respectively after 48 h of fermentation.

Keywords: Cellulosic waste, *Streptomyces sp*., bioconversion, cellulase, fermentation.

.

انتاج الوقود الحيوي من تحلل المخلفات السليلوزية بوساطة عزلات محلية من بكتريا *Streptomyces sp.*

ناظم حسن حيدر*، بنان محمود
قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

*E-mail: Nadhemwandawy@yahoo.com

الخلاصة

هدفت الدراسة الحالية الى انتاج انزيم السليليز من بكتريا الستربتومايسس ودراسة تاثير بعض الظروف الزرعية على انتاج الانزيم; انتاج الوقود الحيوي من المخلفات السليلوزية من خلال التحلل المائي للمخلفات بالاحماض والانزيمات المايكروبية. تم غربلة 74 عزلة لبكتريا *Streptomyces* sp لانتاجها للسليليز في الوسط الصلب والسائل، اظهرت النتائج قدرة عالية للعزلة *Streptomyces* sp B 167 في انتاج انزيم السليلز والتحويل الحياتي للسليلوز، واختيرت العزلة في بقية الدراسة. كما اظهرت نتائج الظروف المثلى، بان انتاجية انزيم السليليز من قبل عزلة الستربتومايسس B167 بلغت 2.1 و 2.28 وحدة/مل بعد 48 ساعة من فترة الحضانة وعند الرقم الهيدروجيني 7. تم تحسين انتاجية السليليز من قبل العزلة المختارة بلغت 2.57 وحدة/مل عند تزويد وسط السليلوز السائل بمستخلص الخميرة وبتركيز 1% كمصدر نيتروجيني. اضافة المصادر الكربونية مثل (المانيتول، الكلوكوز، المالتوز، السكروز و النشا) الى التحلل السكري للسليلوز لم يحسن من انتاجية انزيم السليليز. التحويل الحياتي للمخلفات السليلوزية الى السكريات المختزلة بلغ اقصاها (77.78 %) مع قشور الموز ثم اعقبها قشور الرز (75.56 %)، قشور الحمضيات (71.11 %)، قشور عرانيس الذرة (60.0 %) واقل قيمة للتحويل الحياتي (53.33 %) سجلت مع نشارة الخشب. التفكك الحياتي للمخلفات السليلوزية ازداد مع زيادة تركيز المادة المغذية. اقصى انتاجية لانزيم السليليز (3.18 وحدة/مل) والتحويل الحياتي (86.1 %) حصلت عند التركيز 3 % (وزن/حجم) من المخلفات السليلوزية (قشور الموز). تم دراسة عملية التحول السكري للمخلفات السليلوزية بعدة طرق. المعاملة الاولية للمخلفات السليلوزية مع 1 % من حامض الهيدروكلوريك وحامض الكبريتيك انتجت 21 و 15.8 غم من السكريات المختزلة / 100 غم من المخلفات السليلوزية. بالمقارنة مع التحلل المائي لانزيمات بكتريا الستربتومايسس B167 انتج كمية عالية من السكريات المختزلة بلغت (25 غم / 100 غم من المخلفات السليلوزية). درست عملية تخمير السكريات الناتجة من المعاملات المختلفة باستخدام الخميرة سكرومايسس سرفيسيا وباستخدام تخمرات الحالة الثابتة، اقصى انتاج للايثانول بلغ (0.30، 0.19 و 0.10 غم ايثانول / غم كلوكوز) تم الحصول عليه من نواتج المعاملة باستخدام انزيم الستربتومايسس، حامض HCl و H_2SO_4 بالتتالي بعد 48 ساعة من عملية التخمير.

Introduction

Lignocelluloses materials can be utilized to produce ethanol, a promising alternative energy for the limited crude oil [1]. There are mainly two process involved in the conversion hydrolysis of cellulose in the lignocellulosic material to produce reducing sugars and fermentation of the sugars to ethanol [2 and 3]. The hydrolysis of cellulose is usually catalyzed by cellulose enzymes, and fermentation is carried by yeasts or bacteria. During the enzymatic hydrolysis, cellulose is degraded by the cellulase to reducing sugar that can be fermented by yeast to ethanol. The optimization of cellulase enzymes and enzymes loading can also improve the hydrolysis. The saccharification and fermentation effectively removes glucose, which is an inhibitory to cellulase activity, thus increasing the yield and rate of cellulose hydrolysis [4]. Lignocelluloses is a renewable organic material and is the major component of woody plants and non-woody plants such as grasses. It is composed of three major components: cellulose (35 – 50%), hemicelluloses (20 – 35%) and lignin (10 – 25%) [5]. Lignocelluloses wastes include a variety of materials such as sawdust, sugarcane bagasse, and waste paper, switch grass, and straw, stem, stalks, leaves, husks, shells and peels from fruits, wheat, corn, sorghum and barley etc. significant effort have been made to convert these lignocelluloses residues to valuable products such as biofuel, chemicals and feed [6]. In urban areas, a considerable portion of solid waste includes fruit waste generated daily by household consumption, and by food processing industries and restaurants. These industries and establishments usually discard the inedible parts of the fruits which include exocarp commonly referred to as ‘rind’ or ‘peel’. In most cases, these waste materials are dumped in landfills which lead unhygienic conditions. However, utilization of these waste materials in production of biofuels would be of great environmental and economic benefit as it could reduce sources of energy and also get ride of the waste [7].

Lignocelluloses are complex structure difficult to degradation, due to presence of lignin. However, lignocelluloses biomass digestibility may be enhanced by various pretreatment like physical, chemical and biological. Some physio-chemical treatment such as dilute acid, hot water, ammonia recycles

percolation, and lime; but these methods are capital-intensive, slow and inefficient; the overall yield of the fermentation process will be decreased this treatment releases inhibitors such as weak acids, furan and phenolic compounds [8 and 9].

In nature, a variety of bacteria and fungi produce cellulases to hydrolyze these insoluble polysaccharides to soluble oligomers, and subsequently to monomers. However this conversion is quite difficult owing to the complex structure of plant cell wall designed to resist microbial degradation. Therefore, attempts are being made to find the effective enzyme systems from various cellulytic microorganisms. Actinomycetes, one of the known cellulase – producer, has attracted research interest due to its potential applications in recovery of fermentable sugars from cellulose; they are capable of producing different extracellular enzymes including cellulase, chitinase and xylanase [10]. For commercial production, microbial enzymes have the enormous advantage of being scalable to high-capacity production by established fermentation techniques. A wide variety of microorganisms are known for their ability to produce cellulase enzymes. *Streptomyces sp*. is one of the best known enzyme producers [11].

This study focused on the isolation and screening of a high cellulase producing *Streptomyces* isolates from soil and decayed agriculture waste samples collected in Iraqi. The influences of different cultural conditions on production of crude cellulase by *Streptomyces* isolate; produce ethanol as a fuel from cellulosic waste through extracellular enzymatic hydrolysis and fermentation.

Materials and Methods

Collection of samples

Thirty five samples were collected from different locations, including garden soil (25), spoiled fruits (10). Soil samples were collected from the top to 10 cm deep using a sterile scoop, placed in sterile labeled polyethylene bottles and transported to the laboratory. The decayed waste also collected in the sterile container and stored in refrigerator at 4°C for further screening of isolates. The soil samples were heated in oven for 2 h at 70 °C and used for isolation of *Streptomyces sp*. [12].

