International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **6(4),** 21-26, April **(2017)** *Int. Res. J. Biological Sci.*

Statistical optimization of cellulase production in *Pseudomonas* spp and its application in bioethanol production

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Available online at: www.isca.in, www.isca.me
Received 9th February 2017, revised 4th April 2017, accepted 9th April 2017

Abstract

Cellulolytic microorganism converts cellulose into monomeric units which can be used for production of valuable products. This study demonstrates the production of cellulase by Pseudomonas spp VC14. Strain Pseudomonas spp VC14 cellulase activity was increased by optimization of physicochemical parameters by Plackett-Burman statistical design. Optimization showed NH_4Cl and $FeSO_4$ as significant components influencing cellulose production. On optimization the production of cellulase increased 5.41 fold (1.84 U/ml) within 24 hrs compared to unoptimized medium. Simultaneous saccharification and fermentation of reducing sugars with Saccharomyces cerevisae showed 5.1% bioethanol production. Thus, the results of the present work clearly revealed that cellulose from strain Pseudomonas spp VC14 can efficiently be used for bioethanol production.

Keywords: Carboxymethyl cellulose, Cellulase, Bioethanol, Saccharification, *Pseudomonas* spp.

Introduction

The major problem in the 21st century is to meet the growing demand of energy for transportation and for industrial processes[1]. Bio ethanol has been an alternative fuel to the current fossil fuels crisis, developing countries have begun to explore on bio fuels like butanol and ethanol[2]. World production of bio ethanol has reached up to 51,000 million with United States and Brazil getting top position and India on the fourth position[3].

Cellulase finds its potential application in various industrial sector such as animal feed, textiles, laundry, biofuel and brewing industry. Due to immense potential cellulase has been great demand by both academic and industrial research group[4]. Microbial conversion of cellulosic biomass into simple reducing sugars is a promising strategy for low cost bio-mass processing. Cellulase due to its increased industrial applicability such as production of bioethanol[5], biomethanation[6]; agricultural and plant waste management[7]; chiral separation and ligand binding studies[8].

A wide range of microorganism, *Bacillus, Pseudomonas, Cellulomonas, Trichoderma*, *Fusarium*, *Penicillum* and *Thermomonospora* have the ability to produce an array of enzymes *viz.,* cellulases, hemicellulases and pectinases which together hydrolyze insoluble polysaccharides into soluble oligormers and to monomers[9]. Cellulose is a linear polymer of β-1, 4-D glucopyranose units. Cellulase enzyme is a combination of three major type of enzymes which exhibit higher collective activity and degrade cellulose into fermentable glucose. The cellulase complex is divided into three main types: i. endoglucanases (endo-1,4-β- glucanases), ii. exoglucanases including cellobiohydrolases and iii. β-glucosidases. Endoglucanases hydrolyze internal bonds and release new terminal ends, cellobiohydrolases acts on the existing or endoglucanase-generated chain ends and β-glucosidases can catalyze the hydrolysis of terminal non-reducing residues in β-D-glucosides with release of glucose[10]. The present work aims at isolation of cellulase producing bacteria from soil samples and Co- culturing of cellulase producing bacteria and yeast was also carried out for simultaneous saccharification and fermentation (SSF) of cellulose into bioethanol.

Materials and methods

Isolation and screening: Five different environmental samples (soil, wood, cow dung) were collected from various parts of Bangalore, Karnataka. Samples were collected, labeled and were stocked at 4° C till further use.

Cellulase producing microorganisms isolation and screening: Soil sample were serially diluted up to 10^{-5} dilutions and 100 µl of 10^{-2} dilution was spread plated onto carboxymethylcellulose agar (g/l) $MnSO_4$ - 0.05; $FeSO_4.7H_2O$ – 0.05; $CaCl_2$–2, NH_4Cl-1.0 containing 1% carboxy methyl cellulose with pH 7. Plates were incubated at 37±2°C for 3 days. Bacterial colonies showing different morphological appearance were selected.

