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Der Pharmacia Lettre, 2016, 8 (17):42-48
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Optimization of cellulase production by bacterial strains, isolated from the soils of the North West of Algeria

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ABSTRACT

The present study was focused on the optimization of the isolated, selected cellulase producing bacteria from the soils of the North West of Algeria. For this purpose, six different samples were collected from four rhizospheric soil, cow dung and bark where cellulose wastes are submitted to natural fermentation. The early screening of cellulase producing bacteria on the used CMS culture medium, which was containing cellulose as sole source of carbon of the collected six different samples (four from rhizospheric soil, cow dung and bark) allowed the selection of sixteen bacterial producer strains. Where the produced cellulase was explored by the measuring of the formed diameter of the zone of hydrolysis around the inoculated, colony in solid used culture medium submerged with 1% congo red solution and the formed reduced sugars was estimated by the using of DNS method. The obtained results indicated that five bacterial strains manifested the highest potential of cellulose production. Furthermore, the study of the effect of several physico-chemicals parameters implicated in the cellulose production such as pH-value and temperature the by the selected EC4' Bacillus circulans indicated that the production of cellulose was at pH-value of 6.6(375 U/ml) and temperature of 40°C (425 U/ml).Further, the identification of isolated, the selected EC4' Bacillus circulans strains has been achieved by the study of the morphological and biochemical characteristics.

Key words: Cellulase producing bacteria, cellulase, enzyme activity, hydrolysis, physical and chemicals parameters.

INTRODUCTION

The cellulosic biomass production, once thought to be an ever increasing unmanageable waste, is now considered as an important renewable source of energy [1].

The annual world biosynthesis of cellulose by both land and marine algae occurs at a rate of $0.85*10^{11}$ tons per years, which was corresponding to more than four times the world's yearly total energy consumption[2]. Cellulose could be employed as an important energy source for the production of useful end products only after its hydrolysis to glucose [3], which can further be used as a substrate for other bioprocess[4].The major factors affecting the hydrolysis of cellulose include porosity(accessible surface area) of waste materials, crystallinity of cellulose fiber and lignin and hemicelluloses content[5]. The production of soluble sugars from cellulose in agricultural residues relies by the sequential/coordination action of individual components of cellulose complex (i.e.,exoglucanase; EC3.2.1.91, endoclucanase; EC 3.2.1.4 and β-D-glucosidase; EC3.2.1.21) derived from cellulolytic microorganisms[6]. For many years, cellulose producing bacteria have been isolated, characterized for the production of more effective cellulases from variety of sources such as soil, plant materials, hot springs, organic matters, feces of ruminants and composts[7].

The aim of the present work was the isolation and the screening of the cellulose producing strains with higher activity with higher activity and the optimization of the physicochemical parameters, such as pH of the culture medium, temperature and the study of the cellulase activity by the using DNS method.

MATERIALS AND METHODS

Isolation of cellulose producing bacteria

The cellulose producing bacteria were isolated on CMS solid culture medium containing cellulose as sole source of carbon and composed of the following components:1% peptone,1% cellulose,0.2%K_2HPO_4, 1% agar,0.03% $MgSO_4$,0.25%$(NH_4)_2SO_4$,0.2% gelatin [8]. For the isolation cellulase producing bacteria, one gram of each different sample was weighed and introduced into 9 mL of sterile distilled water. Serial dilution of 10 of 1 mL of the mixture was introduced into a sterile Petri dish and inoculated on 100 ml CMS culture medium, containing cellulose as sole source of carbon and incubated at 30ºC for 24 hours.

After enrichment in CMS broth, the inoculums were respectively streaked in CMS agar plates with cellulose as unique source of carbon for incubation at 30ºC for 48h.bacterial colonies were purified by repeated streaking.

Screening of cellulase producing bacteria

The isolated, selected cellulase producing bacteria colonies were transferred individually on CMS agar plates, incubated at temperature of 30°C for 48hours. In order to estimate the produced cellulose enzyme by the selected bacteria, the plates were flooded with a solution of 1% congo red, incubated at room temperature for 15 min. Furthermore, a solution of 1M NaCl was added and incubated at room temperature for 15 min. The produced cellulase was explored by the measuring of the formed diameter of the zone of hydrolysis around the inoculated colony in solid used culture medium submerged with 1% congo red solution[9] and the formed reduced sugars was estimated by the using of DNS method[10].

