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Isolation, Identification and Cellulase Production by two Bacillus Species from the Soil under *Dipterocarpus* and *Lagerstroemia* Forests

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Abstract

Cellulase producing bacteria were isolated from two forest soils of Dipterocarpus (Local name Garjon) and Lagerstroemia (Local name Jarul) and were identified to be Bacillus sphaericus (GK1) and Bacillus pumilus (JK1) respectively. Optimization of the fermentation medium for maximum cellulase production was carried out with respect to the culture conditions at pH, temperature, incubation period, substrate concentrations, carbon sources and nitrogen sources. The isolate GK1 showed highest enzyme production after 4 days at pH 6.5 besides the isolate JK1 showed highest enzyme production at 35°C of 1% substrate concentration. Maximum cellulase production was showed by isolate GK1 when asparagine was used as nitrogen source while isolate JK1 showed highest cellulase production using CMC as carbon source. The isolate GK1showed highest CMCase activity using at 40°C, 1.5h optimum incubation period and 1% CMC as substrate concentration and the isolate JK1 showed highest CMCase activity at pH 7.5. In comparative activities of different cellulases the crude enzymes of the isolate JK1 showed highest enzyme activities i. e. CMCase 185.59 U/ml.

Keywords: Cellulase, *Bacillus sphaericus and Bacillus pumilus*.

Introduction

Cellulose is the most common organic compound on Earth. It is well known that plants are the most common source of renewable carbon and energy on the earth. Cellulose is the major component of plant biomass[1]. Plants produce 4×10^9 tons of cellulose annually[2]. Cellulose is a linear polysaccharide which is constructed from monomers of Glucose bound together with β 1-4 glucosidal linkage[3]. Successful bioconversion of cellulosic materials mainly depends on the nature of cellulose, sources of cellulolytic enzyme and optimal conditions for catalytic activity and production of enzymes. Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi[4-7].

The bioconversion of cellulose to soluble sugars and glucose is catalyzed by a group of enzymes called cellulases that are produced by microorganisms[8]. These cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, the most abundant and renewable biopolymer on Earth. The demand for microbial cellulases and related enzymes is growing more rapidly than ever before[9]. They are either cell bound or extracellular. Although a large number of microorganisms can degrade cellulose, only a few of them produce significant quantities of free enzymes capable of completely hydrolyzing crystalline cellulose [10].

Experimental Methodology

Screening and Isolation: *Dipterocarpus* (Local name Garjon) and *Lagerstroemia* (Local name Jarul) forest soil were collected to isolate cellulolytic bacteria using Czapek's agar medium. Again the isolates were tested for cellulolytic activities in Winstead's medium. Among the numerous isolates *Bacillus sphaericus* (GK1) and *Bacillus pumilus* (JK1) were found promising cellulose degrader. After isolation the organisms were purified through repeated plating in Nutrient Agar Media. The isolates were screened for its enzyme producing ability by clear zone around the colonies, staining with 0.1% Congo red solution in CMC agar plate.

Bacterial Identification: The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations. The results were compared with Bergey's Manual of Determinative Bacteriology" 8th edition[11].

Media: Cellulolytic bacterial strains were grown in Winstead's media containing carboxymethyl cellulose (CMC) and asparagine-0.3% as growth supplement; the mineral constituents are K_2HPO_4-0.3% and $MgSO_4.7H_2O$-0.25%. Single colonies were isolated and maintained on nutrient agar.

Production of sugar: Cellulolytic microorganisms were allowed to grow on cellulosic materials, they degrade the cellulose into sugar. So estimation of reducing sugar in the

culture filtrate by Nelson's modification of Somogyi method[12] indicates the rate of degradation of cellulosic substances.

Saccharification: For saccharification the enzyme preparation (crude) was adjusted to pH 4.0 and sodium azide (0.2%) was added to inhibit the microbial growth. Hydrolysis was carried out under stationary condition in 25 ml screw cap test tubes at 50°C with substrate (cellulose) concentration of 7.5% (w/v) for 24 hour and 48 hour of intervals Begum[13]. The sugar content in the hydrolysate was measured. Saccharification percentage was calculated by applying the following equation[14]:

$$\text{Saccharification \%} = \frac{\text{mg of reducing sugar per ml}}{\text{mg of substrate per ml}} \times 100$$

Biomass yield: Bacterial biomass was determined by measuring absorbance at 600nm [15].

