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ISOLATION AND CHARACTERIZATION OF KOCURIA ROSEA FROM PAPER INDUSTRY WASTE AND PRODUCTION OF CELLULASE ENZYME

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ABSTRACT Cellulose is polymer; main constituent in cellulosic waste contributes to environmental waste. Cellulose degrading bacteria (CDB) are organisms which selectively secretes cellulase enzyme which degrade cellulosic, lingocellulosic waste and cellulose fibers. Bioremediation principles of using microorganisms in degrading waste were used for paper industry waste to isolate CDB. Carboxy methyl cellulose agar (CMCA) was used for isolating CDB and qualitative analysis with 1% Congo red and 1M NaCl was done. Identification of Kocuria rosea was done morphologically by using Gram staining and biochemically by VITEK 2 system screening unit. Fermentation media with glucose as carbon source and submerged fermentation technique was used to extract crude cellulase enzyme. Crude cellulase activity was determined by using DNSA and estimated by Miller's method and found to be very efficient. Different parameters like effect of pH, salt, temperature on cellulase production were also studied.

KEYWORDS : Cellulose Degrading Bacteria, Cellulase, Kocuria Rosea, Millers Method.

INTRODUCTION

On earth cellulose is most a bundant and renewable natural polymer and the generate from paper and pulp industry, textile industry, agriculture **(Sukumaran, 2005).** Cellulase is enzyme synthesizes by numerous microorganisms, commonly by bacteria and fungi that breakdown cellulose molecule into monosaccharides such as beta-glucose or shorter polysaccharides and oligosaccharides **(Barkalow, 2000 and Immanuel, 2006).** In growth rate comparison bacteria has high growth rate than fungi and it has good potential to use in cellulase production but in cellulase production bacterial application extensively not used. A few bacterial species like Cellulomonas, Cellovibrio, Pseudomonas sp. etc. cellulolytic property was reported. **(Nakamura, 1982).** Cellulose is polysaccharide connected by β-1, 4 glycosidic linkages so that it is most abundant carbohydrate in nature **(Saha, 2006).** Therefore, it has tendency to tremendous commercial interest to developed effective treatment process for utilization of cellulosic waste as cost-effective carbon sources **(Nishida, 2007).** Cellulase enzyme used in food processing industries for extraction, clarification and stabilization fruit and vegetable juices. Cellulase enzyme has various applications in textile, detergent, dye, paper and pulp industries **(Karmakar, 2011, Parshetti, 2006).** Bioremediation principles make use of living organisms to degrade environmental waste. Here, organism isolated from paper refines and mills waste was use to degrade cellulosic waste which mainly composed of polymeric units of cellulose. Thus there is need to have CDB which follow bioremediation principles by secreting cellulose enzyme and will selectively degrade CDB'S **(Jeeva, 2017 and Verma, 2012).** The isolated CDB on CMCA were then used for qualitative screening usng 1% Congo red and 1M NaCl to show clear zone colonies **(Kaur, 2012. Shaikh, 2013 and Shanmugpriya, 2012).** Upon biochemical and morphological screening the organism was identified as *Kocuria rosea* **(Ligozzi, 2002)**. Crude cellulose enzyme extract were prepared by using submerged fermentation techniques and then it is used as a sample for assaying enzyme activity **(Sethi, 2013).** Carboxy Methyl Cellulase (CMC) was used as a substrate and using Miller's method determination of enzyme activity was done and results are represented in respective units. International Unit (IU) can be used to measure enzyme activity **(Shuanggui, 2011., Miller, 1959 and Wood, 1988).** Standard glucose estimation was done by using DNSA method. Also the effect of parameters like pH, salt concentration and temperature CDB and cellulose enzyme activity were studied **(Muhammad Irfan, 2012 and Dumorne, 2017).** The given study was targeted to isolate *Kocuria rosea,* a CDB isolate from paper industry waste in degrading cellulosic waste in-situ at application site by optimizing surrounding environmental conditions.

MATERIALS AND METHODS

I] Sampling

The paper industry waste was collected as a sample for isolating cellulose degrading bacteria. It was collected from paper refines and mill industries, MIDC phase-1, Dombivli (E) in sample collecting bags and stored at room temperature **(Shaikh, 2013).** It was collected on during December 2018 and stored at room temperature and use for further processing and screening.