Isolation of *Streptomyces sp.*

One gram of each soil sample and decade fruits were dissolved in 100 ml sterilized normal saline (NS) water, mixed vigorously and left to stand for a few minutes. Thereafter, 1ml was taken from each samples and added to 9 ml of sterilized N.S, mixed well, then 100 µL serial dilutions of (10, 10^{-1},10^{-2}) were spread plated in duplicate on soybean agar (Difco USA) plates. Antibiotic nystatine was added to minimize contamination; the plates were incubated for 7 days at 30 °C. Various *Streptomyces* isolates, based on their special morphological characteristics as different colored aerial mycelium with sitting colonies, were selected and purified by sub-culturing (4-5) times on soybean agar plates and stored at 4 °C for further use [13].

Identification *of Streptomyces* sp. Isolates.

Morphological characteristics of isolates

Morphological characterization which includes colony and spore morphology was performed. A primary step for identification of *Streptomyces* spp. was done by incubation the isolates on soy bean agar medium at 30°C for 14 days. The isolates were examined according to the colony color, colony form, colony surface, spore formation, substrate mycelium, and diffusion pigment production [12].

Identification of *Streptomyces* sp isolates B 167

Morphological characterization of screening isolate

Identification of the Isolate B 167: The potential isolate that showed higher cellulase activity was inoculated on soybean agar and the aerial spore mass color and reverse side and soluble pigment production were studied. The spore chain morphology of the selected isolate B 167 of 14 days old was examined by light microscope after staining with gram stain, by (one or two drop at most of stain was added and Immerse the specimen/material was immersed in to the drop. The cover slip was holed, touch of one edge of the drop of mountains, and lower gently to avoiding any air bubbles). The colors of mature sporulating aerial mycelium and substrate mycelium were monitored for the 7, 14 and 21 days old cultures grown on the soybean agar [14].

The physiological and biochemical characteristics were determined according to the method of Holt *et al.* [15] and Lakshmipathy *et al*. [16].

Raw materials

In the current study ligninocellulosic waste of plant origin including: Orange peel, Banana peel, Corn steep peel, Rice husks and Saw dust were obtained from local shops and washed twice with

distilled water and then utilized as substrate. The ligninocellulosic waste were dried in the sun and shredded to small – size particles and then crushed by a grinding miller to powder form in order to increase its susceptibility to chemical pretreatment. The resulted powder was sieved with a fine – mesh sieve and stored in a clean vials at room temperature for further using.

Screening of cellulose degrading *Streptomyces* isolates for cellulase production

Cellulase production was determined for pure culture of *Streptomyces* isolates by two different methods:

Cellulase Production in solid media

This test was carried out according to the method described by [17]. Medium CMC agar (carboxymethylcellulose 0.5 g/L, $NaNO_3$ 0.1 g/L, K_2HPO_4 0.1 g/L, $MgSO_4$ 0.05 g/L, yeast extract 0.05 g/L, agar 15 g/L) and Modified Cellulose agar replacing carboxymethylcellulose in CMC agar with cellulose for cellulose degrading efficiency test.

Screening of cellulose degrading microorganisms was conducted by using Congo red dye. The isolates were grown on cellulose and CMC Agar (pH 7.0) were spot inoculated with spore suspension of pure cultures and incubated at 30 °C for 5 days to allow for the secretion of cellulase. The agar medium was flooded with an aqueous solution of Congo red (1 % w/v) for 15 min to visualize the hydrolysis zone. The Congo red solution was then poured off and the plates were further treated by flooding with 1 *N* HCl for 15 min. To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on cellulose and CMC agar were measured. The culture producing the largest clear zone was selected for further studies.

Screening of isolates for cellulase production in liquid media

The isolates which showed largest clear zone (more than 10 mm) were screened for cellulase production in liquid media. The modified Mineral Salt Medium (MSM) described by Waldron and Eveleigh [18] was used for cellulase production containing (g/l): K_2HPO_4 2; KCl, 0.5; $MgSO_4$. $7H_2O$, 1; NaCl, 0.5; NH_4NO_3, 1; $CaCl_2$. $2H_2O$, 0.1; yeast extract, 2 and cellulose powder, 10. Final pH was adjusted to 7 ± 0.2. Sterile medium (50 ml) was inoculated with 2% of 48 h old seed culture of *Streptomyces* sp. prepared by inoculation of MYE broth with 7 day agar plate culture of the isolates and submerged batch culture was carried out in shaker incubator (180 rpm/min) at 30 °C for 72 h. After the incubation period, the culture broths were centrifuged at 10000 g for 15 min, and filtered through previously weighed container. The containers with the residues were dried in the hot air oven at 70 °C for a constant weight and reweighed. The difference between the initial and the final weights gave the amount of cellulosic substrate degraded by the isolates. The supernatant was used as crude enzyme solution for detection of reducing sugar, cellulase activity and soluble protein.

Analytical Techniques

Cellulase activity (CA)

Cellulase activity of the culture sample was determined according to the method described by Wood and Bhat [19] with some modifications. 0.2 ml of culture filtrate was added to 1.8 ml of 1% carboxymethylcellulose prepared in 0.05M sodium citrate buffer (pH 4.8) in a test tube and incubated at 40°C for 30 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in a water bath at 100°C for 15 min. One ml of 40% Rochelle salt solution was then added to stabilize the color. Absorbance was recorded at 575 nm against the blank (0.05 M sodium citrate buffer).One unit of enzyme activity was defined as the amount of enzyme that released 1 μM of glucose per min.

Reducing Sugars Content

Reducing sugars analysis was conducted by using 2 ml of sample which was added to 3 ml of DNS and boiled for 15 min. After cooling, 1 ml of Rochelle salt was added. The absorbance was recorded at 575 nm using a spectrophotometer against the blank of distilled water [19].

Protein determination

Protein content was determined according to lowery *et al*., [20]. In this assay, one ml of crude enzyme supernatant was used and 5 ml reaction mixture was added in a clean test tube. The tubes were kept at room temperature for 10 min. the 0.5 ml of Folin reagent was added to the previous mixture. The tubes were incubated for 20 min at room temperature and the absorbance was measured at 720 nm.

Optimization of Cellulase Enzyme Production

Cellulase production depends upon the composition of the fermentation media. Medium optimization for over production of the enzyme is an important step and involves a number of physio-chemical parameters such as the incubation period, pH, effect of nitrogen sources, and supplemental substrate in submerged fermentation. For the initial optimization of the medium, the traditional method of 'one variable at a time' approach was used by changing one component at a time while keeping the others at their original level. The selected isolate was grown in MYE broth for 48 h at 30 ˚C. This culture was used as stock culture inoculums at concentration of 2% (v/v). For optimum conditions of cellulose production, MSM as mentioned previously, supplemented with 1 % of cellulose powder as a sole source of carbon. The pH of the medium was adjusted to 7.0. Cultivations were performed in 250 ml flasks containing 50 ml medium at 30 ˚C, and stirred in a rotary shaker incubator (180 rpm/min).

Effect of incubation period on cellulase production

Time course (incubation period) of cellulase production was studied. The isolate was grown on mineral salt medium containing 1% cellulose powder, at different incubation period (1-5) days at pH 7 and 30 ˚C in shaker incubator at 180 rpm/min.