Screening for cellulase producing Microorganisms: *Congo red staining*: Incubated plates were flooded with 0.1% for 15 to

20 minutes, the stain was poured off and then flooded with 1N NaOH so as to destain for another 15 to 20 minutes and finally 1 N NaOH was also discarded. Colonies showing clear zones around the bacterial colonies indicates cellulose hydrolysis[11].

***Gram's Iodine staining*:** Iodine solution (1%) was flooded for 2-3 min to observe the cellulolytic activity. clear zone of hydrolysis around the colonies indicated cellulose degradation[12].

***Cellulase activity*:** 50 ml of minimal salt media containing 1% carboxymethylcellulose (CMC) was inoculated with 100 µl of bacterial suspension (10^8 cfu/ ml). The flask was incubated for 72 h at 37°C in a shaking condition (120 rpm) and centrifuged at 10,000 rpm for 10 min[13]. The resulting supernatant was subjected to cellulose activity. Uninoculated broth served as control. The cellulase activity was measured by determining the amount of reducing sugars liberated by using a DNS method[14] using glucose as standard (0 to 100 mg/ml). One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1μmol of glucose per minute under standard assay conditions. A bacterial isolate with high cellulose activity was selected further for cellulose production optimization.

Characterization of bacterial isolate VC14: Isolate VC14 exhibiting maximum cellulase production was presumptively identified by cultural, morphological and biochemical test[15].

Statistical optimization of cellulase production: To determine the variables that significantly affect cellulase activity, Plackett-Burman design was used. Seven variables were screened in 12 experimental runs with two level of concentration of each variable and cellulase activity was used as a response[16] (Table-1). Each column represents a different experimental trial, and each row represents different variables. Each variable was tested at high (+) and low (-) levels. Analysis of the experimental data was performed with Design Expert version 10.0 (State-Ease, Minneapolis, MN, USA).

Table-1: Variables representing medium componants used in Plackett – Burman design.

Variables	Medium components (factors)	Low level (-)	High level (+)
X_1	CMC (g/l)	0.1	1.0
X_2	$CaCl_2$	0.3	3.0
X_3	pH	4	7
X_4	$MnSO_4$(g/l)	0.05	0.5
X_5	Yeast extract (g/l)	0.1	0.5
X_6	$FeSO_4$ (g/l)	0.05	0.5
X_7	NH_4Cl(g/l)	0.2	2.0

Each experimental run was carried out in 100 ml conical flasks containing 50 ml of production media, 100 µl of bacterial isolate VC14 was transferred to the production media. Flasks were incubated at 37°C for 24 hours at 120 rpm.

Saccharification by *Pseudomonas* VC14: Conical flasks containing 100 ml of optimized media was inoculated with 100 µl of *Pseudomonas* VC14 isolate to initiate saccharification process. The flasks were incubated at 37°C for 72 h in a rotary shaker (120 rpm).

Fermentation Conditions for Bioethanol Production: After 3 days of incubation, the above culture broth was conditioned for co-culturing of *Saccharomyces cerevisae* by adding filter sterilized salt solution (KH_2PO_4, 0.4 g; $CaCO_3$, 0.05 g; $MgSO_4$, 0.02 g and NaCl, 0.01 g to 1 l culture broth). Culture broth was inoculated with 5% (v/v) of cells of *S. cervisiae*. After 5 day incubation at 25°C, the culture broth was subjected to alcohol production by $K_2Cr_2O_7$ test[17].

Results and discussion

Isolation of cellulolytic bacteria: In the present study out of 38 isolates were isolated from 5 different samples. Out of 38 isolates screened for cellulase activity, 17 isolates (49.73%) were positive congo red staining. By Iodine staining, 23 isolates (60.52%) were positive for cellulase production.

These results were similar in the context of cellulolytic activity with that of the work of Gomashe *et al.*[18] and Gupta *et al.*[19]. Thus indigenous microbes could be a potential source of cellulase which can be explored for use in many applications.

Gohel *et al.*[20] in his study reported grams iodine gives the best results followed by congo red staining. Gopinath *et al.*[21] characterized the isolates based on gram's iodine staining. Despite its wide use, the plate assay based on CMC agar is of low specificity, presence of small amounts of contaminating starch in commercial agars makes plate clearing assay unreliable.