Enzyme assay: Cellulase activity was measured by the method of Nelson's modification Somogyi method[12]. The amount of reducing sugar in culture filtrate was measured using by a spectrophotometer with the absorbance set at 550 nm.

Production of protein: When microorganisms are allowed to grow on cellulosic waste material they convert cellulose into protein, popularly known as single cell protein. Soluble protein contents of each enzyme extract were determined by the Lowry method[16].

Optimization of medium pH, temperature, incubation period and substrate concentration: Determination of optimum condition medium isolate were inoculated at different pH such as 3.5, 4.5, and 5.5. 6.5, 7.5 and 8.5, temperature was at 27°C, 35°C, 40°C, 45°C and 50°C, incubation period was 2 to 7 days and substrate concentration at 0.5%, 1.0%, 1.5% and 2.0% were observed respectively.

Carbon and nitrogen sources: The effect of various carbon sources such as CMC, rice bran, rice straw and saw dust were examined in the production medium. Various nitrogen sources like asparagine, beef extract, ammonium sulphate, peptone and urea were examined for their effect on enzyme.

Effects of temperature, pH, incubation period and substrate concentration on enzyme activity: Effects of temperature (25°C, 30°C, 35°C, 40°C, 45°C and 50°C), pH (4.5, 5.5. 6.5, 7.5 and 8.5), incubation period (2, 3, 4, 5, 6, and 7 days) and substrate concentration (0.5%, 1.0%, 1.5% and 2.0%) on the crude enzyme of the culture were studied and recorded.

Results and Discussion

Isolation and screening of the cellulase producing microorganism: Cellulose degrading bacteria were enriched and isolated by inoculating in Winstead's media. The bacterial culture showed growth as the medium turned cloudy. Bacteria isolates showed result positive on screening media by producing clear zone. Physiological and biochemical characteristics are shown in Table-1. Further indicating the presence of cellulase. Both the isolates GK1 and JK1 showed positive results in Catalase, Casein hydrolysis, Starch hydrolysis, Gelatin hydrolysis and in acid fermentation of Arabinose, Xylose Mannitol respectively.

Table-1
Physiological and biochemical properties of the isolate GK1 and JK1

Characteristics	GK1 Reactions	JK1 Reactions
Gram staining	+	+
Citrate	+	+
Catalase	+	+
H_2S production	+	+
Nitrate reduction	-	-
Voges-Proskaur test (V.P.test)	+	-
Methyl red	-	-
Casein hydrolysis	+	+
Indole test	-	-
Motility	+	-
Hydrolyzing ability		
Starch	+	+
Gelatin	+	+
Acid fermentation	+	+
Arabinose	+	+
Mannitol	+	+

"+": positive reaction; "–": negative reaction

Effects of incubation period, medium pH, temperature and substrate concentration: The Effects of incubation period, medium pH, temperature and substrate concentration were shown in following Tables. Isolate GK1 was showed highest production of CMCase (251.69 U/ml) at 4 days of incubation periods shown in (Table-2). Isolate no. GK1 maximum production of CMCase (255.08 U/ml) was recorded pH at 6.5 as shown in (Table-3), temperature at 35°C isolate JK1 was showed highest production of CMCase (272.03 U/ml) shown in

(Table-4). During substrate concentration isolate JK1 showed highest production of CMCase (210.17 U/ml) shown in (Table-5) were reported by many workers[17-20] which are in accordance with our observation. Similar observation has also been made by other previous work findings[17,18,20-25]. Production of CM Case by fungi at 25-28°C were reported[13,26], 40°C was reported[26]. Our results are concurrence with the above reports.

Table-2
Effects of incubation period of different parameters by the isolates GK1 and JK1

Incubation Period (Day)	Isolate No	Extracellular Protein µg /ml	Reducing Sugar µg /ml	CMCase activity U/ml	Biomass Yield (absorbance at 600 nm)	Saccharification (%)
2	GK1 JK1	68.84 80.62	55.08* 122.03	85.16 82.20*	0.168 0.159	0.46 1.06
3	GK1 JK1	385.87** 365.94	75.42 123.73	146.61 161.02	0.142 0.221**	0.67 1.05
4	GK1 JK1	278.99 238.22	100.00 201.69**	251.69** 156.78	0.169 0.310	1.02 1.46
5	GK1 JK1	168.48 192.93	152.54 137.29	250.85 235.59	0.267 0.251	1.28** 1.18
6	GK1 JK1	76.99 137.68	144.07 66.10	131.36 162.71	0.217 0.114	1.22 0.52
7	GK1 JK1	56.16 155.94	61.85 57.63	122.03 107.63	0.101* 0.112	0.32* 0.41

