ISOLATION, PRODUCTION AND OPTIMIZATION OF CELLULASE BY *BACILLUS PUMILUS* UNDER SUBMERGED FERMENTATION USING LABORATORY MEDIUM BY SMF METHOD

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Abstract:

Cellulolytic waste is superfluous in lithosphere and its conversion is one of the basic necessities. In current research, we have screen out potential bacterial isolates that were found capable for degradation of cellulose material. They can easily convert it into simple usable form of sugar. All isolates were capable to use cellulose in natural environmental condition such as pH, humidity and moderate temperature range. Isolation was done using to carboxymethylcellulose agar medium. While, the isolates were cultivated by submerged fermentation method (Smf). Among all isolates *Bacillus pumilus* was found most potent strain for cellulose degradation and for production of cellulase at large scale.

Key words

Cellulolytic waste, *Bacillus pumilus*, carboxymethylcellulose agar, Submerged Fermentation Method (Smf)

Introduction

Enzyme are delicate protein molecules necessary for life. Lignocellulosic biomass accounts for 50% of all the biomass available in world (**singhania et al.,2010**). Lignocellulosic biomass is mainly composed of plant cell walls, the structural carbohydrates cellulose and hemicellulose and heterogeneous phenolic polymer lignin is the main component (**Goyal,2014**). Recent times cellulosic waste is accumulated by agricultural, industrial and municipal in enormous form and inefficiently utilized due to high cost. Lignocellulosic Waste from agricultural and industrial have great potential to renewable source for regeneration of energy but looking for inexpensive and regenerate in abundance form. (**Annamalai et. al., 2014**)

Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms commonly by bacteria and fungi (**Immanuel et al., 2006**). The complete

enzymatic hydrolysis of cellulosic materials needs different types of cellulase; endoglucanase (1,4- β -d-glucan-4-glucanohydrolase), exo cellobiohydrolase (1,4- β -d-glucan glucosylhydrolase) and β -glucoside glucosylhydrolase. (Azadian et. al., 2017) Cellulases are observed as the most important renewable resource for bioconversion. It has become the economic interest to develop an effective method to hydrolyze the cellulosic biomass (Chander Kuhad et. al., 2016).

Cellulase is an important and essential enzyme to depolymerize cellulose into fermentable sugar and utilized for renewable sources (Goa et. al., 2012). Compared with fungi, bacteria have a high growth rate and good potential for cellulose production. Some bacterial species, viz *Cellulomonas Species*, *Pseudomonas Species*, *Bacillus Species* and *Micrococcus* have cellulolytic property (Abou-Taleb et. al., 2009)

Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for bio-polishing of fabrics as well as in house hold laundry detergents for improving fabric softness and brightness. Application of enzyme in textile, food, detergent, leather and paper industries demands identification of highly stable enzyme active at extreme pH and Temperature. Cellulase is used in the fermentation of bio mass into biofuels, fibre modification and they are even used for pharmaceutical applications (Sadhu et.al., 2013)

The purpose of this present study is to screen out potential cellulase producing bacteria and extensively discover its degradation capacity of cellulose and hemicelluloses containing waste materials.

[2] MATERIAL AND METHOD

2.1. Primary screening for cellulase producing bacteria

78 different soil samples were collected from industrial and agricultural zones for primary screening of cellulase producing bacterial species. The collected soil sample were suspended and 1 gm of sample was serially diluted in 10 ml of sterile distilled water and mixed properly upto 10^{-7} . while from each tube 100 μ l of sample was spread on agar plates. The medium for isolation contains 1.0 gm peptone, 0.3 gm meat extract, 0.5 gm NaCl, 3.0 gm agar powder, 100 ml distilled water. The plates were incubated for 24 hours at 37 °C whereas pH was maintain 7.4

2.2 Secondary screening for cellulase producing bacterial

The isolated colonies were transferred on to carboxymethylcellulose agar plates. The CMC agar plate contain 3.0 gm carboxymethylcellulose, 1.0 gm peptone, 0.3 gm meat extract, 0.5 NaCl, 3.0 gm agar powder, 100 ml distilled water. The plates were inoculated at 37 °C for 48 hours. The

incubated CMC agar plates were flooded with 1 % Congo red and allow to stand for 15 min at room temperature. 1 M NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having clear zone were selected for identification and cellulose production. Further bacterial straining was purified by repeated streaking. The purified colonies were preserved at 4°C.(Shaikh et. at., 2013)

2.3 Production of cellulase

2.3.1 Development of inoculums

The selected bacterial cultures were individually maintained on CMC agar slant at 4°C. The selected bacterial culture was inoculated in broth medium containing 1.0 gm peptone, 0.3 gm meat extract, 0.5 gm NaCl and 100 ml distilled water. After the incubation period these bacterial cells were used as inoculum.