Cellulytic activity of *Pseudomonas spp, Bacillus spp, Bacillus polymyxa,* and *Bacillus brevis* from mangrove soil of Bhitarakanika, Odisha was reported by Thatoi *et al.*[22]. Tabo and Monsalud[23] reported the occurrence of *B. cereus*, *B licheniformis* and *B. pumilus* from philipines mangrove soil similar to our finding.

Cellulase activity: Among the 17 isolates which showed positive result by both congo red and iodine staining, VC14 exhibited the maximum extracellular cellulase activity (0.36 U/ml) compared to other isolates as shown Figure-1.

Rastogi *et al.*[24] reported highestcelluase activity of 0.02 and 0.058 U/ml by *Brevibacillus* sp. DUSELG12 and *Geobacillus* sp. DUSELR7 on days 10 and 7 days of incubation. Gupta *et*

al.[19] isolated several cellulose-degrading bacteria exhibiting cellulase activity in the range of 0.162–0.400U/ml. The highest enzyme activity of *A. Anitratus* and *Branhamella* sp., culture supernatant were 0.48 and 2.56 U/ml for CMC respectively[25].

Morphological, biochemical identification of VC14 isolate: The morphological and biochemical characteristic of VC14 isolate produced diffusible green pigment with smooth translucent raised colonies on King's B agar. On gram staining, Gram negative rods, motile, catalase and oxidase positive. It utilized citrate and arginine dihydrolase, arabinose, galactose, glucose, fructose, xylose. Methyl red and Voges-Proskauer, Nitrate reduction and urease were negative. Based on morphological, cultural and biochemical analysis, the isolate was identified as genus of *Pseudomonas* spp.Talia *et al.*[26] in hiswork on cellulolytic bacteria showed that the dominance of *Pseudomonas* spp in the soil samples collected from native Chaco soil[27].

Optimization of culture media conditions by Plackett – Burman experimental design: Statistical methods for medium optimization have proved to be a powerful and useful tool for biotechnology. Medium components screening using Plackett-Burman design (PBD) results are given in Table-2. Table-3 shows results of main effects calculated at confidence level of 95%. Among the tested variables, the factors that appeared to be of positive effects are NH_4Cl and $FeSO_4$. Presence of high levels of NH_4Cl and $FeSO_4$ in the growth medium affects cellulase production positively. On the other hand, the presence of CMC, $CaCl_2$, $MnSO_4$, yeast extractand pH at their lowest levels would result in high cellulase production.

$NH_4Cl$is the most significant factor with positive effect for cellulase production with 24.88% contribution followed by $FeSO_4$ (10.52%) within the tested levels. From the calculated *t-test*, NH_4Cl_2 is the most significant variable affecting cellulase production. Among the selected factors, increase of CMC and $CaCl_2$ showed decrease in cellulase production indicating indirect relationship between these two factors for high yield of cellulase production Figure-2.

In accordance with our results Yan-Ling Liang*et al.*[28] noted maximum CMCase activity with NH_4Cl as the sole source of nitrogen. Kumar *et al.*[29] and Kalogeris *et al.*[30] also observed a similar phenomenon in their studies. On the contrary Vyas *et al.*[31] demonstrated $(NH_4)_2SO_4$ as best inorganic nitrogen source for endo and exoglucanase activity. Similar findings was observed by Balamurugan *et al.*[32].

According to the experimental data obtained, the optimum media composition (g/100ml): CMC, 1.0; $CaCl_2$, 0.3; $MnSO_4$, 0.5; $FeSO_4$, 0.5; yeast extract, 0.5; NH_4Cl_2, 0.2; pH, 4 incubating at 37°C for 24 hrs. On optimization cellulase production increased by 5.41 fold (1.84 U/ml) within 24 hrs compared to unoptimized medium. Singh *et al.*[33] reported carbon and nitrogen sources to be the significant and inorganic salts as insignificant factors for cellulase production.

