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HALOPHILIC BACTERIAL STRAIN *BACILLUS CEREUS* IND 2: A BIOLOGICAL TOOL FOR BIODEGRADATION OF PAPER PULP EFFLUENT

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ABSTRACT

The paper industries generate effluent with several types of solid waste, sludge, organic and inorganic substances. These waste composed of various toxic ingredients which affect the physico-chemical characteristics of water bodies, soil texture, microbial population, flora and fauna of the aquatic and land ecosystem. To reduce the pollutants present in paper pulp effluent, the novel halophilic bacterial strains were used. These bacteria are able to tolerate wide range of physico-chemical parameters and produced various types of extracellular enzymes. Therefore, in the present study, the halophilic bacterial strain *Bacillus cereus* IND-2 isolated from sediment samples of hyper saline environment was optimized with physico-chemical parameters and nutritional sources for *x*ylanase production. Maximum xylanase and biomass (2.16 U/ml; 87 mg/ml) were produced by the strain *B. cereus* IND 2 at 35°C temperature, pH 7.5, 1.5 mM NaCl concentration and 48 hours of incubation. Nutritional sources like sugarcane bagasse, gelation and calcium also induced maximum *x*ylanase and biomass production in *B. cereus* IND2. The raw paper pulp effluent was treated with the Xylanase optimized bacterial strain *B. cereus* IND 2 for 5 days. As a result, pH, temperature, BOD, COD, TDS, TH and chlorides content of *B. cereus* IND 2 treated and pre treated paper pulp effluent were recorded and the percentage of biodegradation was determined. The bacterial strain *B. cereus* IND 2 treated paper pulp effluent showed remarkable biodegradation. In addition, FTIR analysis of bacterial strain treated effluent showed transformation of complex chemical components into simple chemical components. Thus, bacterial strain B. cereus IND 2 act as a novel bacterial strain for biodegradation of pollutants in the paper pulp effluent.

Keywords: *Bacillus cereus* IND-2, Paper pulp effluent, FTIR analysis.

1. INTRODUCTION

Bulk of effluent discharged by pharmaceutical, textile, paper mill industries etc. constitute one of the major causes of environmental pollution and significant public health hazard in developing countries [1]. Paper mill effluent composed of chlorinated substances [2, 3] and highly toxic recalcitrant compounds like dibenzo-p-dioxin and diabenzenefuran, that accumulate in the water bodies and causes severe environmental pollution [4]. The paper mills also generate black effluent with very high biological oxygen demand, chemical oxygen demand, toxic substances, recalcitrant organics, turbidity and high temperature [5]. At present, bleaching process for kraft pulp is undertaken by chlorine based chemicals and sodium hydrosulphite, which are highly toxic, colour persistent and also causes many harmful disturbances in biological system [6]. Therefore, biological method superior to physical

method is used for the treatment of paper-pulp effluent in order to mitigate the toxic substances. The microbial communities present in the hypersaline marine sediments play an important role for the mineralizabron of complex organic matter into simple form in the paper mill effluent [7]. Various studies showed that, xylanase can be produced by the bacterial strains through the optimization by physico- chemical parameters and cheap nutritional sources such as agricultural wastes, carbon, nitrogen and metal ions sources [8].

In the present investigation, *B. cereus* IND 2 strain was optimized with physico-chemical parameters and nutritional sources to enhance the xylanase production. The optimized strains were inoculated into the black color paper pulp effluent for biodegradation studies.

2. MATERIAL AND METHODS

2.1. Collection and screening of bacterial strain for xylanase activity

The sediment sample was collected from the crystallizer ponds of solar salt pans situated at Thoothukudy, Tamilnadu. The collected sediment sample was serially diluted as per standard procedure [9]. From these, 1 ml of the sample (Dilution factor 10^3) was inoculated into the halophilic agar medium and incubated for 48 hours at 37°C. The isolated bacterial strain was sub cultured and preserved. The bacterial strain was identified by morphological and biochemical test and 16S rRNA sequence. Further, the isolated colonies with bacterial density 15×10^3 CFU ml^{-1} were screened for xylanase activity by using brich wood xylan agar medium [10].

