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Effect of Physico-Chemical Parameters on Cellulase Production from Banana Pseudostem Waste Bacteria

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A B S T R A C T

The use of microorganisms in the bioconversion of cellulose containing waste led to elaborate study of cellulases producing microbes. The current study was carried out to find out best suitable physico-chemical parameters for maximum enzyme production from bacteria isolated from banana pseudostem waste. Out of 18 morphologically distinct isolates, B 1.2 and B 1.3 were most potent and consistent over other isolates in terms of cellulase production on solid medium. Identification was performed by Biolog method and B 1.2 was identified as *Enterococcus faecium* and isolate B 1.3 as *Aeromonas hydrophila*. Isolate B 1.2 was found to secrete maximum cellulase under submerged condition at 2 % substrate concentration while B 1.3 was found at 1 % substrate concentration. Both the Isolates had shown maximum enzyme production at 7 pH, 37° C temperature and preferred peptone as nitrogen source.

Introduction

Banana (*Musa* sp.) is the second most important fruit crop in India, with output of about 14.2 million tons per annum (Mahalakshmi and Linnett Naveena, 2016). Among different parts of the banana plant, the trunk-like part is called pseudostem or false stem. Banana pseudostem waste generates approx 70-80 MT per hectare, generally disposed of in the environment that causes severe problems pertaining to the pollution, ecosystem imbalances, exhaustion of soil nutrients, and so forth. It is rich in lignocellulosic materials, often referred to as plant biomass. Lignocellulose is a complex carbon (carbohydrate) polymer, composed of polysaccharides like cellulose and hemicellulose and may have complex aromatic compound like lignin.

Cellulose is the most abundant biomass on earth (Tomme *et al.,* 1995) and considered to be the most

feasible renewable biological resource on the globe which is produced by the plants by photosynthesis process (Zhang and Lynd, 2004). Cellulases are inducible enzymes that could be synthesized by a large number of diversified microorganisms during their growth on cellulosic material (Sang-Mok and Koo, 2001). Use of cellulases began in early 1980s, initially in the animal feed and then food applications (Chesson, 1987).

Subsequently, cellulases were used in various industries like textile, laundry as well as in the pulp and paper industries (Godfrey and West, 1996), as well as in the agriculture for waste decomposition and maintenance of soil fertility (Han and He, 2010). Apart from this, cellulases are also useful in various bio-processes like bio polishing, bio finishing, alcohol fermentation, malting, brewing, extraction and processing of vegetable juices, etc.

Cellulases production in the microbes is a complex system and depends on physico-chemical parameters such as size of inoculum, medium temperature and pH, presence of inducers, aeration, growth time, etc. (Immanuel *et al.*, 2006).

Materials and Methods

Sample Collection and Isolation

For the isolation of cellulose decomposing organisms, different samples of fully decomposed and partially decomposed banana pseudostem were collected from Navsari Agricultural University, Navsari, Gujarat, India. Isolation was performed on cellulose agar medium after enrichment in the broth culture containing cellulose as sole carbon source. Pure culture of isolates was preserved at 4°C temperature.

Screening for Extracellular Cellulases Production

For primary screening, cellulose agar plates were prepared. Actively growing pure cultures of different isolates were inoculated by spot test method and plates were incubated. Efficiency of isolates was judged on the basis of zone ratio (Zone diameter/colony diameter). Two most potent bacterial isolates showing highest zone ratio were further studied in liquid medium.

Enzyme Production

Activated culture of the isolates was inoculated in the cellulose broth @ 5%. At the interval of each 24 hours, samples were withdrawn aseptically from each flask and measured for cellulases production in the medium to know the time required for maximum cellulases secretion in the liquid medium.

Enzyme Assay

Activity of Cellulases was measured by the carboxy methyl cellulase (CMCase) assay. A0.5 ml of 2% buffered carboxy methyl cellulose (CMC) solution (sodium citrate buffer 0.2 M, pH 4.8) was incubated with 0.5 ml of the enzyme preparation and 0.5 ml of phosphate buffer for 30 min at 50°C.

The liberated glucose was estimated by 3, 5-dinitrosalicyclic acid (DNS) method (Miller, 1959). The amount of glucose (reducing sugar) produced was estimated from the standard plot of glucose solution. One unit of cellulase activity was defined as the amount of enzyme required to liberate one µmole of glucose $min^{-1}ml^{-1}$under the standard assay conditions.