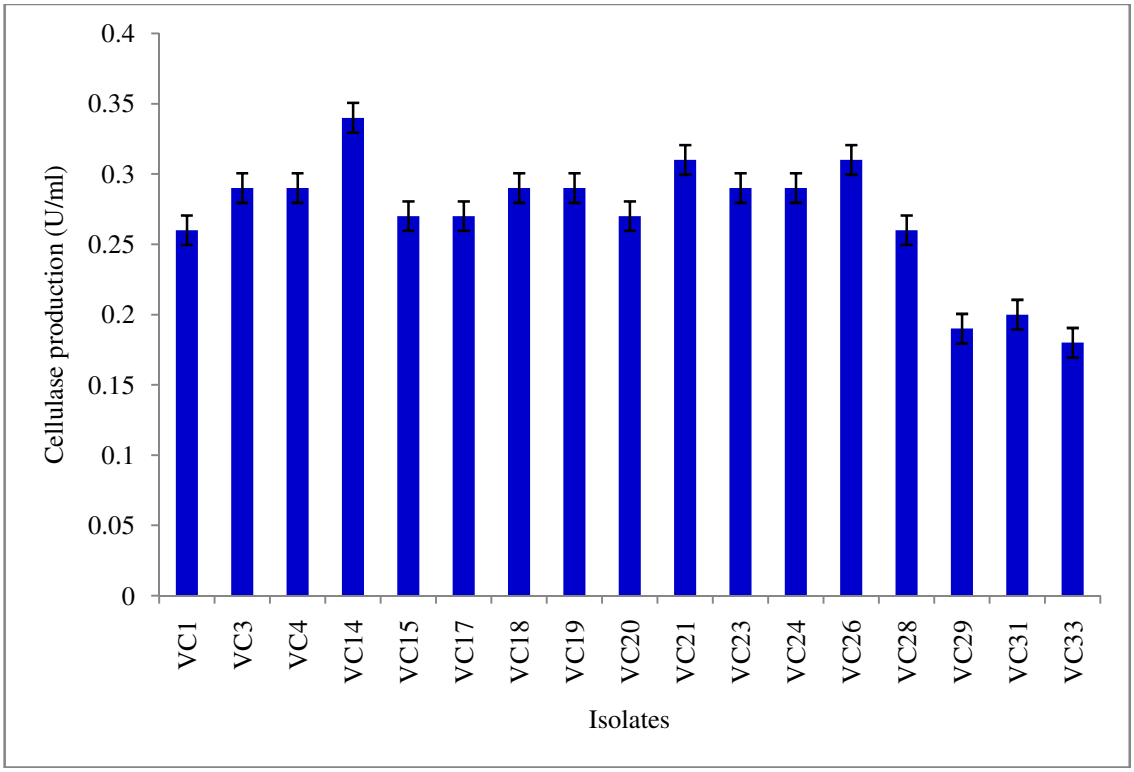

Figure-1: Cellulose degradation bacterial isolates.

Table-2: Plackett- Burman experimental design matrix with the observed response.

Run	Variables											
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	D1	D2	D3	D4	Cellulase activity (U/ml)
1	-	+	-	+	+	-	+	-	-	-	-	0.236
2	+	+	-	+	+	+	-	-	+	-	+	0.0
3	+	-	-	-	+	-	+	+	-	+	+	0.180
4	+	-	+	+	-	+	+	-	+	+	-	0.569
5	-	-	-	+	-	+	+	+	+	-	+	0.083
6	+	+	-	-	-	+	-	+	-	+	+	0.013
7	-	-	+	-	+	+	-	-	+	+	+	0.22
8	-	-	-	-	-	-	-	+	+	+	-	0.013
9	-	+	+	+	-	-	-	+	+	-	-	1.25
10	-	+	+	-	+	+	+	+	-	-	-	0.402
11	+	-	+	+	+	-	-	-	-	-	+	1.70
12	+	+	+	-	-	-	+	-	-	+	-	1.22

X_1- X_7 refers to assigned varaiables. '+' refers to high concentration of the variable; '-' refers to low concentration of the variable.

Table-3: Analysis of media components as per Placket- Burmann design for cellulase production.