II] Isolation and screening of bacteria

Cellulase producing bacteria was isolated from paper industry waste by isolation technique using Carboxy methyl cellulose (CMC) agar media. It provides proper nutrients for growth of cellulose degrading organism. The plates were incubated at 37º C for 72 hours **(Verma, 2012 and Jeeva Raj, 2017)**. To visualize the hydrolysis zone, plates were flooded with an aqueous solution of 1% Congo red for 15 minutes and washed with 1M NaCl **(Manmeet Kaur, 2012).** Then the CMC agar plate was selected, which shows clear zone around colony. CDB was selected for further use that degrades cellulose on CMC plate and showed larger clear zone **(Shaikh, 2013).**

III] Morphological and biochemical identification

The morphological identification was done by Gram staining. The bacterial isolate was identified by VITEK 2 system. The VITEK 2 system was originated in 1970's as an automated biochemical identification system, which performs the entire step required for bacterial identification **(Ligozzi, 2002)**.

IV] Production of cellulase enzyme

In production of cellulase enzyme the fermentation media contained components in g/L. These include Glucose 0.5 gm, Peptone 0.75 gm, $FeSo_4$ 0.01 gm, $KH_2.PO_4$ 0.5 gm, $MgSo_4$ 0.5 gm. Ten milliliters of medium were taken in 100 ml sterile conical flask. Bacterial culture was inoculated in sterile fermentation media. The inoculated medium was incubated at 37 ° C for 7 days. At the end of the fermentation period, the culture medium was centrifuged at 2000 rpm for 25 minutes to obtain the crude cellulose extract, which used as enzyme source **(Sethi, 2013).**

V] Enzyme Assay

Cellulase activity was measured by miller method. Dinitrosalisic acid (DNS) reagent was used to assay cellulase enzyme activity by estimation of reducing sugars released from CMC. In this reaction mixtures was composed of crude cellulase enzyme solution(1.0 ml) plus 0.5% carboxymethyl cellulose (1.0 ml) and 50 mM sodium phosphate buffer (pH 7) (1.0 ml) respectively. These reaction mixtures were incubated at 37 ° C for 30 minutes. The reaction was terminated by adding 3 ml DNSA reagent. The colour was then developed by boiling the mixture for 5 minutes. The absorbance of sample was measured at 530 nm against a blank containing all reagents but without the crude enzyme **((Miller, 1959 and Sethi, 2013).** Glucose calibration curve was used to estimate cellulase production. One International

Unit (IU) of enzyme is the quantity of enzyme, which is required to convert 1 µmol of glucose per minute under standard assay conditions **(Muhammad, 2012)**

VI] Ability of microorganism to grow in different pH, salt concentration and temperature conditions

In determination of ability of organism to grow in different pH and salt concentration, different CMC broth with different pH range 3-9 pH was used. Te temperature range selected was 5°C- 55°C and salt concentrations used were in the range from 5- 30 mg/ml. the respective solutions were prepared and autoclaved at 121°C for 15 minutes. Then loop full of culture was added in each sterile CMC broth test tubes with different pH, temperature and salt concentration. Then all tubes were incubated at respective temperature for 72 hours. And growth was observed and measured colorimetrically at 530nm **(Dumorne, 2017)**.

RESULTS

I] Sampling: Three samples were collected from 3 locations of paper mills and refineries of Dombivli MIDC area, further the best one was selected for further work after isolation.

II] Isolation of cellulose degrading bacteria

Isolation of CDB from paper industry sample was done using CMCA plate of all three isolates. Qualitative analysis shows positive growth with clear zone around colony respectively indicating presence of cellulose degraders. The colony observed was of size 1 mm, circular, white, smooth, elevated and opaque in characteristics (**Figure 1).**

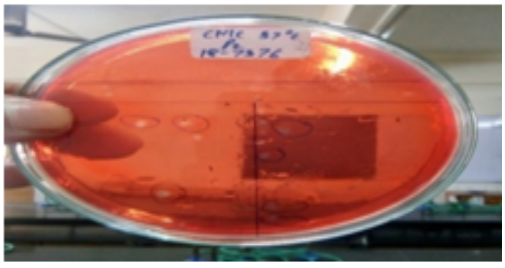

Figure 1: Clear zone around cellulose degrading bacterial colony.

III] Morphological and Biochemical Identification of CBD

Morphological identification was carried out using Gram staining, the isolates was Gram positive cocci and further identification was done by biochemical VITEK 2 system. The identified organism was *Kocuria rosea.* The results of important biochemical test are as listed in Table 1.