Effect of pH on cellulase production

The effect of pH on cellulase production and activity was studied. MSM containing 1 % cellulose powder was adjusted to different initial pH (5, 6, 7, 8, 9 and 10), and incubated at optimized incubation period (48 h) in shaker incubator at 180 rpm/min at 30 ˚C.

Effect of Nitrogen Sources on cellulase production

To evaluate the most appropriate organic and inorganic nitrogen sources for cellulase production, MSM containing 1 % cellulose powder was supplemented with different nitrogen sources NH_4Cl, NH_4SO_4, trypton, peptone, yeast extract, beef extract and urea were employed at a concentration of 1%.

Effect of Carbon Sources on cellulase production

For appropriate carbohydrate as carbon source for cellulase production, MSM containing 1 % cellulose powder was supplemented with different carbohydrate sources including (manitol, glucose, maltose, sucrose and starch) at concentration of 1% (w/v).

Effect of different cellulosic waste on cellulase production

Optimization of different natural waste sources for cellulase production was conducted. MSM (50 ml) without cellulose powder was dispensed into 250 ml flasks. Duplicate flasks were prepared containing the following as sole carbon sources at concentration of 1 % (w/v): Orange peel, Banana peel, Corn steep peel, rice husk and Saw dust.

Effect of different concentration of cellulosic waste (Banana peel) on cellulose production

Different concentration of Banana waste for cellulase production was conducted. MSM containing different concentration of (0.5, 1, 2, 3, 4, 5 and 6 %) (w/v) were used. In all experiments above, fermentation was carried out for 48 h, in shaker incubator (180 rpm/min) at 30 ˚C. Clarified cell-free culture supernatants were assayed for reducing sugar, enzyme activity and total protein production.

Fermentation and ethanol production

Microorganisms and cultivation

The isolate *Streptomyces sp*. which showed higher cellulose production, used for hydrolysis of cellulosic (Banana) materials. The isolate maintained on soybean agar plate at pH 7.0 and stored at 4˚C.

Saccharomyces cervisiae: the yeast *S. cervisiae* was isolated from decayed fruits. Samples were suspended in sterile containers and transferred to laboratory. The decayed fruit samples were suspended in sterile distilled water and allowed to settle, the supernatant was diluted by serial – 10 fold dilutions and the samples were inoculated on to medium containing 2 % (w/v) dextrose, 1 % (w/v) peptone and 0.5 % (w/v) yeast extract plates. The plats were incubated at 30 ˚C for 48 h. The grown yeast isolates were identified as *S. cervisiae* by studying some of the morphological and physiological characteristics [21].

Pretreatment and Saccharification of cellulosic material for fermentation

Saccharification of ligninocellulosic waste (Banana Pell) was carried out with acids and by microbial hydrolysis with *Streptomyces sp.*

Acid saccharification: acid pretreatment was achieved with 1 % (v/v) of hydrochloric and sulfuric acid for 30 min at a solid to liquid ratio of 1:10. The mixture was filtered with whatman No. 1 filter paper and then the filtrate was further hydrolyzed by autoclaving at 121˚C for 15 min for saccharification. After pretreatment, the cellulosic residue was soaked in distilled water for 30 min, and then filtered. The filtrate collected from the acids and autoclave pretreatment were used to determine the reducing sugar content [22].

Hydrolysis and saccharification by *Streptomyces sp*. : for saccharification of substrate (Banana peel), locally isolated culture *Streptomyces sp*. B 167 was employed. MSM (100 ml) pH 7.0 was dispensed into 250 ml flasks. Duplicate flasks were prepared containing optimum concentration of Banana peel (3 % w/v) in optimum condition. The mixture was then pretreated by autoclaving at 121 ˚C for 15 min and then filtered with whatman No. 1 filter paper. The culture medium was inoculated with 2 % (v/v) of *Streptomyces sp.* B 167 and incubated at 30 ˚C in shaker incubator of 180 rpm/min for 48 h, samples at the end of incubation period was analyzed for total reducing sugar.

The pretreated samples obtained after the saccharification using acid and microbial culture, were taken, and the culture media were autoclaved for 15 min and filtered using whatman NO.1filter paper and adjusted to pH 5.5 using 0.1 N NaOH and HCl and the fermentation was carried out by *S. cervisiae*.

Fermentation Conditions for Bioethanol Production

Fermentation conditions were performed as described by Ciani and Maccarelli [23] with some modification. After saccharification with different treatments, the substrate was allowed to ferment with yeast isolate *S. cervisiae*. The fermentation medium (100 ml) from each treatment was supplemented with peptone 0.5 and yeast extract 0.25 % (w/v) at pH 5.5. The cultures were inoculated by transferring 5% (v/v) of cells of *S. cervisiae* containing (OD = 0.5, 1.5×10^8 CFU/ml) yeast cells. The fermentation process left under aerobic conditions for 24 h for multiplications of cells and then under anaerobic conditions for 5 days at 28 ± 1 ˚C. During fermentation, the samples were withdrawn after 0, 24, 48, 96 and 120 h of incubations. The samples for different period of fermentation in duplicates were taken and centrifuged at 6000 rpm/min for 15 min for estimation of total reducing sugar remaining and ethanol production. Ethanol concentration was determined by gas chromatography (GC) using 120/180 6.6 % carbowax column. The GC was set at 90 ˚C oven temperature, 170 ˚C injection temperatures and 175 ˚C detection temperatures. Standard was prepared by using 5 g ethanol. Ethanol concentration was calculated based on the peak areas of the samples and standard solutions [24].

Results and Discussion

Isolation and screening of cellulose producing *Streptomyces*:

A total 74 *Streptomyces* isolates were isolated from the soil and decayed agriculture waste by dilution plate technique on soybean media, with pH 7 at 30 ˚C for 5 days. The isolates were sub-cultured to obtain pure cultures and were stored at 4 ˚C for further use.

Screening of cellulase producing *Streptomyces* were conducted using Congo red test as preliminary study for identifying cellulose degraders. After 4 days of incubation, all isolates showed different growth and hydrolytic zones on cellulose agar as the sole source of carbon, the clear zone in the medium indicated cellulose degradation by the isolates. Out of 74 isolates screened for cellulase production in solid media, 10 isolates showed higher growth and clear zones table- 1. The isolates gave higher growth and clear zones (over 10 mm) were selected for further screening in liquid media.

Out of 10 isolates tested, the isolate B 167 gave the clear zone diameter of 15.5 mm, and showed maximum bioconversion % and cellulase activity table- 1. Therefore the isolate B 167 was selected for further identification and for saccharification process in the current study.

Table 1- Screening of isolates for cellulose degradation and cellulase activity

No. of isolates	Isolate name	Growth zone (mm)	Hydrolytic zone (mm)	Bioconversion %	Cellulase activity (U/ml)
1	B 65	10	10.5	12.93	0.37
2	B 224	10	13	34.15	1.49
3	B 130	5	10.5	25.85	0.62
4	**B 167**	**11.5**	**15.5**	**63.41**	**2.11**
5	B 138	8	12	21.95	1.49
6	B 119	15	12.5	29.27	1.62
7	B 95	8.9	11.1	24.39	1.99
8	B 245	17	13	58.54	1.74
9	B 90	7.4	14.7	60.98	1.92
10	B 77	7.3	11.35	17.07	1.37

Morphological and Biochemical Characterization of the Selected Isolate

The selected cellulose degrader was examined for its morphology. The colonies of the isolate were large, regular, circular, raised, gray, and slimy on soybean agar surface. The aerial mycelium appeared gray in color where as dark brown pigmentation was observed on the reverse side of the colonies figure-1. The microscopic view showed Gram-positive filaments with non-motile, straight chain spores. The cultural characteristics of the isolate B 167 showed inability of isolates to grow in temperature at 4 °C, while exhibited good growth of temperature at 37 and 45 °C. The isolate showed good growth at pH value ranged from 3 to 10.