Identification of the selected cellulose producing bacteria

The identification of the isolated, selected cellulose producing bacteria was mainly based on the determination of the morphological analysis such as macroscopic and microscopic observation after the coloration of Gram and the determination of further biochemical characteristic by the using the (galerie API20E) was carried out according to the manufacturer's instructions (bioMerieux, Marcy l'Etoile, France).The determination of the physiological and biochemical criteria of the of the isolated, selected cellulase producing bacteria was based on the study of the bacterial growth on the above described culture medium CMS, containing cellulose as sole source of carbon, incubated at temperatures of 30°C for 5 days. The typical colonies were isolated and purified. The short-time conservation of the pure isolates has been achieved on a solid CMS, culture medium; the obtained culture was maintained at 4°C and renewed every month.

Crude enzyme production

The isolated cellulase producing bacteria strains were inoculated in fermentation broth culture medium composed of the followed ingredients: 1%cellulose,0.2% K_2HPO_4,0.03% $MgSO_4$, 1% peptone,0.25% $(NH_4)_2SO_4$[10], incubated under agitation speed of 100 rpm at room temperature for 24 hours. The crude extract enzyme was harvested by centrifugation of the inoculated isolated, selected bacterial strain in the fermentation broth at 1000×g for 10min at temperature 4°C.

Cellulase activity assay

The Cellulase activity was evaluated by the measure of the reduced sugar obtained from the hydrolysis of the used solubilized cellulose in phosphate buffer 0.05 M at pH 8 according to the described DNS method [12]. For this purpose, a volume of 0.5 ml of the crude extract enzyme obtained by the centrifugation of the inoculated fermentation broth culture medium with the selected cellulose producing strains, was added to a volume of 1ml 1% cellulose in phosphate buffer and incubated at temperature of 50°C for 30min. Furthermore, the reaction was stopped by the addition of a volume of 1ml of DNS solution, incubated at temperature of 100°C for 10min. The obtained reducing sugar was determined by measuring of the absorbance at 540nm by the using of the glucose as standards, where 1 unit(U) of crude extract enzyme was estimated as the amount of enzyme that release 1µmol reducing sugars per mL per minute.

Hydrolysis of cellulose

The hydrolysis of cellulose by the cellulase was determined by the using DNS method [11]. Briefly, 5 sterilized tubes containing 0.5ml of crude enzyme and 1ml of 1% cellulose solution prepared in phosphate buffer pH8 were incubated at 50c in different times courses respectively for 1,2,3,5,10,15 min. The reaction was stopped by adding 1ml of DNS reagent. The obtained reducing sugar was determined by measuring of the absorbance at 540 nm by the

using of the glucose as standards, where 1 unit (U) of crude extract enzyme was estimated as the amount of enzyme that release 1µmol reducing sugars per mL per minute.

Effect of pH on the cellulase activity

In order to explore the effect of pH value on the cellulose activity, a volume of 0.5 ml of the inoculated broth was introduced in different test tubes, added to a volume of 1ml of 1% cellulose, the pH value (5.8, 6.2, 6.6, 7, 7.45, 8) was adjusted by the using of 0.05M phosphate buffer solution. The reaction mixture of crude extract cellulase and cellulose solution was incubated at 50°C for 30 min. A blank consisting of 1 ml of 1% 0.5 ml soluble cellulose (1%, w:v) in 0.05 M phosphate buffer (pH 6.4) was also incubated in a water bath at the same temperature and time with the other test tubes. The reaction was stopped by adding 1 mL of DNS reagent in each test tube and then immersing the tubes in a boiling water bath for 5 min after which they were allowed to cool and 5 mL of distilled water was added and the reducing sugars liberated were estimated according to the described DNS method [10]. The absorbance for all the test tubes was measured at 540 nm with spectrophotometer. An enzyme unit is defined as the amount of enzyme that release 1µmol reducing sugars per mL per minute at 50°C.