Variables	Medium components	Effect	SE	$t(X_i)$	*p*- value	Significance level
X_1	CMC (g/l)	-0.11	0.17	0.64	0.5366	Non-significant
X_2	$CaCl_2$	-0.27	0.17	1.58	0.1452	Non-significant
X_3	pH	-0.13	0.17	0.76	0.4648	Non-significant
X_4	$MnSO_4$(g/l)	-0.12	0.17	0.70	0.499	Non-significant
X_5	Yeast extract (g/l)	-0.045	0.17	0.26	0.80	Non-significant
X_6	$FeSO_4$ (g/l)	0.36	0.17	2.11	0.06	Non-significant
X_7	NH_4Cl_2 (g/l)	0.55	0.17	3.23	0.0090	Significant

Bioethanol production: Researchers have studied various raw material and different methods for bioethanol production. During the fermentation process, after 5 days sample was taken for the estimation of remaining ethanol production. Bioethanol concentration in the present study was found to be 5.1%. Anshika *et al.*[34] reported 5.20% and 4.04% bioethanol with Saccharomyces cerevisiae and *Klebisella oxytoca* respectively.

Narasimha *et al.*[35] observed the ethanol production of 4.6% using *Saccharomyces cerevisiae*. *Pseudomonas* strain produced 4.032 % and 11.0% of ethanol with cellulose and wood powder.[1]. Vaithanomsat *et al.*[5] observed lower yield of bioethanol (0.02 g ethanol / g glucose) from enzymatically saccharified sunflower stalks as cellulosic waste.3.36 g/l of ethanol concentration was reported by *Pachysolen tannophilus* MTCC 1077 using RSM by SSF using sugarcane bagasse Sasikumar and Viruthagiri[36]. Using cashew apple juice waste as substrate highest ethanol concentration of 12.64 g/l was obtained under anaerobic batch fermentation by Karuppaiya *et al.*[37].

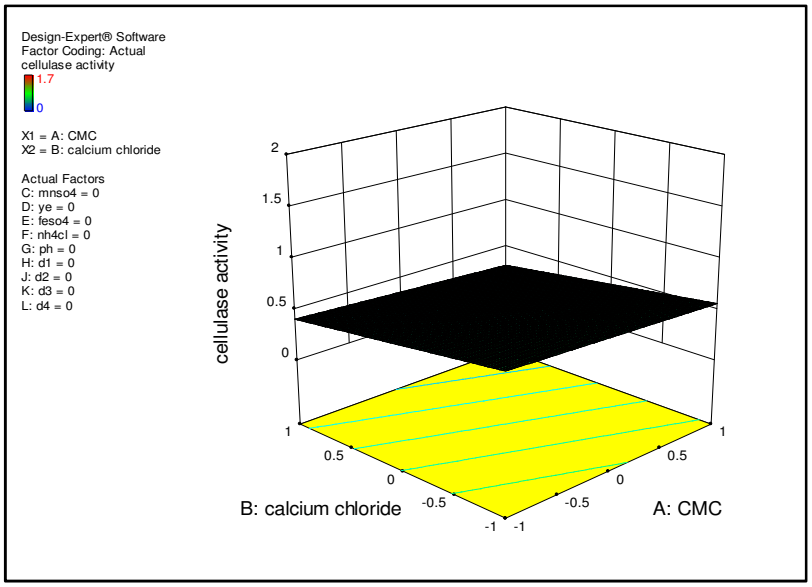

Figure-2: Interaction of CMC with $CaCl_2$ with respect to cellulase activity based on Plackett Burmann experimental results.

Conclusion

In this research study, main focus was on isolation of maximum cellulolytic activity showing microorganism in soil which will further help in conversion of cellulose present in agricultural / municipal (organic) waste into other useful products. On optimization, NH_4Cl and $FeSO_4$ were found to be the significant factors for enzyme production, both physical and chemical components on optimization showed 5.41 fold increases in cellulolytic activity. These microorganisms play a significant role in the biosphere by simplifying complex polymer cellulose into various economically important products.

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