The isolate showed capability of assimilating all 9 sugars tested table-2. The morphological and cultural characteristics of the isolate were compared with known Actinomycetes species described in Bergey's Manual of determinative Baceriology [15] and Bergey's Manual of Systematic Bacteriology [25], and was identified as *Streptomyces sp*. B 167.

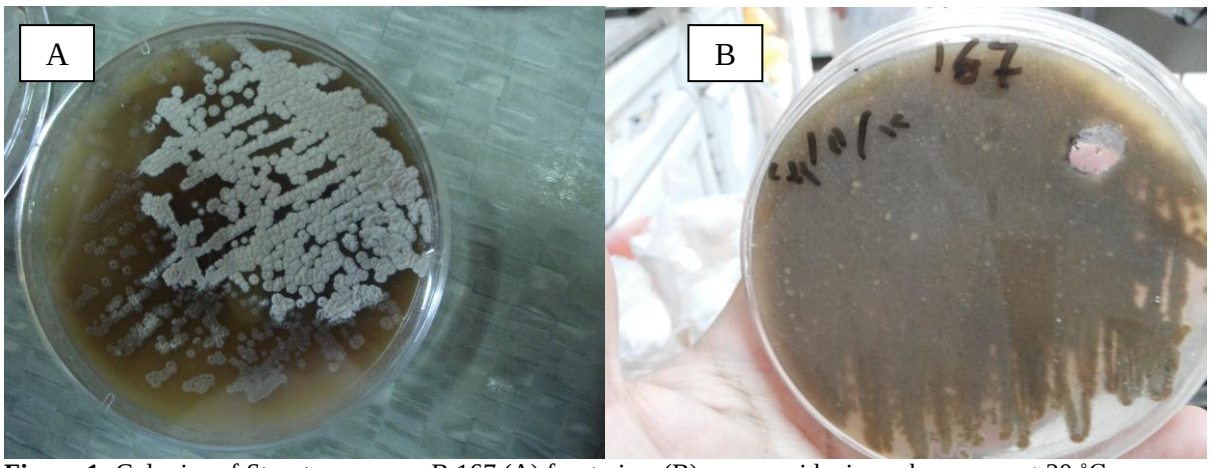

Figure 1- Colonies of *Streptomyces sp*. B 167 (A) front view (B) reverse side, in soybean agar at 30 ˚C.

Table 2- Morphological, cultural and biochemical characteristics of *Streptomyces* sp. B 167.

No.	Morphological and biochemical characteristics	Streptomyces SS 20
1.	Colony color and shape	Gray and circular
2.	Colony diameter	2 mm
3.	Presence of spores	Aerial mycelium
	Spore surface	slimy
	Aerial spore mass color	silvery gray
4.	Pigmentation	
	a. Reverse side pigment	Brown
	b. Diffusible pigment	No pigment
	c. Melanin pigment	-

5.	Starch hydrolysis	+
6.	Growth at 4 °C	-
	Growth at 37 °C	+
	Growth at 45 °C	+
6.	Growth range pH 3-10	+
7.	Sugars	
	a. Arabinose	+
	b. Xylose	+
	c. Inositol	+
	d. Manitol	+
	e. glucose	+
	f. Fructose	+
	i. Rhamnose	+
	g. Sucrose	+
	k. Raffinose	+

Kinetics of Enzyme Production

Streptomyces species have always been a source of thousands of bioactive compounds. Enzymes are one of the important products of this unusual group of bacteria. In the current study *Streptomyces* sp. B 167 with potential cellulolytic activity was subjected to produce cellulase in submerged culture. The results in figure-2 showed that, total cellulase activity, total reducing sugar and protein yield reached maxima after 48 h of incubation period. This pattern of extracellular enzyme production is similar to that reported for *Streptomyces sp*. EC 22, with maximal activity of xylanase and cellulase reached after 48 and 72 h respectively [26]. In comparison, in *S. flavogriseus*, grown of microcrystalline cellulose, cellulase activity was maximal after 26 h [27]. Total reducing sugar and extracellular protein concentration reached maximum after 48 h, which were followed by a sharp decrease. These results were in agreement with that reported by Okeke and Paterson [26], they observed maximum reducing sugar and protein yield after 48 h of incubation in culture of *Streptomyces sp*. grown in microcrystalline cellulose.

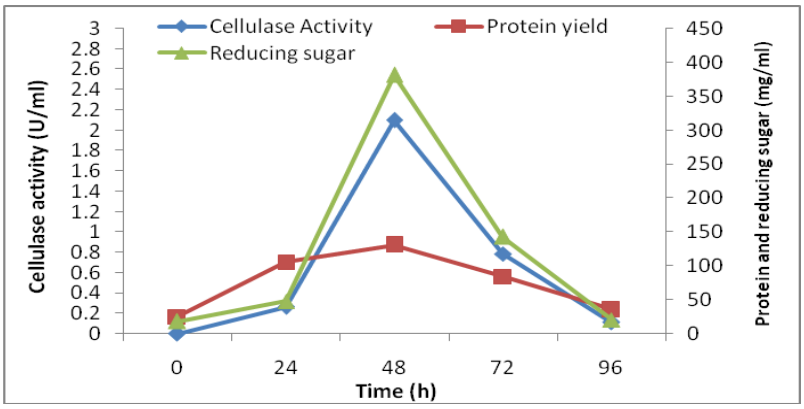

Figure 2- Time course for production of extracellular cellulase, total reducing sugar and total protein by *Streptomyces sp*. B 167 grown on cellulose powder as sole source of carbon at 30 °C.

Effect of pH

The modified cellulose broth medium was prepared with a wide range initial pH (5 – 10). Good cellulase productivity was obtained at pH 6 – 9, with highest level 2.28 U/ml at initial pH 7. Also the results showed that the highest total reducing sugar and extracellular protein observed at pH 7 figure-3. The pH of fermentation medium modulates microbial growth and enzyme production. Also cellulase enzyme produced by *Streptomyces flavogriseus* exhibited maximum activity at pH 7 [27]. However, some *Streptomyces sp.* has an optimal cellulase production at pH 8 [28]. While Yassien, et al., [29] observed highest level of enzyme production in case of *Streptomyces* strain C 188 at initial pH 6.5.

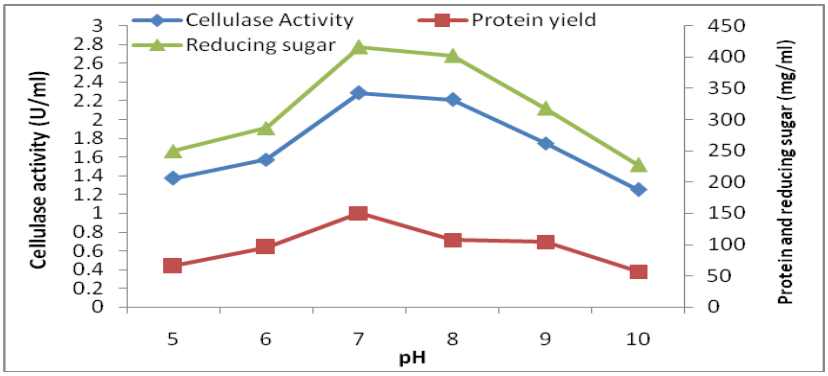

Figure 3- Effect of pH on cellulase activity, total reducing sugar and protein production by *Streptomyces sp*. B 167 grown on cellulose powder as sole source of carbon at 30 °C for 48 h.