2.3.2 Cellulase enzyme (crude form) production by submerged fermentation process.

The selected bacterial isolates were studied for cellulase enzyme production in submerged fermentation process. Tack 250 ml Erlenmeyer flask containing 50 ml production medium. The product on medium containing 3.0 gm Carboxymethylcellulose, 1.0 gm peptone, 0.3 gm meat extract, 0.5 gm NaCl, 100 ml distilled water and autoclaved at 121 °C for 15 min. After autoclave, the medium was inoculated with 3.0 ml of bacterial isolates and inoculated in a rotary shaker at 37±2 °C for 48 hours of fermentation period with agitation speed of 100 rpm. The enzyme activity checked at an interval of 24 hours. After centrifugation served as crude enzyme source.

2.3.3 Estimation of cellulase enzyme

Estimation of cellulase enzyme activity was assayed using dinitrosalic acid (DNSA) reagent (Miller, 1959) by estimation of reducing sugars released from CMC. Crude enzyme was added to 2.0 ml of 1 % CMC solution to the tube as substrate. Incubate the tube at 55 °C for 30 min. now add 1 ml DNSA solution to stop the enzyme activity. Then keep it in boiling water bath for 15 min. add 6 ml distilled water and measured O.D at 540 nm absorbance. Cellulase production was estimated by using glucose calibration curve. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1 µmol of glucose per minute under stander assay condition (Yang et al.,2014).

2.4 Identification for cellulase producing bacteria

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations.

2.41 Morphological and Biochemical characterization examination staining techniques

All isolates were stained using different staining techniques for morphological studies such as Gram staining, nucleus staining and endospore staining. While, biochemical studies included indole test, methyl-red test, vogues proskauer test, citrate utilization test, catalase test, oxidase test, gelatin test, casein hydrolysis test, congo red test, motility test, amylase test, nitrate reduction test, carbohydrate fermentation test by standards method. The various media was prepared as per standard protocol in sterile distilled water and pH was adjusted accordingly.

2.5 Optimization of culture condition on cellulase activity

a) Effect of pH, Temperature, incubation period, carbon and nitrogen source on cellulase production

In order to determine all effect on cellulase production, the selected bacterial isolate was grown in CMC broth and incubated at various parameters. The influence of all factors on enzyme activity was determined by measuring cellulase activity at varying pH values from 4 to 8 and Temperature varying from 28 to 45 °C and incubation period varying from 24 to 120 h at 37 °C. both carbon and nitrogen sources have wide variety of impacts on the various organisms and enzyme production. Carbon and nitrogen sources have been replaced with various substances. Carbone source such as glucose, xylose, lactose, maltose, sucrose and nitrogen source such as sodium nitrate, urea, beef extract, yeast extract, ammonium phosphate. All factor influence on enzyme activity was determined by measuring cellulase activity.

3.0. RESULT AND DISCUSSION

3.1. SAMPLE COLLECTION

A total of 78 samples were collected from composting sites, paper and pulp industries, different farms as well as decaying wood, garden soil sample were also collected in ziplock bag. As shown in Fig 3.1

Fig 3.1 Image of collected soil sample

3.2.Primary screening

Among all isolates 53 bacterial isolates were obtained in pure form with positive results. This isolates were showing cellulase production. All isolates were purified in pure culture form on Nutrient agar

3.3. Secondary screening

Seconday screening of cellulase producing bacteria from pure culture was carried out using CMC medium. 17 potent isolates were collected showing zone of hydrolysis surrounding their

colonies. (Figure 3.3). From which highly potential eight isolates were selected for further studies.

Table. 3.1 Potent isolation, source and morphology character.

Isolate	Location	Gram reaction
Ps-1	Timber soil, Geeta mandir, Ahemdabad.	Gram positive
Ps-2	Timber soil, Geeta mandir, Ahemdabad	Gram positive
Ps-3	Timber soil, Geeta mandir, Ahemdabad	Gram negative
Ps-4	Timber soil, Geeta mandir, Ahemdabad	Gram positive
ps-6	Wheat growing soil, Vasna, Kathlal	Gram positive
Ps-7	Timber soil, Geeta mandir, Ahemdabad.	Gram positive
Ps-8	Cotton soil, Vasna, Kathlal	Gram positive

3.4. Study of cultural characteristics of the isolates

As shown below table 3.4 on the basis of visual observation character were noted and gram staining (table 3.3) was performed to study morphology characters.