2.2. Xylanase enzyme assay

The xylanase activity of the bacterial strain *B. cereus* IND 2 was measured by using 1% brich wood xylan as substrate [11]. Xylanase activity was assayed in 3.0ml of a reaction mixture containing 1.0 ml of crude extracellular enzyme source, 1 ml of 1% birch wood xylan (prepared in 0.05 M Na-citrate buffer, pH 5.5) and 1 ml of 0.05 M citrate buffer. The mixture was incubated at 55°C for 10minutes. The reaction was stopped by the addition of 3.0 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 10 minutes [12]. After cooling, the colour developed was read at 540 nm. The amount of reducing sugars liberated was quantified using xylose as standard. One unit of enzyme activity is defined as the amount of enzyme which releases 1 μmol of xylose in 1 minute under assay conditions [13].

2.3. Optimization of bacterial strain with physico-chemical parameters

The bacterial strain *B. cereus* IND2 (Accession no: MZ088148 IND-2) was optimized with various physico-chemical parameters for maximum xylanase production. The effect of pH on xylanase production was studied in pH ranged from 5.5 to 9.5(1 pH interval) under different temperatures ranged from 25°C to 45°C (5°C interval) using brich wood xylan as substrate. By using the same substrate, sodium chloride (NaCl) concentration between 0.5 mM to 2.5 mM (0.5mM interval) with optimum pH 7.5 and temperature 35°C, maximum xylanase production was recorded. Similarly, at 24 hours interval, incubation time ranged from 24 to 120 hours, maximum xylanase production was observed with the optimum pH 7.5, temperature 35°C and 1.5 mM NaCl concentration by using brich wood xylanase as substrate.

2.4. Biomass determination

The bacterial biomass was determined with the reported method [14]. The dry weight of bacterial biomass was determined by filtering 1 ml of cell medium through pre-weighed Whatman filter paper number 44. It was then dried at 80°C for overnight in a hot air oven and reweighed. The difference in weight showed the biomass of bacteria and it was denoted as dry weight per millilitre.

2.5. Paper pulp effluent collection

The samples were collected from paper manufacturing industry located at Cheranmahadevi, Tirunelveli district. Samples were collected in sterilized glass bottles aseptically and transported to the laboratory in an ice pack condition. The collected samples were preserved at 4°C in refrigerator for further analysis.

2.6. Physico-chemical characters of raw and treated paper pulp effluent

The physico-chemical parameters such as pH, temperature, BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), TDS (Total Dissolved Solids), TH (Total Hardness) and chlorides were determined in the raw paper pulp effluent. Simultaneously, 5 ml of xylanase optimized bacterial strain *B. cereus* IND 2 was mixed with 100 ml of raw paper pulp effluent in a conical flask. Then, the set up was kept for 5 days in an incubator at 35°C. After the incubation period, the physico-chemical parameters of

bacterial strain *B. cereus* IND-2 treated paper pulp effluent were determined. The samples were analyzed as per the standard methods [15].

2.7. Percentage of biodegradation

The percentage of biodegradation of paper-pulp effluent was determined by using the following formula.

Dagradation (%) = {(Initial value - Final value)/ Initial value} x 100

Initial value: Physico-chemical parameters of raw paper-pulp effluent (Control). Final value: Physico-chemical parameters of bacterial strain treated paper pulp effluent (Experimental).

2.8. FTIR analysis

Fourier Transform Infrared Spectroscopy (FTIR) analysis was used for determining the changes in surface functional groups of the samples, before and after microbial degradation. For FTIR analysis, raw paper pulp effluent residue and bacterial strain *B. cereus* IND 2 treated paper pulp effluent residue were obtained separately after evaporation of ethyl acetate extract. Each residue was then mixed with pure KBr and the analysis was carried out individually in the mid infrared region of 400-4000 cm^{-1} with 16 scan speed by using 8400S spectrophotometer [16].

3. RESULTS AND DISCUSSION

The growth of paper pulp degrading bacterial strain *B. cereus* IND 2 was carried out in Halophilic Agar medium with pH 7.5 in shake flasks. At 35°C, a marked increasing optical density (OD) at 540 nm revealed that growth reached maximum at 48 hours of incubation for bacterial strain *B. cereus* IND-2 (OD value 1.795,74 mg/ml) (Table 1).

Table 1: Growth of paper pulp degrading bacterial strain *B. cereus* IND 2

Incubation time (hours)	OD value (540 nm)	Biomass (mg/ml)
24	1.69	49
48	1.795	74
72	1.356	32
96	0.404	19
120	0.095	8

The growth of bacterial strain was gradually reduced from 72 hours of incubation and at 120 hours very less growth was recorded. In contrast, the Bacillus sp. Ay-952465 showed maximum growth at 72 hours of incubation [17].