Effect of Physico-Chemical Parameters on Cellulases Production

Various physico-chemical parameters were studied for its effect on cellulases production by varying one parameter at a time, whereas, all other parameters were kept constant. Different parameters such as pH (5, 6, 7, 8 and 9 and 10), temperature (20°C, 30°C, 40°C, 50°C and room temperature), substrate concentration (0.5, 1.0, 1.5, 2.0 and 2.5%) and nitrogen sources (gelatin, peptone, ammonium sulphate, urea and sodium nitrite) were studied in the liquid medium (Tandel *et al.*, 2014).

Identification of Isolates

Selected isolates were identified at species level by using biolog method.

Results and Discussion

Isolation of Cellulase Producing Bacteria

Total 18 morphologically diverse bacterial isolates were obtained from banana pseudostem waste and preserved on slant at 4°C temperature for further studies. The highest number of morphologically distinct isolates were obtained from partially decomposed banana pseudostem sample-1 collected from pseudostem processing plant (B 1.1 to B 1.8) followed by partially decomposed pseudostem sample-2 collected from organic farm (B 2.1 to B 2.6), fully decomposed pseudostem sample-3 obtained from conventional banana farm (B 3.1 to B 3.3) and fully decomposed pseudostem sample-4 collected from organic farm (B 4.1).

In vitro Screening of Cellulase Producers

All the eighteen isolates were preliminary screened for their cellulolytic potential on medium amended with CMC as sole carbon source and efficacy of the isolate was judged on the basis of halo zone near colony. Only cellulolytic microbes have the capacity to hydrolyze CMC and could give halo zone near colony after incubation and intensity of zone can be correlated with amount of cellulase production (Islam and Roy, 2018).

All isolates were developing variable zone ratios on solid medium in the range of 1.60-4.33, with lowest activity in B 2.1 isolate and highest in B 3.1 isolate. Further solid medium optimization suggested that most of the isolate produced maximum cellulase at 0.5-2.0% substrate concentration, 7.0-8.0 pH, gelatin as nitrogen source and at room temperature. Isolates B 1.2, B 1.3, B 3.1 and B 3.3 were found most consistent on the basis of primary solid media optimization data and were producing maximum enzyme (CMCase) activity at 24 hours of incubation and at the commencement of stationary phase and therefore, opted for further studies.

Enzyme Kinetics of Isolates

Selected four isolates (B 1.2, B 1.3, B 3.1 and B 3.3) were inoculated in the liquid media amended with CMC as sole carbon source and monitored at an interval of 12 hours for its growth and cellulase production. Experimental data evident that all the isolates could possess variable amounts of cellulolytic potential under liquid media. All the isolates were showing same type of kinetics i.e. enzyme activity with growth that started increasing as incubation progressed from 0 hour to 12 hour and showed maximum value at 24 hours of incubation. In terms of enzymatic production abilities, isolate B 1.2 and B 1.3 could secrete cellulases in the liquid medium above 0.5 U/ml and others two i.e. B 3.1 and B 3.3 were inferior over the previous two and could produce cellulases below 0.5 U/ml. Therefore, two most potent isolates B 1.2 and B 1.3 were selected for further optimization study under liquid media conditions.

Biolog Identification of Isolates

Both the isolates i.e. B 1.2 and B 1.3 were identified on the basis of substrate utilization pattern of Biolog. Biolog identification data suggested that isolate B 1.2 was identified as *Enterococcus faecium* and isolate B 1.3 as *Aeromonas hydrophila.* First potential isolate *E. Faecium* is a Gram positive cocci that is commonly known as lactic acid bacteria. (Robert and Bernalier-Donadille, 2003) reported a bacterium with cellulolytic potential and the identification data indicated that the isolate was closely resembled to *E. faecium.* Another potential candidate bacteria selected on the basis of screening was *Aeromonas hydrophila,* Gram negative bacterium. (Chakraborty *et al.,* 2019) reported the role of *A. hydrophila* in the cellulolysis.