Effect of organic and Inorganic Nitrogen Sources

For studying the effect of nitrogen sources, supplementation of the fermentation medium with different organic and inorganic nitrogen sources were carried out. The highest level of cellulase activity (2.57 U/ml) and bioconversion (68.89 %) of cellulose with total sugar and protein production were obtained in the precence of 1 % yeast extract figure- 4. Therefore, the modified cellulose broth containing 1 % yeast extract was used for further studies. The results were in agreement with cellulase productivity in *Streptomyces longispororuber* isolated from Saudi Arabia soil samples. Highest activity was observed by the supplementation of CMC liquid medium with 1 % corn steep liquor and followed by 1 % yeast extract [29].

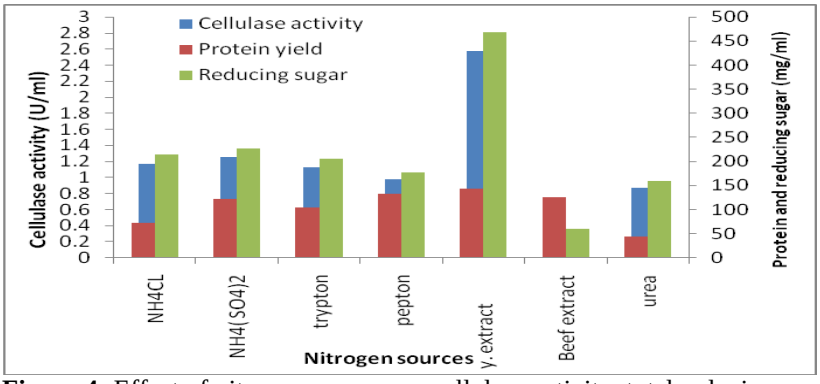

Figure 4- Effect of nitrogen sources on cellulase activity, total reducing sugar and protein yield by *Streptomyces sp*. B 167 grown on cellulose powder as sole source of carbon at 30 °C for 48 h.

Effect of Carbon sources on Cellulase Activity

Cellulase enzymes are inducible, so the presence of cellulose induces enzyme production in addition to its role as a carbon source. An attempt was carried out to improve the cellulase productivity by adding different carbon additives (manitol, glucose, maltose, sucrose and starch) at concentration of 1 % (w/v) to the fermentation medium. The results in figure-5 showed that none of the tested carbon sources improve enzyme production with comparison with the previous optimized parameters. This result is in agreement with that reported by Gautam and Cellegues [30]. Also Okeke and Paterson [26], observed lower cellulases and xylanases activities when *Streptomyces sp.* E 22 was grown on glucose, cellobiose and xylose, while the enzymes activity induces with microcrystalline cellulose. Lazim, *et al.*, [31] studied the effect of various carbohydrates (dextrin, maltose, starch, glucose, fructose and sucrose) on alkaline protease production by *Streptomyces sp.* CN902 in solid state fermentation. The results indicated that the addition of any carbon source to the mixture lead to the reduction in alkaline protease production.

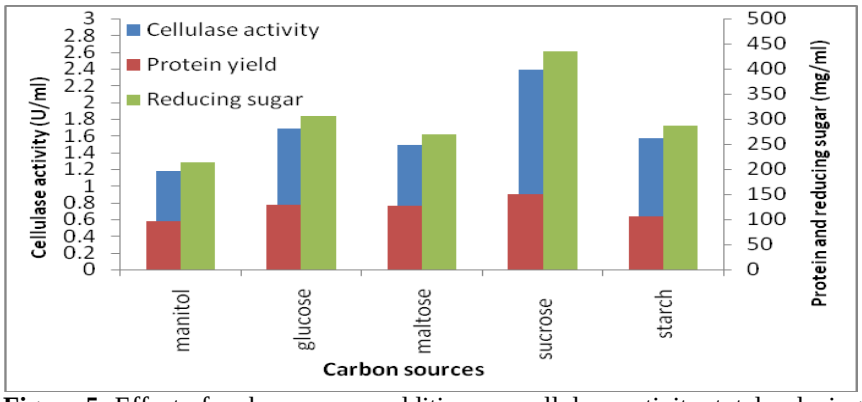

Figure 5- Effect of carbon sources additives on cellulase activity, total reducing sugar and protein production by *Streptomyces sp*. B 167 grown on cellulose powder at 30 °C for 48 h.

Effect of cellulosic waste on cellulase production

Mineral salt medium (50 ml) was dispensed in five 250 ml flasks in duplicate and 1 % of different cellulosic waste (orange peel, Banana peel, corn steep liquor, rice husk and saw dust) were added as sole carbon source in each flasks. The flasks were autoclaved and inoculated with the isolate *Streptomyces sp*. B 167 and kept in the shaker incubator for a period of 48 h at 30 ˚C. After incubation period the cultures broth were filtered through a previously weighed filter paper. The filter papers with the residue were dried in the oven at 70 ˚C for constant weigh and re-weighed. The difference between the initial and the final weights gave the amount of cellulosic waste degraded or bioconverted to reducing sugar. From the results obtained, it was observed that the bioconversion of the Banana peel waste was maximum (77.78 %) followed by the rice husk (75.56 %), orange peel (71.11 %), corn steep peel (60.0 %) and sawdust (53.33 %) as shown in figure-6. Also the results observed higher cellulase activity (2.99 U/ml), total reducing sugar and protein yield with Banana waste. Prasad et al., [32] studied the effect of different cellulosic waste (vegetable peel, wood powder, and straw powder) at concentration of 1 % on bioconversion process of ligninocellulosic waste by *Streptomyces albospinus* MTCC 8768. They obtained maximum degradation (64 %) of the grated vegetable peels, followed by the straw powder (38 %) and wood powder (28 %).

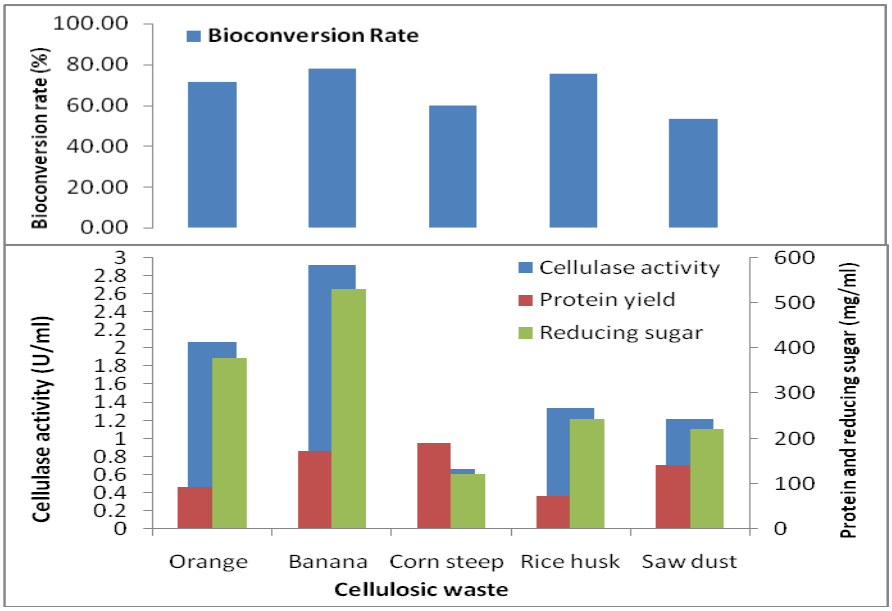

Figure 6- Effect of cellulosic waste on bioconversion rate, cellulase activity, total reducing sugar and protein production by *Streptomyces sp*. B 167 at 30 °C for 48 h.