The bacterial strain *B. cereus* IND 2 was optimized with different physico-chemical parameters such as pH, temperature, NaCl and nutritional sources such as carbon, nitrogen and metal ions. The results revealed that at an optimum pH 7.5, 35°C temperature and 1.5 mM NaCl concentration, maximum xylanase enzyme 2.16 U/ml with biomass of 87 mg/ml was produced. But, below or above the optimum pH, temperature and NaCl concentration, the rate of production of xylanase and biomass were gradually reduced.

Table 2: Effect of pH, temperature and NaCl on xylanase Production in bacterial strain *B. cereus* IND 2

pH	Temperatur (°C)	NaCl (mM)	Xylanase production (U/ml)	Biomass (mg/ml)
5.5	25	0.5	0.86	32
6.5	30	1.0	0.97	36
7.5	35	1.5	2.16	87
8.5	40	2.0	1.93	61
9.5	45	2.5	0.18	10

The halophilic nature of *B. cereus* IND-2 showed an evident that it did not grow without NaCl. Maximum growth and xylanase production (2.16 U/ml) was obtained in 1.5 mM NaCl concentration and decreased above and below the concentration (Table 2). In these respects, the bacterial strain resembled the extremely halophilic bacteria *Holorhabdu sutahensis* [18] and in Chromo halobacter sp. TPSV101 [19].

The effect of nutritional sources on xylanase production in the bacterial strain *B. cereus* IND2 is shown in table 3. *B. cereus* IND 2 preferred rice bran, wheat bran and sugar cane bagasse for xylanase and biomass production. The maximum enzyme and biomass production was observed in sugar can bagasse, (3.14 U/ml; 97 mg/ml) followed by rice bran (2.91 U/ml; 91 mg/ml) and wheat bran (2.27 U/ml; 76 mg/ml). Among the substrate, 1% of sugarcane bagasse showed the maximum xylanase production. Similar results have reported for xylanase production by using sugarcane bagasse, rice bran and wheat bran as substrate in

Staphylococcus sp. [20] *Scytalidium thermophilium* [21]. Earlier studies reported that nitrogen sources have been found to stimulate xylanase production in *Bacillus* sp. [22]. Among the nitrogen utilized for xylanase and biomass production, gelatin, supported maximum xylanase and biomass production (4.13 U/ml; 113 mg/ml) followed by beef extract (3.55 U/ml; 106 mg/ml) and peptone (3.26 U/ml; 98 mg/ml) the substrate used for increased xylanase production with purified xylan was uneconomical. Therefore, in the present study, cost effective agricultural residues were used for xylanase and biomass production. The nitrogen sources yeast extract induced xylanase production in *B. mojavensis* AG 137 [23]. In contrast to the present study, highest xylanase production was observed in *B. cereus* IND 2 by using gelatin as substrate.

The metal ions such as calcium (Ca^{2+}), magnesium (Mg^{+}) and zinc (Zn^{2+}) were involved in enzyme and biomass production in *B. cereus* IND-2. Among the ions tested for xylanase and biomass production, calcium enhanced maximum enzyme and biomass production (2.37 U/ml; 81 mg/ml) followed by magnesium (2.09 U/ml; 69 mg/ml) and zinc (0.50 U/ml; 6 mg/ml). The metal ions such as calcium and magnesium increased the xylanase and biomass production and very negligible loss of enzyme production was observed in metal ion zinc. The metals like calcium enhanced the xylanase production in *Aspergillus sydowii* SBS -45 was in accordance with the present study in *B. cereus* IND 2 [24].

The physico-chemical parameters of the pre treated and bacterial strain *B. cereus* IND2 treated paper pulp effluent were recorded on the 5th day of incubation. The results revealed that the pH of the treated effluent was reduced from 8.28 to 7.58 with 8.45 % of biodegradation, temperature was reduced from 34°C to 32°C with 5.88 % of biodegradation. BOD was reduced from 309 mg/ml to 168 mg/l with 45.63 % of biodegradation, COD was reduced from 855mg/l to 542 mg/l with 36.60 % of biodegradation. TDS was reduced from 567 mg/ml to 291 mg/ml with 47.61 % of biodegradation, TH was reduced from 59 mg/ml to 59 mg/ml to 35 mg/ml with 40.67 % of biodegradation and chloride was reduced from 43 mg/ml to 27 mg/ml with 37.20 % of biodegradation (Table 4).