Table.1 Extent of Cellulase Production among the Isolates

Sr. No	Isolate Designation	Zone Ratio (Zone diameter/ colony diameter)	Sr. No	Isolate Designation	Zone Ratio (Zone diameter/ colony diameter)
1	B 1.1	2.00	**10**	B 2.2	2.20
2	B 1.2	3.75	**11**	B 2.3	2.71
3	B 1.3	3.50	**12**	B 2.4	2.30
4	B 1.4	3.20	**13**	B 2.5	2.50
5	B 1.5	4.20	**14**	B 2.6	3.14
6	B 1.6	3.33	**15**	B 3.1	4.33
7	B 1.7	2.42	**16**	B 3.2	2.70
8	B 1.8	2.85	**17**	B 3.3	2.40
9	B 2.1	1.60	**18**	B 4.1	3.00

Fig.1 Biochemical identification of *Enterococcus faecium* (B-1.2) by BIOLOG

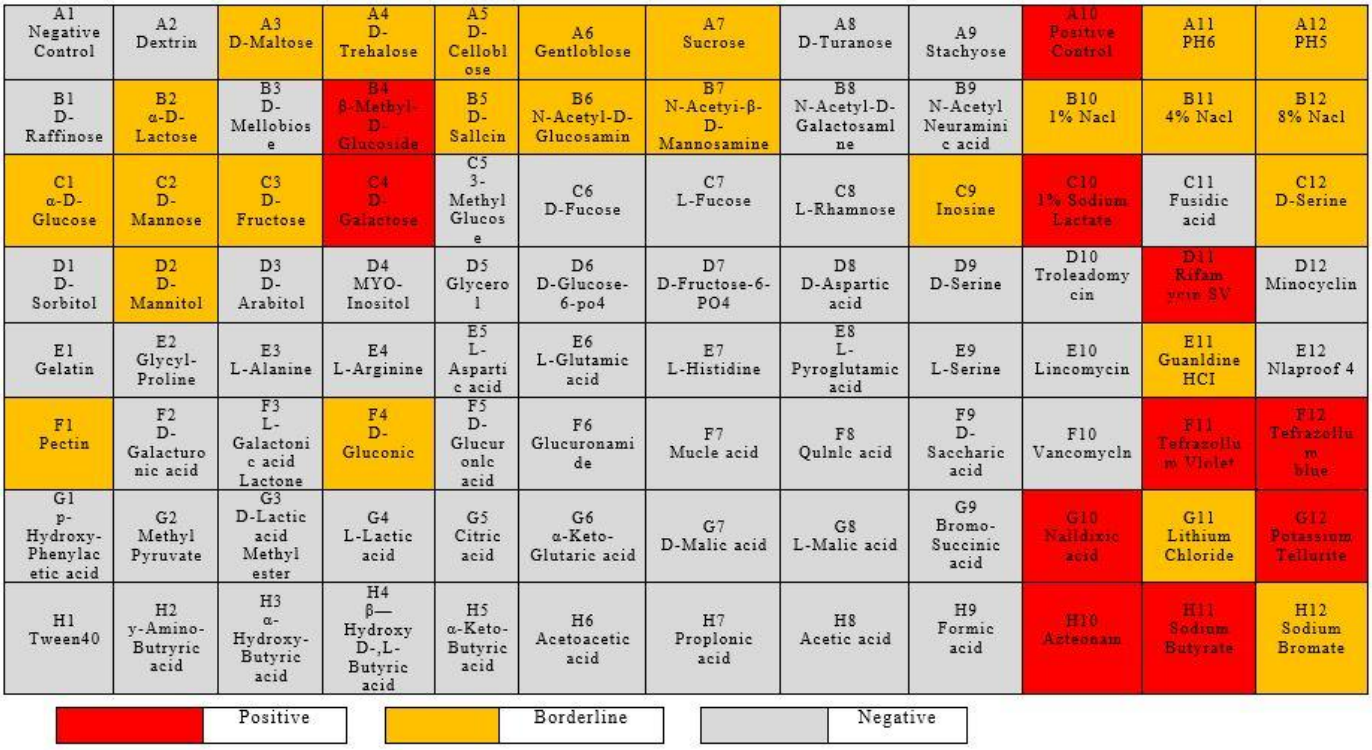
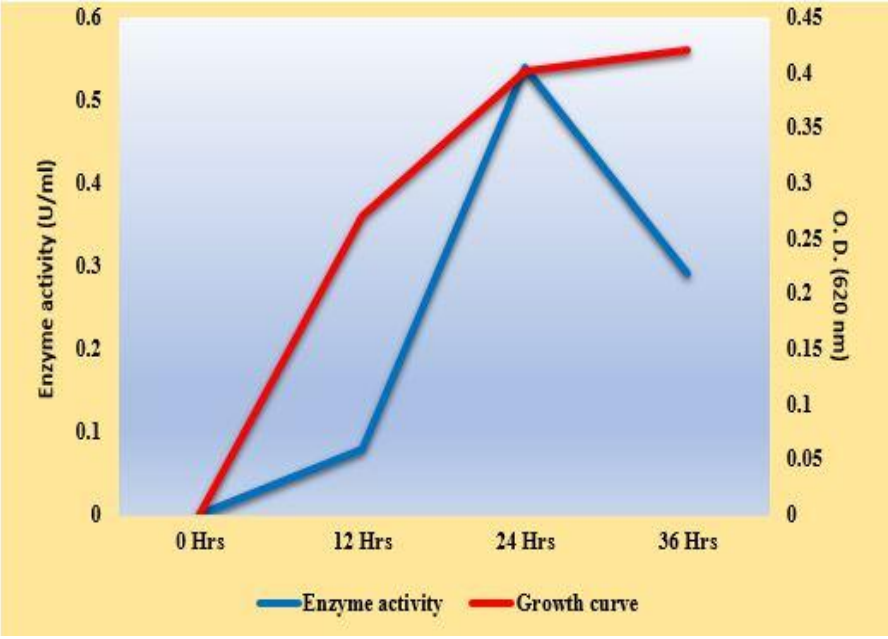