The ability of cellulolytic microorganisms to degrade cellulose varies greatly with the physio-chemical characteristics of the substrate and the crystallinity degree of cellulose is one of the most important structural parameters which affect the rate of enzymatic degradation by hydrolysis [33].

A large proportion of vegetation added to soil is cellulose; therefore, decomposition of cellulose has a special significance in the biological cycle of carbon. Different concentrations of cellulosic waste (Banana peel) were used, to study the effect of substrate concentration on enzyme production. It was observed from results in figure-7 that there was a gradual increase in the degradation of the cellulosic waste and enzyme production. Maximum bioconversion (86.1 %) was obtained at 3 % (w/v) of cellulosic waste, in which enzyme activity reached its higher level (3.18 U/ml), with increase in total sugar in protein yield. Thereafter, the degradation rate and enzyme production were declined as the substrate concentration increased. This may explain why the increase in concentration of cellulose acts as inhibitory and a resistance to the process of cellulose hydrolysis, as a result, decreases in enzyme activity at higher concentration [34].

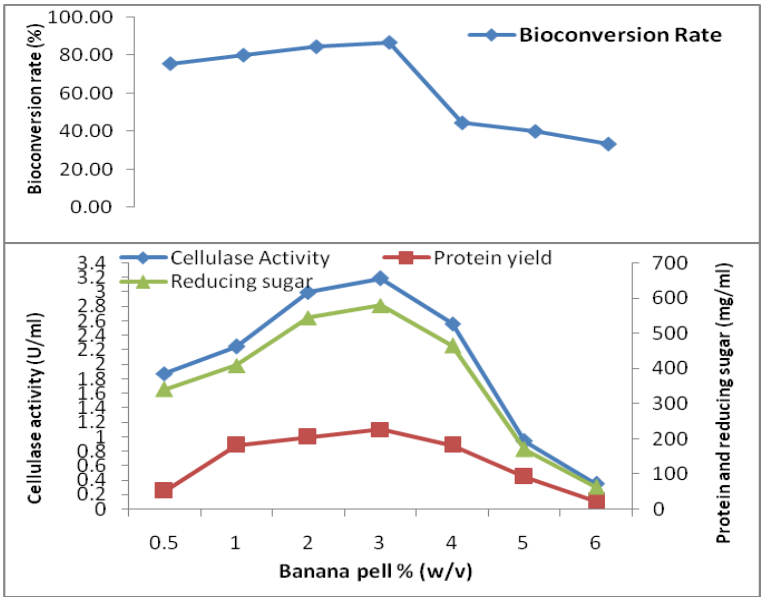

Figure 7- Effect of substrate concentration on bioconversion rate, cellulase activity, total reducing sugar and protein yield by *Streptomyces sp*. B 167 at 30 °C for 48 h.

Saccharification of Cellulosic Waste (Banana Pell)

Acid Saccharification

The effect of acid treatment was studied first; the results are shown in figure-8. By treating the cellulosic waste with 1 % of hydrochloric and sulfuric acid for 30 min and autoclaving for 15 min, approximately 0.638 and 0.475 g of reducing sugar / 3 g of dried cellulosic waste (21 and 15.81 g reducing sugar / 100 g dried waste) were produced respectively. Although acid treatment helps the hydrolysis of cellulosic material, however, it has been reported that after acid treatment some by-product, e.g., furfural and 5 – hydroxyl furfural, will be produced where these degradation compounds are known to inhibit the fermentation step [35]. To avoid formation of the by-products in the hydrolysis process, an alternative pretreatment method without acid addition should be performed. Hsu *et al.*, [22] observed approximately 27.1 g/100 g dried solid of reducing sugar from corn cob cellulosic waste treated with 0.5 % sulfuric acid for 20 min. Also they achieved highest yields of reducing sugar (58.1 g/ 100 g dried solid) when the cellulosic waste after being treated with 0.5 % sulfuric acid and autoclaved for 60 min.

It can be seen from results that out of two commercial acids used as saccharifying agents, hydrochloric acid was found to be more effective for higher recovery of reducing sugar then sulfuric acid. The results obtained in the current study were in agreement with Subashini *et al.*, [36]. They observed maximum amount of reducing sugar (14.8 and 13.2 g/ 100 g dried solid) of Sago waste when treated with 0.3 N of HCl and H_2SO_4 for 60 min respectively.

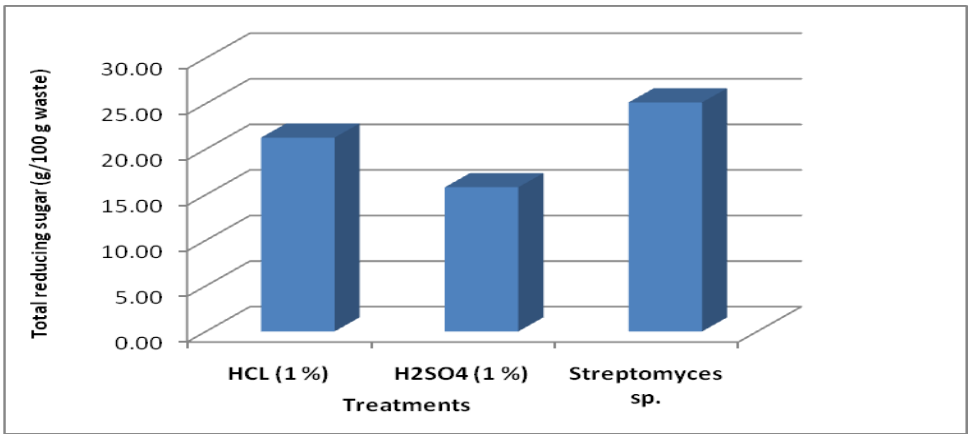

Figure 8- Total reducing sugar production after treating cellulosic waste with 1 % of hydrochloric, sulfuric acid and *Streptomyces sp*. B 167 and autoclaving for 15 min.

Hydrolysis of cellulosic waste with *Streptomyces sp*. B 167

After being autoclaved, the cellulosic waste (Banana peel) was hydrolyzed with the culture of *Streptomyces sp*. B 167 at optimum condition for 48 h. The results in figure-8 showed that the yield of total reducing sugar was 0.754 g/ 3 g of dried solid (25 g/ 100 g dried solid). In addition, the production of cellulase enzyme was associated with the growth of bacteria. Maximum activity of cellulase recorded was 4.145 U/ml. However, the amount of the resulting reducing sugar was higher compared to those of acid pretreatment process.