Table 3: Effect of Nutritional sources on xylanase production in bacterial strain *B. cereus* IND 2

S. No	Nutritional Sources (1%)	Xylanase production (U/ml)	Biomass (mg/ml)
	Carbon sources		
I	a. Rice bran	2.91	91
	b. Wheat bran	2.27	76
	C. Sugar cane bagasse	3.14	97
	Nitrogen sources		
II	a. Beef extract	3.55	106
	b. Peptone	3.26	98
	c. Gelatin	4.13	113
	Metal ions (1 mM)		
III	a. Ca+	2.37	81
	b. Mg+	2.09	69
	c. Zn	0.50	6

Table 4: Physico-chemical parameters of pretreated and treated effluent

Parameters	Pre treated effluent	Treated effluent	Biodegradation (%)
pH	8.28	7.58	8.45
Temperature (°C)	34	32	5.88
BOD (mg/l)	309	168	45.63
COD (mg/l)	855	542	36.608
TDS (mg/l)	567	291	47.61
TH (mg/l)	59	35	40.67
Chloride (mg/l)	43	27	37.20

The bacterial strain *B. cereus* IND-2 was involved in degradation of paper pulp effluent at 5 days of incubation. These optimized strains were used for the treatment of paper pulp effluent at an optimized condition for degradation. The optimized bacterial strain *B. cereus* IND-2 and decreased the level of pH at the end of 5 days incubation. Similar observations during the microbial degradation of paper pulp effluent were reported in *Bacillus* sp. [25, 26]. The results of present study showed that the reduction of BOD and COD were observed when the effluent was treated with *B. cereus* IND-2 the reduction of these parameters due to the degradation of chlorinated organic compounds and lignin present in the effluent [4, 27]. The bacterial strain *Pseudomonas aeruginosa* DSMZ 0.3504 has been successfully reduced TDS of paper pulp effluent was resembled with the study [28]. In the present study, TH was decreased due to the influence of bacterial strain *B. cereus* IND-2. The total hardness of the effluent may be due to the presence of heavy metals. Similar result reported by scientists [29] added support to the present findings. The chloride content of the paper pulp effluent was reduced by the presence of the optimized bacterial strain *B. cereus* IND-2.

The FTIR spectrum of the raw paper pulp effluent displayed a medium peak appeared at 3438.5 cm^{-1} for C≡C-H stretching vibration group of alkynes compound. The C-H bending group of alkane's compound formed as a strong peak at 1429.0 cm^{-1}. The alcohol and phenol compound of C-O stretching vibration group appeared as strong peak showed at 1118.5 cm^{-1}. A strong peak visualized at 875.5 cm^{-1}-C-H- out of plane bend group of olefin compound. The strong peaks appeared 636.4 cm^{-1} and 617.1cm^{-1} both were belongs to ≡C-H bending group of alkynes compounds. A strong peak appeared at 470.5 cm^{-1} 470.5 cm^{-1} C=O stretching vibration group of chungamide (Table 5 and fig. 1).

Table 5: FTIR analysis of raw paper pulp effluent

Peak (cm^{-1})	Appearance	Group	Compound class
3438.5	Medium	C≡C-H stretching vibration	Alkynes
1429.0	Strong	C-H bending	Alkanes
1118.5	Strong	C-O stretching vibration	Alcohol, Phenol
875.5	Strong	=C-H out of plane bend	Olefin
636.4	Strong	≡C-H bending	Alkynes
617.1	Strong	≡C-H bending	Alkynes
470.5	Strong	C=O stretching vibration	Chung-amide