IV] Extraction and characterization of cellulase enzyme

Extraction and characterization of cellulase enzyme was carried out for the isolate by submerged fermentation technique. Growth was seen in the form of turbidity in the selective media CMC fermentation broth and upon centrifugation supernatant was separated from pellet which serves as a source of crude cellulase enzyme extract **(Figure 2).**

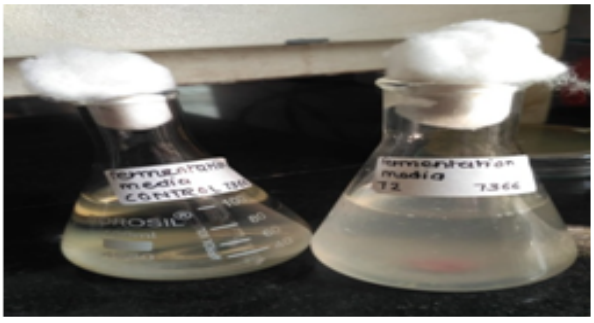

Figure 2: Turbidity in fermentation media, source of crude cellulase enzyme extract.

V] Cellulase enzyme activity

Estimation of cellulase enzyme activity isolate was done by using DNSA and Miller's method. Glucose reduced from CMC by *Kocuria rosea* isolate was found to be 20.5 mg/ml. Enzyme activity of produce crude cellulase enzyme from *Kocuria rosea* was 3.79 µmol /ml/ min **(Figure 3).** The formula used was Enzyme activity = Glucose liberated µg/ml / Molecular weight of Glucose x Incubation time X Dilution factor

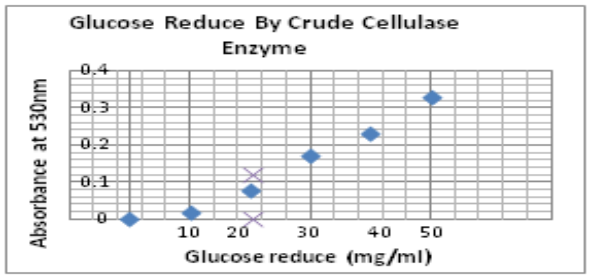

Figure 3.Glucose reduced by crude cellulase enzyme extract from paper industry sample.

VI] Effect of different parameters like pH, salt concentration and temperature on growth of bacteria

Effect of different parameters like pH, temperature and salt concentration were studied on growth of *Kocuria rosea.* The isolate *Kocuria rosea* was able to grow within pH range 5.0 - 7.0, maximum Salt tolerance is to 20 mg/ml and Temperature range 28°C - 37°C (**Dumorne, 2017**).

DISCUSSION

The aim of present work was to isolate cellulose degrading bacteria and produce cellulase enzyme. Isolated CDB isolate from paper industry waste was found to be *Kocuria rosea* and it was able to produce cellulase enzyme. It synthesize cellulase enzyme to breakdown cellulose to glucose and glucose produced was used as nutrient source for their growth. It was also able to grow in 20 mg/ml salt concentration, pH range 5.0 - 7.0 and temperature range 28°C - 37°C. Similar results were obtained by **Dumorne, 2017**. Enzyme activity of produced crude cellulase enzyme was 3.79 µmol /ml/ min or 3.79 IU. The produced cellulase enzyme from *Kocuria rosea* did not change their activity at salt concentration 20 mg/ml and 5.0 pH condition**.** So that *Kocuria rosea* can be used in production of cellulase enzyme which can be further used in industries such textile, detergents, pulp and paper industries, improving digestibility of animal feeds, in food industry and production of bio-ethanol **(Karmakar, 2011)**.

The world wide consumption of paper and paper products is increasing at a very high rate, the global production in pulp, paper and publishing sector is expected to increase by 77% from 1995 to 2020 **(OECD, 2001**) and also *Kocuria rosea* has ability to biodegrade Malachite green, Azo dye, Triphenylmethane as well as other industrial dye **(Parshetti, 2006).**

CONCLUSION

In the review it can be concluded that the isolated *Kocuria rosea* has effective ability to degrade cellulose from paper waste industry and having simple growth and nutritional requirements. Thus the isolated CDB can be used in bioremediation of paper industry, textile industry waste because it is eco-friendly and cost effective process than other processes. Future scope of given study include use of *Kocuria rosea* isolate or its consortium with other organisms in "In-Situ" bioremediation studies at large scale.

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Table 1. Biochemical details for Kocuria rosea isolate.

Sr. no	Biochemical Details	Test	Sr. no	Biochemical Details	Test	Sr. no	Biochemical Details	Test
1	Leucine ARYLAMIDASE	+	6	LACTOSE	-	11	D-SORBITOL	-
2	Alanine ARYLAMIDASE	+	7	D-MALTOSE	-	12	SUCROSE	-
3	ALPHA-GALACTOSIDASE	+	8	D-MANNOSE	-	13	POLYMIXIN B RESISTANCE	-
4	ALPHA-GLUCOSIDASE	+	9	D-MANNTOL	-	14	BACITRACIN RESISTANCE	-
5	UREASE	-	10	D-AMYGDALIN	-	15	NOVOBIOCIN RESISTANCE	-

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