Legend: * Indicates minimum ** Indicates maximum

Table-3
Effects of pH of different parameters by the isolates GK1 and JK1

Incubation pH	Isolate No	Extracellular Protein µg /ml	Reducing Sugar µg /ml	CMCase activity U/ml	Biomass Yield (absorbance at 600 nm)	Saccharification (%)
3.5	GK1 JK1	86.96 70.65	66.95 50.85	49.03 36.14*	0.136 0.145	0.57 0.32
4.5	GK1 JK1	88.77 52.54*	67.80 74.58	138.98 104.24	0.210 0.234	0.61 0.73
5.5	GK1 JK1	167.57 92.39	84.75 83.05	166.95 142.37	0.155 0.149	0.82 0.68
6.5	GK1 JK1	290.76** 115.94	162.71 134.47	255.08** 166.95	0.320** 0.289	1.48 1.31
7.5	GK1 JK1	172.10 224.64	44.92 142.37	144.07 236.44	0.168 0.278	0.37 1.28
8.5	GK1 JK1	118.66 63.54	35.59* 60.17	58.81 63.03	0.131 0.179	0.31* 0.58

Legend: * Indicates minimum ** Indicates maximum

Table-4
Effects of temperature of different parameters by the isolates GK1 and JK1

Incubation temperature °C	Isolate No	Extracellular Protein µg /ml	Reducing Sugar µg /ml	CMCase activity U/ml	Biomass Yield (absorbance at 600 nm)	Saccharification (%)
27°C	GK1	78.80	76.27	97.46	0.102*	0.84
	JK1	90.57	71.14	111.02	0.108	0.77
35°C	GK1	144.93	207.63	150.85	0.429**	1.69
	JK1	233.70**	197.46	272.03**	0.243	1.73**
40°C	GK1	168.48	235.59**	206.78	0.369	0.67
	JK1	176.63	88.14	171.19	0.168	1.23
45°C	GK1	105.07	147.46	149.32	0.249	0.81
	JK1	125.91	98.31	146.61	0.126	0.46*
50°C	GK1	68.84*	80.51	105.08	0.106	0.62
	JK1	76.09	49.15	67.46*	0.104	0.59

Legend: * Indicates minimum** Indicates maximum

Table-5
Effects of substrate concentration on extracellular protein, reducing sugar level, CMCase activity, saccharification (%) and biomass yield by the isolates GK1 and JK1

Nitrogen Sources	Isolate No	Extracellular Protein µg /ml	Reducing Sugar µg /ml	CMCase activity U/ml	Biomass Yield (absorbance at 600 nm)	Saccharification (%)
0.5%	GK1	155.79**	119.49	76.27	0.243	1.23
	JK1	84.24	123.42	109.32	0.210*	1.31
1.0%	GK1	132.25	125.42	192.37	0.354**	1.87
	JK1	115.94	139.83**	210.17**	0.323	1.76**
1.5%	GK1	100.54	94.92	103.39	0.245	1.21
	JK1	107.74	109.32	83.05	0.287	1.29
2.0%	GK1	91.48	82.20	70.34*	0.232	0.94
	JK1	71.56	78.81	90.68	0.231	0.89*

Legend: * Indicates minimum ** Indicates maximum

Table-6
Effects of different nitrogen sources on extracellular protein, reducing sugar level, CMCase activity, saccharification (%) and biomass yield by the isolates GK1 and JK1

Nitrogen Sources	Isolate No	Extracellular Protein µg /ml	Reducing Sugar µg /ml	CMCase activity U/ml	Biomass Yield (absorbance at 600 nm)	Saccharification (%)
Asparagine	GK1	72.46	84.75	233.05**	0.242	1.45
	JK1	126.81	148.31**	222.88	0.576**	1.77
Beef extract	GK1	144.30**	142.37	152.54	0.355	2.32**
	JK1	100.18	93.22	156.78	0.310	1.39
Peptone	GK1	113.05	80.51	133.89	0.237*	1.22
	JK1	69.75	54.24	137.29	0.239	1.14*
Ammonium sulphate	GK1	75.18	125.42	150.00	0.369	1.67
	JK1	93.29	120.34	161.02	0.327	1.63
Urea	GK1	65.22*	92.37	127.12	0.249	1.45
	JK1	80.62	100.85	96.61*	0.271	1.71