Fig.2 Biochemical identification of *Aeromonas hydrophila* (B-1.3) by BIOLOG

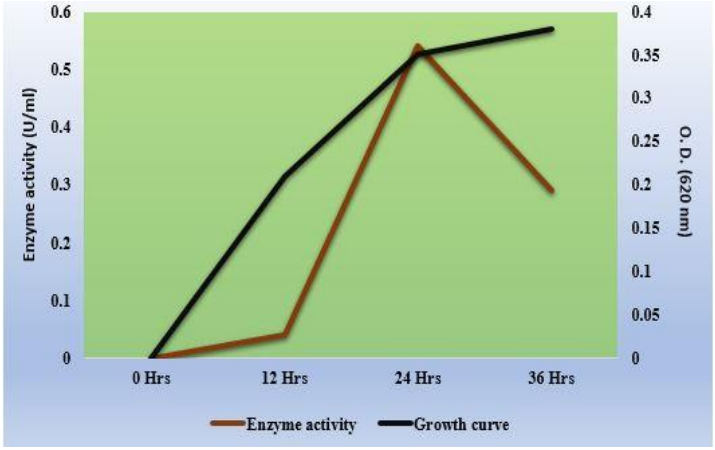
A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Celloblose	A6 Gentloblose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 PH6	A12 PH5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Mellobiose	B4 β-Methyl-D-Glucoside	B5 D-Sallcin	B6 N-Acetyl-D-Glucosamin	B7 N-Acetyi-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamln e	B9 N-Acetyl Neuraminic acid	B10 1% Nacl	B11 4% Nacl	B12 8% Nacl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 MYO-Inositol	D5 Glycerol	D6 D-Glucose-6-po4	D7 D-Fructose-6-PO4	D8 D-Aspartic acid	D9 D-Serine	D10 Troleadomycin	D11 Rifam ycin SV	D12 Minocyclin
E1 Gelatin	E2 Glycyl-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic acid	E6 L-Glutamic acid	E7 L-Histidine	E8 L-Pyroglutamic acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCI	E12 Nlaproof 4
F1 Pectin	F2 D-Galacturonic acid	F3 L-Galactonic acid Lactone	F4 D-Gluconic	F5 D-Glucuronlc acid	F6 Glucuronamide	F7 Mucle acid	F8 Qulnlc acid	F9 D-Sacchari c acid	F10 Vancomycin	F11 Tefrazollum Vlolet	F12 Tefrazollum blue
G1 p-Hydroxy-Phenylacetic acid	G2 Methyl Pyruvate	G3 D-Lactic acid Methyl ester	G4 L-Lactic acid	G5 Citric acid	G6 α-Keto-Glutaric acid	G7 D-Malic acid	G8 L-Malic acid	G9 Bromo-Succinic acid	G10 Nalldixic acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween40	H2 y-Amino-Butryric acid	H3 α-Hydroxy-Butyric acid	H4 β—Hydroxy D-,L-Butyric acid	H5 α-Keto-Butyric acid	H6 Acetoacetic acid	H7 Proplonic acid	H8 Acetic acid	H9 Formic acid	H10 Azteonam	H11 Sodium Butyrate	H12 Sodium Bromate