Effect of temperature on the cellulase activity

In order to investigate the effect of temperature on the cellulose activity, a volume of 0.5 ml of the inoculated broth was introduced in different test tubes, added of a volume of 1ml of 1% cellulose, adjusted at pH value of 8, incubated at different temperature.

The reaction mixture of crude extract cellulase and cellulose solution was incubated at different temperature (20, 30, 40, 50, 60, 70, 80, 90, 100°C) for 30 min. A blank consisting of 1 ml of 1% 0.5 ml soluble cellulose (1%, w:v) in 0.05 M phosphate buffer (pH 8) was also incubated in a water bath at the same temperature and time with the other test tubes. The reaction was stopped by adding 1 mL of DNS reagent in each test tube and then immersing the tubes in a boiling water bath for 5 min after which they were allowed to cool and 5 mL of distilled water was added and the reducing sugars liberated were estimated according to the described DNS method [10].The absorbance for all the test tubes was measured at 540 nm with spectrophotometer. An enzyme unit is defined as the amount ofenzyme that release 1µmol reducing sugars per mL per minute at 50°C.

RESULTS AND DISCUSSION

Nowadays, there is rekindled enormous worldwide interest in the development of new and cost-efficient processes for converting plant-derived biomass to bioenergy in view of fast depletion of our oil reserves and food shortages [6].Several microorganisms have been discovered for decades which have capacity to convert cellulose into simple sugars [13].In this study, sixteen bacteria strains have been isolated from different samples; the primary screening gave five strains with high potential of cellulose hydrolysis. Among this five bacterial strains;EC4' showed better yield of sugar degradation with a clear hydrolysis zone at 52 mm (Figure 1), many researchers on cellulytic bacteria isolated from different environmental sources have been done[14] aerobic bacteria *Cellulommonas sp.YJ5* [15] and *Cellulomonas sp.* ASN2 isolated from soil samples and cellulose activity was measured after 48 hours of incubation .The identification of the new isolate EC4' was determined by morphological observation and biochemical test , colonies of EC4' on cellulose medium salt were yellow green color, small and partially shin; microscopic observation of this isolate demonstrated Gram positive, long, thin and sporuledbacilli(Figure 2), biochemical tests of EC4' strain allowed bringing to *Bacillus circulans.*

Exploration of cellulase activity

Cellulase activity of the isolated, selected EC4' *Bacillus circulans* was estimated according to the described DNS method by to be 0.81 IU/ml. Where, Ekperigin and their co-workers [16]have reported that the maximum cellulase by *A.anitratus* and *Branhamellasp* were (0.48 and 2.56IU/ml)respectively.

Hydrolysis of cellulose by crude cellulose enzyme

The assay of hydrolysis of cellulose by crude extract cellulose has been investigated by the estimation of the potential of crude cellulase enzyme to hydrolysis of insoluble cellulose such as crystalline which is used for this experimental approach. The obtained result led to the conclusion that both of the enzyme activity and concentration of cellulose is related to incubation time. The present study proofed that the reducing sugar formed during time courses is increasing until the yield of cellulase efficiency obtained after 30 min of time incubation(Figure 3).

pH optimum for cellulose production

In order to explore the effect of the pH value of the used CMS culture medium on the cellulose production by the isolated, selected EC4' *Bacillus circulans,* the crude extract of enzyme has been investigated at different pH incubated in the value in the presence of cellulose, incubated at temperature 40°C.The initial pH of the used culture

medium influences the rate of cellulose production. It was inferred from the obtained results that the isolated, selected EC4' *Bacillus circulans* is capable to induce the cellulose production from the initial pH value of the used culture medium from pH 5.5 to pH 8.0. Where, the enzyme production varied considerably from 2.1 U/ml to 375 U/ml (Figure 4). The isolated, selected EC4' *Bacillus circulans* has optimum cellulose production atpH 6.6(375 U/ml). However, it was noted that the cellulase production was declined with increase in pH value from pH7.0 to pH 8.0. Close and co-workers [17]indicated that that cellulase production *Bacillus subtilissubsp* has a pH optimum of 6.5. Where, Ogura J et al. [18] has reported that *Lysobactersp* manifested a high enzyme production at pH range of enzyme production at pH range of 5.0-7.0. Furthermore, Mawadza, Kim and co-workers [19, 20] has found important enzyme production at pH range of 5.0 to 6.5 and 4,5-7 by the investigated *Bacillus* strains, *Bacillus circulans*, respectively .