Legend: * Indicates minimum ** Indicates maximum

Table-7
Effects of different carbon sources on extracellular protein, reducing sugar level, CMC-ase activity, saccharification (%) and biomass yield by the isolates GK1 and JK1

Carbon Sources	Isolate No	Extracellular Protein µg /ml	Reducing Sugar µg /ml	CM Case activity U/ml	Biomass Yield (absorbance at 600 nm)	Saccharification (%)
CMC	GK1	107.79	205.93**	163.56	0.251	1.14
	JK1	148.55	127.12	176.27**	0.426	1.55
Rice bran	GK1	105.98	71.19	134.75	0.248	1.32
	JK1	95.11	55.76	135.59	0.236	1.17
Saw dust	GK1	71.56	45.76	144.92	0.413	2.75**
	JK1	137.68	62.31	136.44	0.247	1.57
Rice Straw	GK1	85.14	146.64	127.12*	0.334	2.71
	JK1	70.65*	58.47	172.88	0.230	1.52

Legend: * Indicates minimum ** Indicates maximum

Table-8
Effects of relative cellulolytic activities of crude enzymes at different pH by selected isolates GK1 and JK1

Incubation pH	Isolate no	CMCase activity U/ml
4.5	GK1	71.16
	JK1	82.53
5.5	GK1	87.25
	JK1	93.32
6.5	GK1	93.54
	JK1	103.15
7.5	GK1	55.17
	JK1	120.15**
8.5	GK1	41.61*
	JK1	54.26

Legend: * Indicates minimum ** Indicates maximum

Table-9
Effects of relative cellulolytic activities of crude enzymes at different incubation temperature by selected isolates GK1 and JK1

Incubation temperature	Isolate no	CMCase activity U/ml
25ºC	GK1	120.34
	JK1	89.83
30ºC	GK1	128.81
	JK1	88.14
35ºC	GK1	111.86
	JK1	214.41
40ºC	GK1	261.86**
	JK1	149.15
45ºC	GK1	109.32
	JK1	58.47
50ºC	GK1	60.17
	JK1	45.76*

Legend: * Indicates minimum ** Indicates maximum

Table-10
Effects of relative cellulolytic activities of crude enzymes at different incubation period by selected isolates GK1 and JK1

Incubation period (hour)	Isolate no	CMCase activity U/ml
0.5	GK1	72.88
	JK1	44.41*
1.0	GK1	106.78
	JK1	178.65
1.5	GK1	239.83**
	JK1	80.51
2.0	GK1	77.97
	JK1	67.63

Legend: * Indicates minimum ** Indicates maximum

Table-11
Effects of relative cellulolytic activities of crude enzymes at different Substrate concentration by selected isolates GK1 and JK1

Substrate concentration (%)	Isolate no	CMCase activity U/ml
0.5%	GK1	105.41
	JK1	117.23
1.0%	GK1	259.16**
	JK1	251.23
1.5%	GK1	244.67
	JK1	243.41
2.0%	GK1	102.22*
	JK1	115.18

Legend: * Indicates minimum ** Indicates maximum

Table-12
Effects of relative cellulolytic activities of crude enzymes at different nitrogen source by selected isolates GK1 and JK1

Sources of nitrogen	Isolate no	CMCase activity U/ml
Asparagine	GK1	116.21**
	JK1	92.13
Beef extract	GK1	81.31
	JK1	104.53
Peptone	GK1	77.15
	JK1	55.10
Ammonium sulphate	GK1	89.23
	JK1	45.27*
Urea	GK1	68.75
	JK1	47.11

Legend:* Indicates minimum ** Indicates maximum

Table-13
Effects of relative cellulolytic activities of crude enzymes at different carbon source by selected isolates GK1 and JK1

Sources of carbon	Isolate no	CMCase activity U/ml
CMC	GK1	114.27**
	JK1	49.52
Rice bran	GK1	61.22
	JK1	109.31
Rice Straw	GK1	66.13
	JK1	45.28*
Saw dust	GK1	78.15
	JK1	52.17

Legend: * Indicates minimum ** Indicates maximum

Table-14
Effects of different optimum conditions on relative cellulolytic activities of crude enzymes produced by selected isolates GK1 and JK1