Positive Borderline Negative

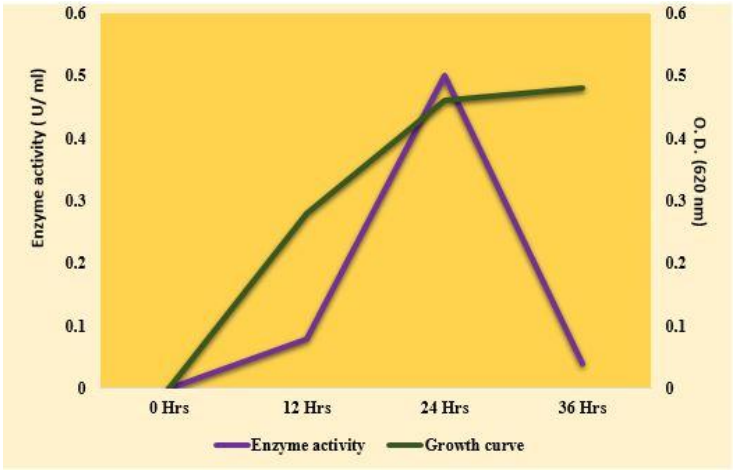
Graph.1 Enzyme kinetics and growth curve of isolate B 1.2



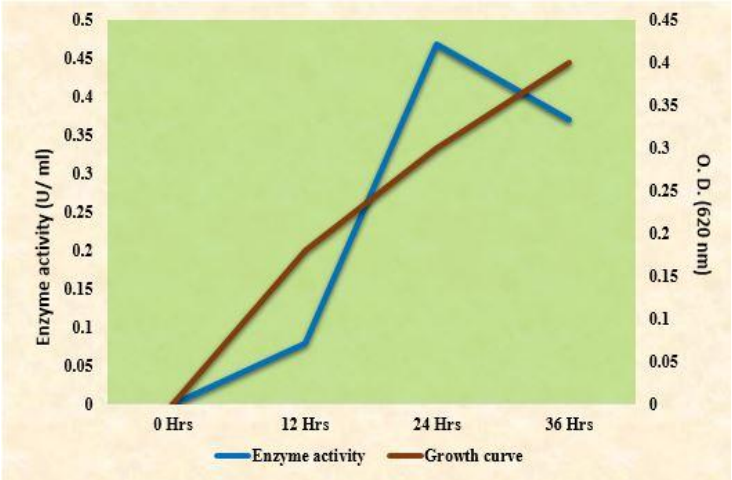
Graph.2 Enzyme kinetics and growth curve of isolate B 1.3



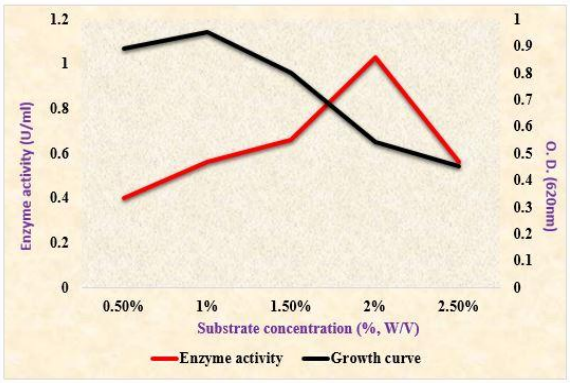
Graph.3 Enzyme kinetics and growth curve of isolate B 3.1