Figure 1:Illustration of hydrolysiszone of the isolated, selected EC4' *Bacillus circulans*, inoculated in the CMS cumture medium, containing cellulose as source of carbon, incubated at temperature of 30°C for 48 hours

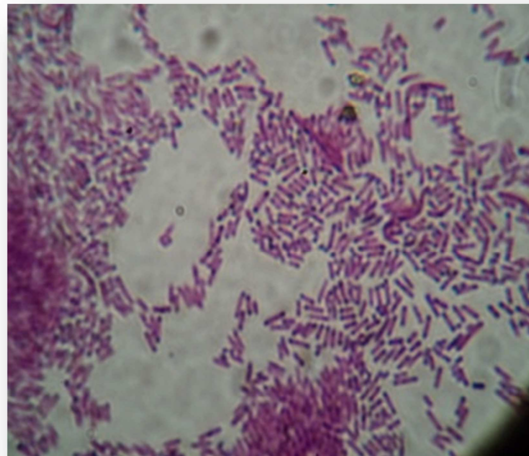

Figure 2: Gram coloration and microscopic observation of the isolated, selected EC4' *Bacillus circulans*, inoculated in the CMS cumture medium, containing cellulose as source of carbon, incubated at temperature of 30°C for 48 hours.

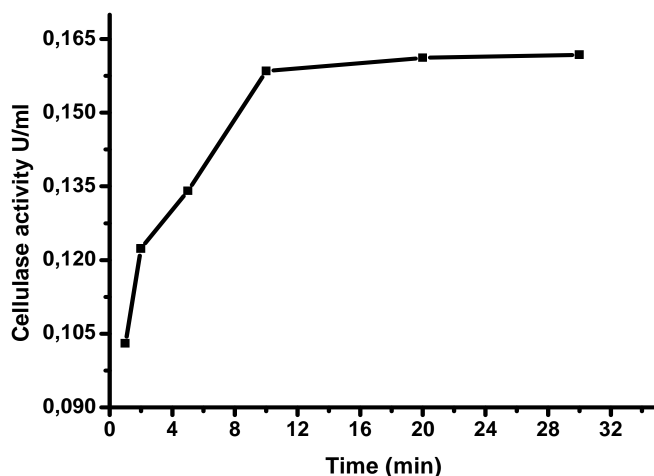

Figure 3: Time courses of reducing sugar formed during hydrolysis processes of 1ml of cellulose by 0,5 ml of crude enzyme

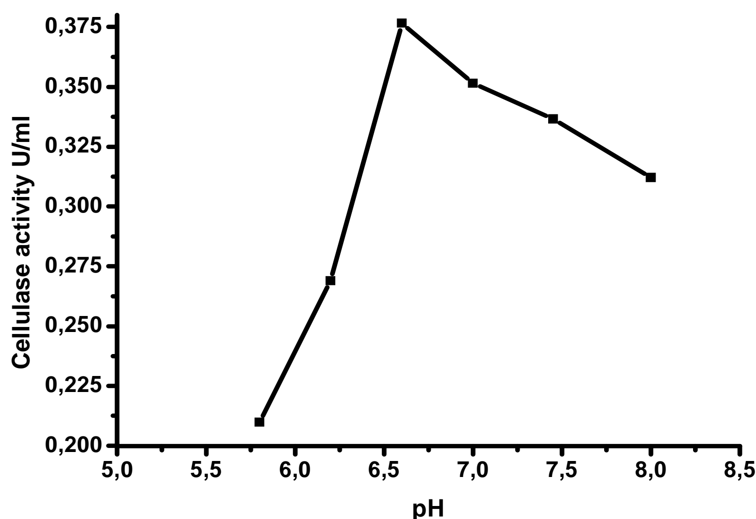

Figure 4:Display of the pH optimum for cellulase production by the isolated, selected EC4' *Bacillus circulans*, inoculated in the CMSculture medium, adjusted pH value (5.8, 6.2, 6.6, 7, 7.45, 8), incubated at 30 °C for 48 hours