Figure- 8 shows the comparison of the highest yields obtained from various hydrolysis method, i.e., pretreated and autoclaved with 1 % HCl and H_2SO_4 and *Streptomyces sp*. B 167 hydrolysis. From the results, 21 and 15.8 g of reducing sugar from 100 g of cellulosic waste was produced respectively after acid treatment. In comparison pretreatment with *Streptomyces sp*. B 167 hydrolysis (without acid treatment) resulted significantly higher amount of reducing sugar yield (25 g / 100 g dried solid). The results suggest that samples treated with the combination of autoclaving and microbial hydrolysis is highly effective for ethanol production with higher conversion of monosaccharide's in the mixture. These results also indicated that cellulase activity produced by *Streptomyces sp*. B 167 cultures has the potential to improve the hydrolysis efficiency of cellulosic materials markedly without acid treatment.

It has been reported that the acid pretreatment had negative effects on the yield of ethanol. However, this approach is expensive, slow and inefficient. The overall yield of the fermentation process will be decreased because this pretreatment releases inhibitors such as weak acids, furan and phenolic compounds [37].

Fermentative Production of Bioethanol

Bioethanol production is a widely studied process for biofuel production. Different researchers have studied various raw material and different methods for bioethanol production. However, it has recently observed that ligninocellulosic wastes under focus for bioethanol production.

For fermentative production of bioethanol, stationary fermentation condition was employed. The process was carried out for a period of 5 days at 28°C. During the fermentation process every 24 h samples were taken for the estimation of remaining reducing sugar and ethanol production. The results in figure -9 show the time course of ethanol production using the hydrolysates of acid and microbial enzyme as the substrate.

In the fermentation using the hydrolysates, the reducing sugar concentration decreased rapidly from (754.5, 638.6 and 475.0 mg/l) to (545.5, 429 and 200.3 mg/l) respectively for microbial, HCl and H_2SO_4 hydrolysate after 48 h of incubation figure-9. The ethanol production rate in the early phase of the culture was relatively slow but rapidly increased after 24 h. Maximum concentration of ethanol was observed after 48h of fermentation reached (162, 84 and 18.3 mg/l) respectively. These indicate that the consumption of glucose by yeast cells was associated with ethanol production. In addition, the ethanol yield was (0.30, 0.19 and 0.10 g ethanol/ g glucose respectively. The yield of ethanol obtained in current study was lower than the ethanol yield (0.45 g ethanol / g glucose) reported by Yu and Zhang [38]. In contrast Vaithanomsat et al., [21] observed lower yield of bioethanol (0.02 g ethanol / g glucose) from enzymatically saccharified sunflower stalks as cellulosic waste.

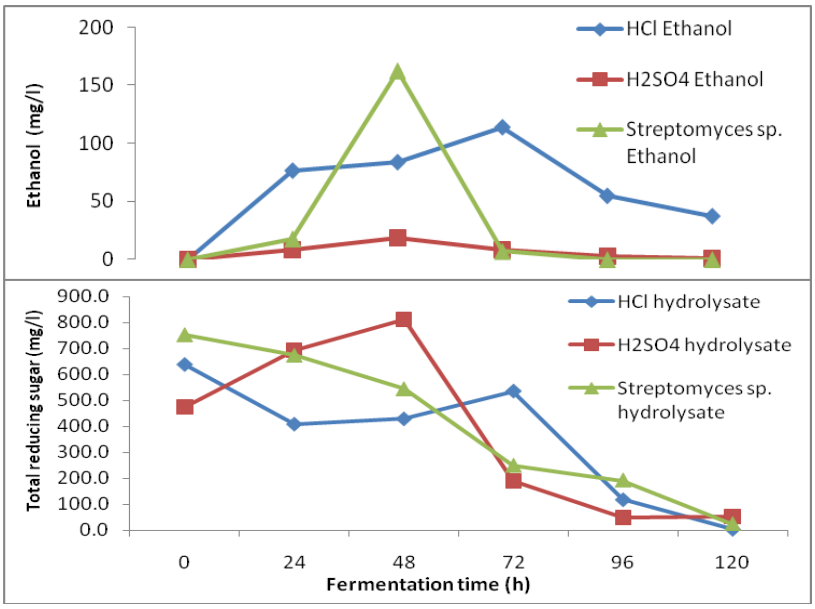

Figure 9- Time course of total reducing sugar and ethanol production in fermentation process with *S. servisiae* using different hydrolysate medium.

Conclusions

This study was focused on the potential of microbial biotechnology to enhance bioconversion of cellulosic waste to sugar and biofuels, thus solving the problem of environmental pollution, and at the same time, producing biofuels that are renewable sources of energy. Cellulase productions in tested isolate improved (2.57 U/ml) by supplementation of cellulose liquid medium with 1 % of yeast extract as nitrogen source. Additives of carbon sources like (manitol, glucose, maltose, sucrose and starch) to the process of saccharification did not improve the cellulose productivity. The bioconversion of cellulosic waste to reducing sugar was maximum with Banana peels (77.78 %) followed by the rice husk (75.56 %), orange peels (71.11 %), corn steep peels (60.0 %) and lowest bioconversions (53.33 %) with starch. The degradation of cellulosic waste increases with an increase in substrate concentration. Maximum cellulase productivity (3.18 U/ml) and bioconversion (86.1 %) was obtained at 3 % (w/v) of cellulosic waste (Banana peels). The pretreatment of cellulosic waste with 1 % HCl and H_2SO_4 produces 21 and 15.8 g of reducing sugar/ 100 g of cellulosic waste. In comparison, hydrolysis with *Streptomyces sp*. B 167 produced a significantly higher amount of reducing sugar yield (25 g / 100 g cellulosic waste). Further fermentation of cellulosic hydrolysates were preformed using *Saccharomyces cerevisiae* in stationary fermentation condition. Maximum yield of ethanol were (0.30, 0.19 and 0.10 g ethanol / g glucose) observed with *Streptomyces sp.* B 167, HCl and H_2SO_4 hydrolysates respectively after 48 h of fermentation.

References

1. Sun, Y. and Cheng, J. **2000**. Hydrolysis of ligninocellulosic materials for ethanol production a review. *Biores. Technol*., 83, pp: 1-11.
2. Gusakov, A.V. Sinitsyn, A.P. Manenkova, and Prostas, O.V. **1992**. Enzymatic saccharification of industrial and agriculture lignocellulosic wastes. *Appl. Biochem. Biotechnol.*, 34, pp: 65.
3. Reshamwala, S. Shawky, B. T. and Dale, B. E. **1995**. Ethanol production from hydrolysates of AFEX-treated coastal Bermuda grass and switch grass. *Appl. Biochem. Biotechnol.*, 51-52, pp: 43-55.
4. 4. Itelima, J. Ogbonna, A. Pandukur, S. Egbere, J. and Saami, A. **2013**. Simultaneous saccharification and fermentation of corn cobs to bio-ethanol by co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae*. *Inter. J. Environ. Sci. Develop*., 4(2) , pp: 239-242.
5. Saha, B. **2003**. Hemicellulose bioconversion. *J. Industr. Miocrobiol. Biotechnol*. 30, pp: 279-291.
6. Howard, RL. Abotsi, E. Jansen, van Rensburg, EL. And Howard, S. **2003**. Lignocellulose biotechnology: Issues of bioconversion and enzyme production. *Afr. J. Biotechnol.*, 2, pp: 602–19.