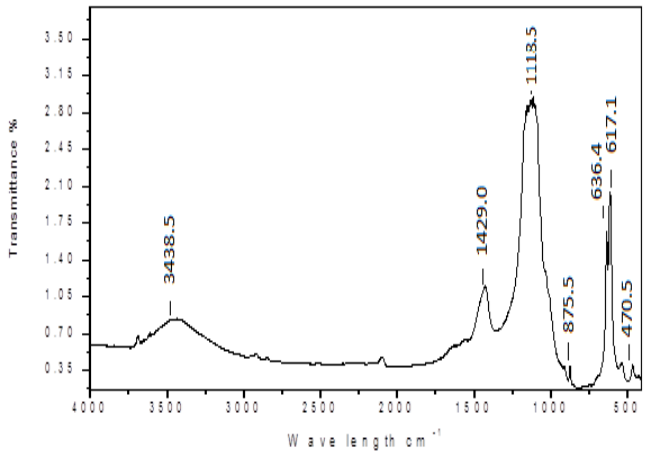

Fig.1: FTIR analysis of raw paper pulp effluent

The FTIR spectrum of the paper pulp effluent treated with xylanase optimized bacterial strain *B. cereus* IND-2 displayed a medium peak appeared at 3426.9 cm^{-1} for C ≡ C-H stretching vibration group of alkynes compound. The C-H bending group of alkanes compound formed as a strong peak at 1423.2 cm^{-1}. The ether compound of C-O-C (Saturation) the asymmetrical stretching vibration group appeared as strong and sharp peak showed at 1151.3 cm^{-1}. The strong peaks appeared at 1128.2 cm^{-1} and 1108.9 cm^{-1} both were belongs to α-side chains on carbon compounds. The alkaynes compound of ≡ C-H bending group showed as strong peak at 617.1cm^{-1} (Table 6, fig.2).

The results of FTIR analysis of bacterial strain *B. cereus* IND-2 treated paper pulp effluent showed the reports of Xu QH et al. [30]. There were differences between the intensities before and after bacterial strain treatment and these changes could be due to degradation of components in the raw paper pulp effluent [31]. The obtained changes in the bacterial strain *B. cereus* IND-2 treated effluent were similar to previous observation and pulp modifications by using xylanase optimized strain *Trichodermobiride* VKF3 [32].

Table 6: FTIR analysis of bacterial strain *B.cereus*IND-2 treated paper pulp effluent

Peak (cm^{-1})	Appearance	Group	Compound class
3426.9	Medium	C≡C-H Stretching vibration	Alkynes
1423.2	Strong	C-H bending	Alkynes
1151.3	Strong and sharp	C-O-C (Saturation) Asymmetrical stretching vibration	Ether
1128.2	Strong	α-Side chains on carbon	Ether
1108.9	Strong	α-Side chains on carbon	Ether
617.1	Strong	≡C-H bending	Alkynes

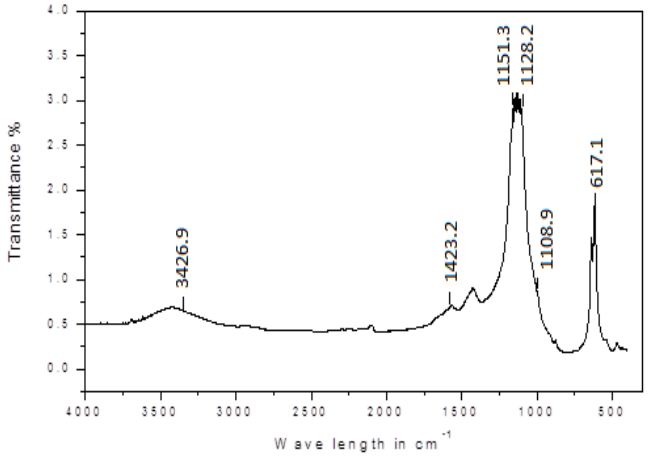

Fig. 2: FTIR analysis of *B. cereus* IND-2 treated effluent

4. CONCLUSION

A salt tolerant extra celluar alkaline xylanase produced by *B. cereus* IND2 was isolated from hyper saline environment from solar saltpan. The effect of physiochemical factors such as pH, temperature, NaCl and incubation time was studied effectively for a maximum alkaline xylanase production at pH 7.5, 35°C, 1.5mM NaCl concentration and 48 hours of incubation. The biodegradation study was performed by the treatment of raw paper pulp effluent with the xylanase optimized bacterial strain *B. cereus* IND2 and the results showed the reduction of contaminants. The FTIR analysis of raw paper pulp effluent and bacterial strain treated effluent further revealed the degradation of contaminants in the paper pulp effluent. The results obtained in the study illustrated the viability of using halo tolerant bacterial strain for the biodegradation of paper pulp effluent.

Conflict of interest

None declared

Source of funding

None declared

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