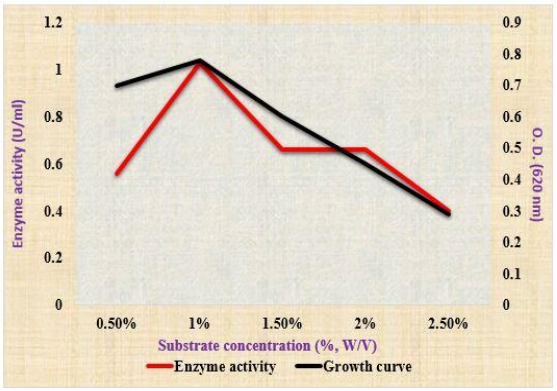
Graph.4 Enzyme kinetics and growth curve of isolate B 3.3



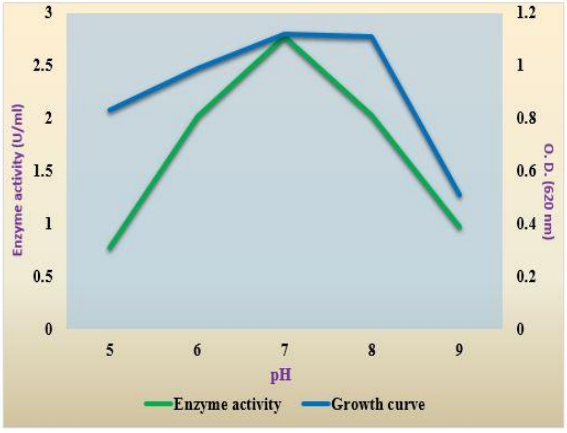
Graph.5(A) Optimization of substrate concentration for B 1.2



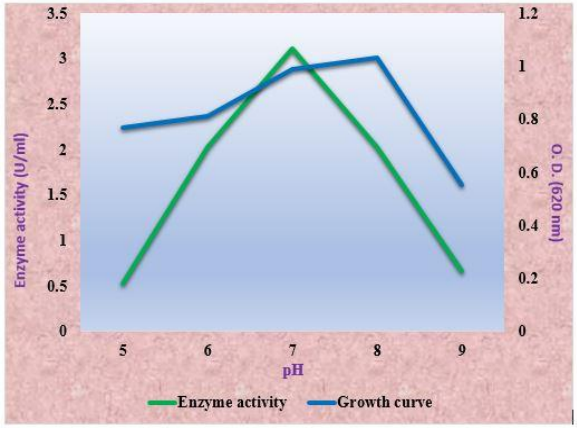
Graph.5(B) Optimization of substrate concentration for B 1.3



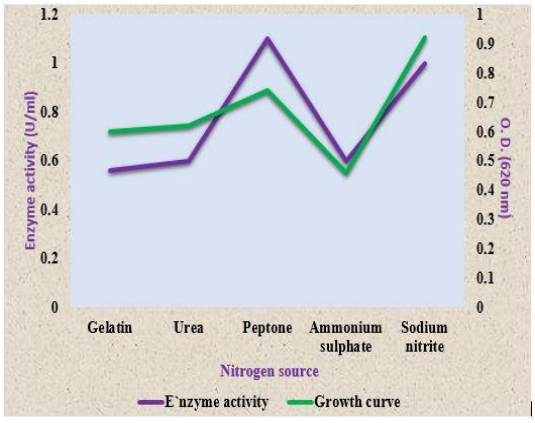
Graph.6(A) Optimization of pH for B 1.2 isolate



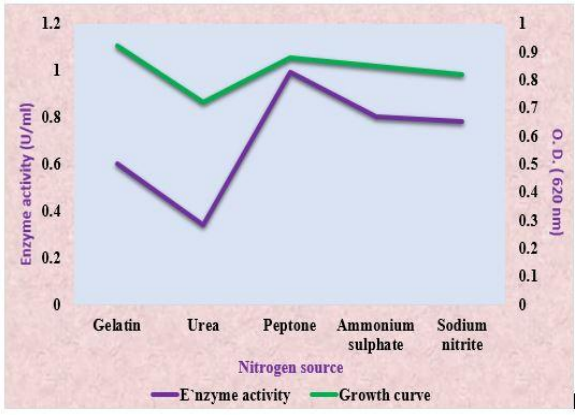
Graph.6(B) Optimization of pH for B 1.3 isolate