Table 3.2: Study of cultural characteristics of isolates

Isolates	Size	Shape	Margin	Elevation	Texture	Opacity	Pigment
Ps-1	Big	Round	Undulate	Raised	Smooth	Transparent	White
Ps-2	Small	Round	Concentric	Raised	Smooth	opaque	Off white
Ps-3	Big	Irregular	Undulate	convex	Granular	Opaque	White
Ps-4	Big	Irregular	Entire	Slightly raised	Granular	Opaque	Off white
Ps-6	Medium	Round	Entire	Flat	Smooth	Opaque	White

Ps-7	Big	Iregular	Undulate	Raised	Rough	Opaque	White
Ps-8	Medium	Irregular	Erose	Flate	Punctuate	Opaque	Off white

3.5. Identification of potent cellulase producing isolate Ps-7

3.5.1. Study of Morphology characteristic of isolates Ps-7

Fig 3.4 image of Gram staininig

Table 3.3: morphological characterization of selected isolate ps-7

Gram reaction	Gram positive
Shape and Arrangement	Big and rod shape
Spore staining	Spore forming
Motility test	Positive

3.5.2. Study of biochemical activity of potent isolate Ps-7

Biochemical characteristics of isolate Ps-7	
Biochemical test	Isolate Ps-7
Sugar utilization test	Acid only
Catalase test	+ve
Voges-proskauer test (V-P test)	+ve
Indole production test	+ve
Ammonia production test	+ve
Starch hydrolysis	+ve

Casein hydrolysis	+ve
Nitrate reduction test	+ve

Table 3.4: Biochemical characteristics of Ps-7

3.6. Zone of hydrolysis of isolate Ps-7 and bacterial identification report

Fig 3.5 : CMC agar plate

Image 3.5 is the *Bacillus* sp. Ps-7 on CMC agar plate showing fine clear zone and maximum hydrolysis the substrate carboxymethylcellulose compare to remaining seven *Bacillus* culture. So from cultural, microscopic, and biochemical characteristics the culture ps-7 shows good results. Therefore this culture was taken for further identification by VITEK 2 SYSTEMS.

Fig 3.6 bacterial identification report

bioMérieux Customer: Laboratory Report Printed Mar 18, 2020 12:24 IST
System #: Printed by: Labadmin
Report Version: 1 of 1
Patient ID: C00125707
Bench: Other

Patient Name: PAYAL-1 DILIPSINH RATHOD, .
Isolate: C00125707-1 (Approved)
Last Updated: Mar 17, 2020 16:47 IST By: LabTech
Card Type: BCL Bar Code: 2391170203621183 Testing Instrument: 00001924160B (16664)
Setup Technologist: Laboratory Technician(LabTech)

Bionumber: 1254504614466260
Organism Quantity: Selected Organism: *Bacillus pumilus*

Comments:

McFarland: 1.86 (1.80 - 2.20)

Identification Information	Card:	BCL	Lot Number:	2391170203	Expires:	Feb 8, 2021 12:00 IST
	Completed:	Mar 18, 2020 06:36 IST	Status:	Final	Analysis Time:	13.83 hours
Organism Origin	VITEK 2					
Selected Organism	89% Probability <i>Bacillus pumilus</i> Bionumber: 1254504614466260 Confidence: Good identification					
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Bacillus pumilus LeuA(79),APPA(17),ELLM(88),TyrA(99),dGAL(12).						

Biochemical Details																	
1	BXYL	+	3	LysA	-	4	AspA	-	5	LeuA	-	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	-	11	AGAL	+	12	AlaA	-	13	TyrA	-	14	BNAG	+
15	APPA	+	18	CDEX	-	19	dGAL	+	21	GLYG	-	22	INO	-	24	MdG	-
25	ELLM	-	26	MdX	-	27	AMAN	+	29	MTE	-	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	-	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	-	47	dTAG	+	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	-
60	OLD	-	61	ESC	+	62	TTZ	+	63	POLYB R	-						

3.7. Result for estimation of reducing sugar by DNSA method

Graph: 3.1: standard graph of glucose by DNS method

3.8. Cellulase production from optimized medium

Isolation and screening are the rate limiting in search and discovery of previously unknown natural products from microorganisms (Sethi et. al., 2013).

Seven isolates Ps-1, Ps-2, Ps-3, Ps-4, Ps-6, Ps-7 and Ps-8 of cellulolytic bacteria from different soil sample were selected for optimization process for production of maximum cellulase. In this study cellulase production was done by submerged fermentation method (Sonia sethi et al, 2013).

As seen in graph 5.1 endoglucanase activities were in different bacillus spp Ps-1, Ps-2, Ps-3, Ps-4, Ps-6, Ps-7, Ps-8 at shaking condition 1.716, 2.004, 2.967, 1.996, 3.418, 3.568, 2.676 U/mL/min for 48 hours at room temperature, respectively. Maximum cellulase activity was observed in Ps-7 for 48 hours, which was 3.568 U/mL/min.