Effect of incubation temperature on cellulase production

Temperature is a critical parameter that has to be controlled and it varies from organism to organism. Temperature influences secretion of extracellular enzymes by changing the physical properties of the cell membrane. In order to explore the effect of the temperature on the cellulose production by the isolated, selected EC4' *Bacillus circulans,* the crude extract of enzyme has been incubated in the presence of cellulose at the following temperature (20, 30, 40, 50, 60, 70, 80, 90, 100°C). The obtained results for the optimization of temperature for enzyme production showed that the isolated, selected EC4' *Bacillus circulans* produces cellulose in wide range of temperature from 20°C to 100°C, where the cellulose was produced from 5 to 425 U/ml (Figure 5). The optimum temperature for cellulase enzyme production was at 40°C (425 U/ml)and the enzyme production was decreased after increase of temperature above 40°C to 100°C.In early study, Yin and Mawadza their co-workers [21, 19] has reported that cellulase production by *Bacillus subtilis* YJ1,*Bacillus* strains RH68 and CH43 has optimum temperature of 60, 70, 65 °C respectively.

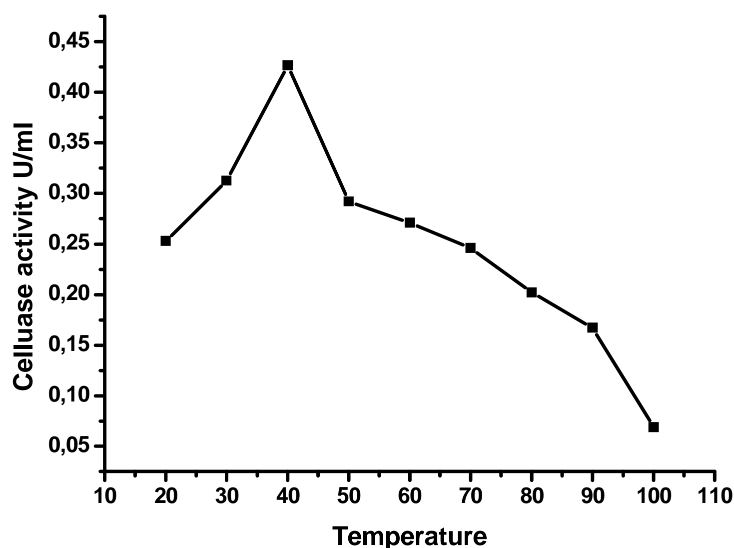

Figure 5:Display of the temperature optimum for cellulase production in the used CMS culture medium by the isolated, selected EC4' *Bacillus circulans* incubated at the following temperature (20, 30, 40, 50, 60, 70, 80, 90, 100°C) for 48 hours

CONCLUSION

Cellulase is among the most important used enzymes in the industrial processes. With the advent of new frontiers in the biotechnology, the spectrum of cellulase has a large application in the industrial application such as production bioethanol and therefore, it is of interest to search for new sources for the production of enzyme. Newly, the isolated strain *Bacillus circulans* presented as excellent candidate producer for cellulase in the investigated CMS culture medium composed of the followed ingredients: 1% cellulose, 0.2% K_2HPO_4, 0.03% $MgSO_4$, 1% peptone, 0.25% $(NH_4)_2SO_4$, with a maximum enzyme yield of 11.747 U/mL, which increase the yield of the cellulase yields by 6 folds. Furthermore, the obtained results indicated the existence of other interesting bacterial strains with important potential for cellulase activity. Therefore, further studies characterization of the enzyme such as purification of the a-amylase and characterization of metal containing protein will require.

Acknowledgement
We are thankful to the department head of the bacteriology laboratory of hospital of University of SidiBelAbbès Mr. Harrachi Abdelkader for providing us with the bacterial strains. We would like to thank Prof. Dr. MouffokBenali, Director of Laboratory for environmental synthetic information, for your encouragement, your support and your useful discussion. Without the kind cooperation of the above mentioned people the work could not have been possible.

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