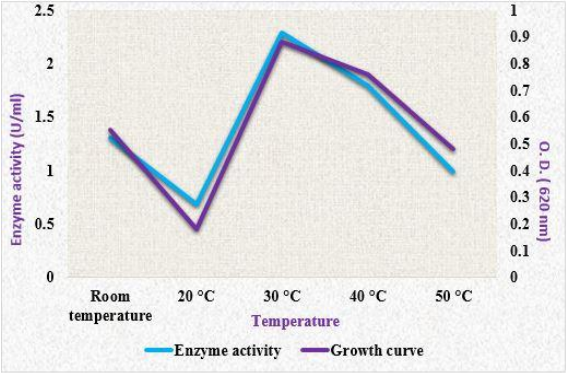
Graph.7(A) Optimization of nitrogen sources for B 1.2 isolate



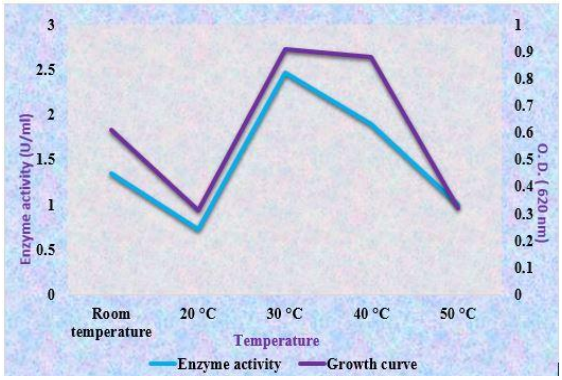
Graph.7(B) Optimization of nitrogen sources for B 1.3 isolate



Graph.8(A) Optimization of temperature for B 1.2 isolate



Graph.8(B) Optimization of temperature for B 1.3 isolate



Effect of Different Physico-chemical Parameters on Growth and Enzyme

Effect of Substrate

Data pertaining to the effect of substrate concentration on growth and enzyme production revealed that optimum substrate concentration for maximal cellulase activity production was found to be at 2 % for B 1.2 and 1 % for B 1.3. Further increase in substrate concentration resulted in decrease in enzyme production. Similar observations were also recorded by (Lugani *et al.*, 2015) for *Bacillus* sp. Y3.

Effect of pH

Effect of pH on the cellulases production was examined at different pH values ranging from 5.0 to 9.0. Experimental data suggested that enzyme activity gradually increased as the pH value increased from acidic to neutral side. Maximum enzyme activity was noted at pH 7.0 for both the isolated. However, maximum growth was recorded at pH 8.0 for both isolates.

An optimum pH is require to maintain three dimensional structure of protein and further increase in pH value may resulted into disruption of ionic bonding of an enzyme and therefore decrease in enzyme activity (Lugani *et al.*, 2015). Several *Bacillus* isolates, including *B. subtilis* and *B. circulans* produced maximum cellulases in the pH range of 7.0-7.5 (Ray *et al.*, 2007).

Effect of Nitrogen Sources

Among four nitrogen sources tested, Isolate B 1.2 preferred peptone followed by sodium nitrate. Whereas, isolate B1.3 preferred peptone followed by ammonium sulphate as nitrogen source for enzyme production in liquid medium. (Sethi *et al.*, 2013) reported ammonium sulphate as the most preferred nitrogen source for cellulase production from different soil bacteria. Nitrogen is essential for cell protein and the nitrogen source incorporation in the medium might directly participate in the protein synthesis process (Mandels, 1975).

Effect of Temperature

Effect of different temperatures on cellulolytic enzyme production was studied by growing both potent isolates at different temperatures. Both isolates preferred 30°C temperature followed by 40°C and very less growth as well as enzyme activity at 20°C and 50°C temperature. Increase in temperature above 40°C may affect extracellular enzyme secretion and/or thermal denaturation of protein. Aboveresults of incubation temperature on enzyme secretion in liquid medium are close to those of (Bakare *et al.*, 2005).

From the investigation and result data it can be inferred that out of total 18 isolates studied, *Enterococcus faecium* (B 1.2 isolate) and *Aeromonas hydrophila* (B 1.3 isolate) were found potent and further, media optimization data could be useful to harvest maximum amount of enzymes from liquid medium. These diverse microbes could be further explored for their applications in various agricultural, industrial, environmental and other biotechnological applications with extensive research and development programs.

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