In the present study Bacillus spp Ps-7 also has been isolated and showed cellulase activity. Similarly kanafusa-shinkai, et. al.,(2012). also reported the cellulase activity of 0.9 U/mL and 0.140 U/mL of certain bacterial and fungal species , respectively, which have high growth rate as compared to fungi, good potential to be used in cellulose production.

Graph 3.2 : Production of cellulase by different isolates.

3.10. Optimization of cellulase enzyme by SmF method

3.10.1. Optimization of temperature for cellulase production

Optimization was carried out by incubating the fermentation flask at 28, 37, 48 °C and the cellulase activity were 1.002, 3.568, 2.448 U/mL/min, respectively. Thus, as show in graph 3.2, maximum activity was observed at 37 °C = 3.568. the reduction of sugar conversion as well as enzyme production was observed at the temperature above 40 °C. **Ray, et. al., 2007** used saw dust for production of cellulase by *Bacillus subtilis* with cellulase activity obtained at 45 °C. the CMAase activity declined when the initial pH 8 for cellulolytic enzyme production by *Paenibacillus* sp (**Yan-ling liang, 2014**). In a previous study, *P. curdlanolyticus* B-6 was cultivated for enzyme production at 37 °C. Furthermore, Kumar et al. reported that the optimal initial pH and temperature for CMCase production by *P. polymyxa* was 37 °C.

Graph 3.2 : Optimization of temperature for cellulase production by *Bacillus pumillus*.

3.10.2. Optimization of pH for cellulase production

As shown in graph 3.3, effect of pH on cellulase production was determined at pH values of 4, 5, 6, 7 and 8 cellulase activity obtained were 3.179, 2.435, 2.370, 2.809 and 2.663 U/mL/min, respectively. Thus, as shown in graph 3.3, maximum activity was observed pH 4= 3.179.it was observed that the cellulase activity has a broad pH range between 3.0 and 9.0. Two major

activity peaks were obtained at 4.5 pH and 7.5 pH (Coa et al., 2002) it was reported that the optimal pH for a cellulase from *Bacillus subtilis* was between 6.0 and 7.0 (Lee, et.al.,2010) and in another report the optimal pH activity of *Bacillus subtilis* cellulase was found to be between 4.0 and 4.5 (Mawadza, et. al.,1996)other report *B.subtilis* produce maximum cellulase activity was found to be at 7. In a previous study, *P. curdlanolyticus* B-6 was cultivated for enzyme production at pH 7.0 . Furthermore, Kumar et al. reported that the optimal initial pH and temperature for CMCase production by *P. polymyxa* was 5.5, respectively. such different results may appear because of differences within the same genus.

3.10.3. Optimization of inoculums size for cellulase production

As shown in graph 3.4, effect of inoculums size on cellulase production was determined at inoculums size of 0.5%, 1%, 1.5%, 2%, 2.5% cellulase activity obtained were 3.033, 1.846, 2.96, 1.464, 1.761 U/mL/min, respectively. Thus, as shown in graph 5.4, maximum activity was observed inoculum size 0.5% = 3.033. The optimum formula of cellulase production from marine isolate *Streptomyces ruber* was KH_2PO_4 1.5 (g/L), MgSO_4 0.1(g/L), MnSO_4 0.05(g/L), NH_4NO_3 0.5(g/L), NaCl 1.5(g/L) with inoculum size of 0.5 ml and in this study isolate Ps-7 have CMCase activity is 2.96 for inoculum size 1.5%. (Shah et.al 2008)

Graph : 3.4. Optimization of inoculum size for cellulase production by *Bacillus pumillus*

Result and Conclusion

To conclude the result obtained in this study clearly shows that certain bacterial isolates obtain from nearby zones and industrial areas have good capacity to produce cellulase in natural environmental conditions without adding any specific supplements and not even much modification in physical parameters are need. Result also clearly indicate *Bacillus pumilus* a Gram positive, rod shaped bacteria yields maximum cellulases activity. The optimum temperature and pH was determined at 37 °C and pH 4. The maximum inoculum size was found using 0.5 %v/v with highest production in medium by SmF method. There are main research work done such as *Bacillus subtilis* MTCC 441 & *Cellulomonas cellulans* MTCC 23 are found potential for conversion of agro wastes for cellulase production by SSF method (Shah et.al 2008)

Our study aim fulfill today's need of potent strain of indigenous flora to remove agriculture and nonagricultural waste containing cellulosic materials. This study also conclude that every region

has its one flora which can helpful to remove waste material and reduce pollution without any change of environmental conditions

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