Isolate no	Sources of carbon	Sources of nitrogen	CMCase activity U/ml	Avicelase activity U/ml	FPase activity U/ml	β-Glucosidase activity U/ml
GK1	CMC	Asparagine	160.17	90.68	107.29	68.64*
JK1	CMC	Asparagine	185.59**	124.58	166.10	95.46

Legend: * indicates minimum, ** indicates maximum

Effects of nitrogen and carbon sources: The influence of various carbon and nitrogen sources on the production of CMCase, extracellular protein, reducing sugar, saccharification (%) and biomass yield by the isolates GK1 and JK1 in the Winstead's broth media as shown in Table 6 and 7. Among the nitrogen sources being used, the isolate GK1 showed the highest enzyme production (233.05 U/ml) when used asparagine used as a nitrogen source. Among the carbon sources being used the isolate JK1 showed the maximum enzyme production (176.27 U/ml) when CMC used as a carbon sources. Similar observation has also been made by other workers[27,28,29]. In the present investigation maximum enzyme activity were found when CMC was used as a carbon source (1.2 %) with Winstead's medium. Similar observation has also been made by other workers[26,30,31].

Effects of Enzyme-Substrate Reaction pH and Temperature on Enzyme Activity: The quantitative CMCase activity of crude enzyme produced by the selected isolates GK1 and JK1 while grown in liquid Winstead's medium having with their suitable carbon and nitrogen sources at different pH (4.5, 5.5, 6.5, 7.5 and 8.5) shown in (Table-8). The highest CMCase activity was showed by the isolate JK1 at pH 7.5 (120.15 U/ml). The optimum temperature during enzyme substrate reaction of crude enzyme of the selected isolates was recorded to be the best at 25°C, 30°C, 35°C, 40ºC, 45°C and 50°C. The highest CMCase activity was showed by the isolate GK1 at 40ºC (261.86 U/ml) is shown in (Table-9). Similar observation with enzyme-substrate reaction temperature and pH was reported[7,26,19].

Effects of Enzyme-Substrate reaction time and substrate concentration on CMCase activity: The quantitative CMCase activity of crude enzyme produced by the selected isolates GK1 and JK1 while grown in liquid Winstead's medium having with their suitable carbon and nitrogen source different incubation periods (0.5, 1.0, 1.5, 2 hour) shown in (Table 10). The isolate GK1 showed highest CMC-ase activity at 1.5 hours (239.83 U/ml). The isolate GK1 highest CMCase activity was recorded at 1% substrate concentration (259.16 U/ml) shown in (Table-11). Our results at optimum conditions during enzyme substrate reaction are in concurrence with another worker[32].

Effects of Different Nitrogen and Carbon Source on Enzyme Activity: The quantitative CMCase activity of crude enzyme produced by the selected isolates while grown in liquid Winstead's medium having CMC as a carbon source and different nitrogen source were determined. The highest CMCase activity (116.21 U/ml) was recorded by the isolate GK1 when asparagine as a nitrogen source is as shown in (Table12). Besides the isolate GK1 highest CMCase activity (114.27 U/ml) was recorded when CMC as a carbon source shown in (Table 13) were reported by many workers[19,27,29,33-36]. Our observation shows similarities with their reports. These data were in accordance with the results who reported that organic nitrogen

sources were more suitable for optimizing the cellulase production by *B. subtilis* and *B. circulans* than inorganic sources[37].

Comparative activities of different cellulose: The isolates GK1 and JK1 produced appreciable levels of CMCase, FPase, Avicelase, and β-glucosidase when CMC and asparagine were used carbon and nitrogen sources respectively. Comparative study of enzyme activity indicated that the isolate JK1 showed CMCase (185.59 U/ml) activity was higher compared to FPase, Avicelase and β-glucosidase as shown in Table-14.

Comparative study of enzyme production by the two isolates indicated that CMCase activity is higher compared to that of FPase activity, Avicelase and β-glucosidase which is in accordance with the findings of many workers[25,26,38-40].

Conclusion

Bacillus sphaericus and *Bacillus pumilus* has been isolated from the *Dipterocarpus and Lagerstroemia* forests soil has the potentiality for using as biofuel producers, organic matters decomposer as well as good producer of cellulases with important economic advantages. These two cellulase producer might be of potential applications in the biotechnology

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