7. Bhandari, S.V. Panchapakesan, A. Shankar, N. Ashok Kumar, H. G. **2013**. Production of bioethanol from fruit rinds by saccharification and fermentation. *Inter. J. Sci. Res. Engin. Technol.* , pp: 362-365.

8. Sun, Y. and Cheng, J. **2002**. Hydrolysis of lignocellulosic materials for ethanol production. *Biores. Technol*., 83: 1-11.

9. Rubin, EM. 2008. Genomics of cellulosic biofuels. *Nature*, 454: 841-5.

10. Rathnan, R. K. and Ambili, A. **2011**. Cellulase enzyme production by *Streptomyces sp.* using fruit waste as substrate. *Aust. J. Basic Appl. Sci*., 5(12): 1114-1118.

11. El-Sersy NA, Abd-Elnaby H, Abou-Elela GM, Ibrahim HAH, Toukhy NM **2010**. Optimization, economization and characterization of cellulase produced by marine *Streptomyces ruber*. *Afr. J. Biotechnol*. 9:6355-6364.

12. Thampayak, I. Cheeptham, N. and Pathom-Aree, W. **2008**. Isolation and identification of biosurfactant producing Actinomycetes from soil. *Research J. Microbiol*. 3(7): 499-507.

13. Maniyar, J. P. Doshi, D.V. Bhuyan, S.S. and Mujumdar. **2011**. Bioemulsifier production by *Streptomyces* sp. S22 isolated from garden soil. Indian *J. of Experimental Biol*, 49: 293-297.

14. Laidi, R. F.; Kansoh, A. L. Elshafei, A. M. and Cheikh, B. **2006**. Taxonomy, identification and biological activities of a novel isolate of *Streptomyces tendae*. *Arab J. Biotechnol*, 9(3) , pp: 427-436.

15. Holt, J G. Sharpe, M. E. and Williams, S. T. **1989**. Bergey's Manual of Systematic Bacteriology.Williams and Williams. Baltimore, London.

16. Lakshmipathy,D. Arun Prasad, A. S. and Kannabiran K. **2010**. Production of Biosurfactant and Heavy Metal Resistance Activity of *Streptomyces Sp*. VITDDK3-a Novel Halo Tolerant Actinomycetes Isolated from Saltpan Soil. *Advances in Biol. Res*., 4 (2) , pp: 108-115.

17. Kasana, R. C. Salwan, R.; Dhar, H. Dutt, S. and Gulati, A. **2008** A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiol*., 57**,** pp: 503-507.

18. Waldron, C.R. and Eveleigh, D.E. **1986**. Saccharification of cellulosics by *Microbispora bispora*. Appl. Microbiol. *Biotechnol.*, 24: 487-492.

19. Wood, T.M. and Bhat, K.M. **1988**. Methods for measuring cellulase activities. *Methods in Enzymol*., 160(9) , pp: 87-112.

20. Lowry, O., N. Rosebrough, A. Farr, and R. Randall, **1951**. Protein measurement with the Folin phenolreagent. *J. Biol. Chem*., 139: 265.

21. Vaithanomast, J.P. Chuichulcherm, S. and Apiwatanapiwat, W. **2009**. Bioethanol production from enzymatically saccharified Sunflower stalks using steam explosion as pretreatment. *World Acad. Sci. Technol*., 49, pp: 140-143.

22. Hsu, CL. Chang, KS; Chang, YH. And Jang, HD. **2011**. Pretreatment and hydrolysis of cellulosic agriculture wastes with a cellulase-producing Streptomyces for bioethanol production.

23. Ciani, M. and Maccarelli, F. **1998**. Oenological properties of non-*Saccharomyces* yeast associated with wine – making. *World J. Microbiol. Biotechnol*., 14, pp: 199-203.

24. Ramos, LP. And Saddler, JN. **1994**. Bioconversion of wood residue: mechanisms involved in pretreating and hydrolyzing lignocellulosic materials. *ACS Symp. Ser.*, 566, pp: 325-341.

25. Williams, S. Sharpe, E. Holt, J. **1989**. Bergy's manual of systematic bacteriology. First Edition. Volume 4: Actinomycetes. Williams and Wilkins, Baltimore, MD.

26. Okeke, B.C. and Paterson, A. **1992.** Simultaneous production and induction of cellulytic and xylanolytic enzymes in a *Streptomyces sp*., *World J. Microbiol. Biotechnol*., 8, pp: 483-487.

27. Kluepfel, D. and Ishaque, M. (1982). Xylan-induced cellulytic enzymes of *Streptomyces flavogriseus*. *Devel. Indus. Microbiol*., 23: 389-396.

28. 28. Solingen, VP. Meijer, D. Kleij, WA. And Branett, C. **2010**. Cloning and expression of an endocellulase gene from a novel Streptomycet isolated from an East African soda lake. *Extremophiles*, 5, pp: 333-341.

29. Yassien, M. AM.; Fatani, A. A. and Asfour, H. Z. **2014**. Production, purification and characterization of cellulase from *Streptomyces sp*., *Afr. J. Microbiol. Res*., 8(4) , pp: 348-354.

30. Gautam, SP.; Bundela, PS.; Pandey, AK; Jamaluddin, M.; Awasthi, MK. And Sarsaiya, S. **2010** Cellulase production by *Pseudomonas sp.* isolated from municipal solid waste compost. *Int. J. Acad. Res.*, 2: 230-233.

31. Lazim, H.; Mankai, H. and Slama, N. **2009**. Production and optimization of thermophilic alkaline protease in solid-state fermentation by Streptomyces sp. CN902. *J. Ind. Microbiol. Biotechnol*. 36, pp: 531-537.
32. Prasad, P.; Bedi, S. and Singh, T. **2012** In vitro cellulose rich organic material degradation by cellulytic Streptomyces albospinus (MTCC 8768). *Malaysian J. Microbiol*., 8(3) , pp: 164-169.
33. Petre, M.; Zarnea, G.; Adrian, P. and Gheorghiu, E. **1999**. Biodegradation and bioconversion of cellulose wastes using bacterial and fungal cells immobilized in radio polymerized hydrogels. *Res. Cons. Rec*., 27, pp: 309-332.
34. Howell, J.A. and Mangat, M. **1987**. Enzyme deactivation during cellulose hydrolysis. *Biotechnol. Bioengin*., 20, pp: 847-863.
35. 34. Palmarola-Adrados, B. Chotěborská, P. Galbe, M. and Zacchi, G. **2005**. Ethanol production from non-starch carbohydrates of wheat bran. *Bioreso. Technol*., 96: 843-850.
36. Subashini, D. Ejilane, J. Radha, A. Jayasri, M.A. and Suthindhiran, K. **2011**. Ethanol production from Sago waste using *Saccharomyces cerevisiae* Vits-M1. *Curr. Res. J. Biol. Sci.*, 3(1) , pp: 42-51.
37. Cara C. Ruiz E. Oliva J.M. Sáez F. and Castro E. **2008**. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. *Biores. Technol.*, 99, pp: 1869–1876.
38. Yu Z. and Zhang, H**. 2004**. Ethanol fermentation of acid-hydrolyzed cellulosic hyrolysate with *Saccharomyces cerevisiae*. *Biores. Technol*., 93, pp: 199–204.