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To study the isolation of enzyme mannanase for reducing chemical use in paper and pulp industry

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Abstract
The present work was carried out to isolate industrially important enzyme producing strain. The soil samples from different areas *viz.* Rhizospheric, Nonrhizospheric and Landfill areas were collected, appropriate dilution of different soil samples were plated & CFU was calculated. The microbial diversity was also checked & mix culture gram (+)ve & gram (-ve) was observed. These isolates were screen for the presence of various enzymes mannanase, xylanase, laccase, protease & amylase, of the various isolates mannanase producing strains was selected for further study. The sample were plated on a medium with LBG to isolate mannanase producing colony of the RN 11 isolates, strain showing maximum zone of clearance was further selected. Selected strain was subjected to morphological & biochemical characterization which showed the *Bacillus species*. On the flask level mannanase activity was checked & after 4 hrs enzyme activity 25 IU was observed. The medium was characterized in terms of pH & temperature. Thereafter the strain was used for the bio bleaching of kraft pulp & it reduced the kappa number by 14.86%.

Keywords: Enzyme, mannanase, kappa, RN 113

Introduction
Enzymes are the known catalytic agents of metabolism. Enzymes have become important tools in biotechnology industry. Enzymes can be produced from various sources like plants, animals and micro organisms. Microbial enzymes are preferred because of their easy and economical production and wide range of properties and applications

The Mannan endo-1,4-β-mannosidase or 1,4-β-D- mannanase, commonly named β-mannanase, is an enzyme responsible for the transformation of heteromannans to manno-oligosaccharides and small amount of mannose, glucose and galactose. Mannanases are involved in catalyzing β-1, 4-mannosidic linkages in the main chain of β-1,4-mannans, glucomannans and galactomannans (Gubitz *et al*., 2001; Sanchez, 2009) [1, 2]. Mannanases are produced by variety of organisms such as bacteria, fungi, higher plants and animals (Dekker and Richard, 1976) [3].

Interest in mannan degrading enzyme systems from different sources has increased in the past decade, especially because of their biotechnological applications (Singh *et al.*, 2003) [4]. Mannanase has been effectively used in pulp bleaching (Lahtinen *et al.*, 1995; Cuevas *et al.*, 1996) [5, 6], in clarification of fruit juices (Christgua *et al.*, 1994) [7], in manufacturing of instant coffee, chocolate and cocoa liquor (Francoise *et al.*, 1996) [8]. In addition, mannanase are potentially applied in the pharmaceutical industry for the production of physiologically active oligosaccharides (Lin and Chen, 2004) [9].

Though mannanases are widely distributed in micro-organisms, higher plants, as well as animals, much attention has currently been focused on the microbial mannanases for their industrial applications. Bacterial mannanases have been reported from various strains in 20 different genera and purified from *Bacillus circulans* (Yoshida *et al.*, 1997) [10], *B. stearothermophilus* (Talbot and Sygusch, 1990) [11], *B.subtilis* (Jiang *et al.*, 2006) [12], *Cellulomonas fimi* (Stoll *et al.*, 2000) [13], *Caldibacillus cellulovorans* (Sunna *et al.*, 1999) [14], *C.tertium* (Kataoka *et al.*, 1998) [15], *Dictyoglomus thermophilum* (Gibbs *et al.*, 1999) [16] and several strains of *Bacillus sp.* (Ma *et al.*, 2004) [17].

For the industrial application mannanases which are active and stable at high temperature and pH are more useful. Although a number of organisms producing mannanases have been reported but still the search is going on for the isolation of microorganisms which can give high yields of alkaline thermo stable mannanase.

Materials and Methods

The present investigation was studied Chandigarh University, Department of Biopharma Sciences, Gharauan. In this research, Borosil and Schott Duran glassware was used throughout the study.These were washed twice with the detergent, rinsed with distilled water, dried in hot air oven at 70 °C and then used. All the chemicals and reagents used in the study were of high purity obtained from Hi-Media, E-Merck, Qualigens and SRL. Locust bean gum (LBG), guaiacol, xylan was purchased from Sigma. Different media for screening of organism for production of different enzymes that is Casein (for protease), CMC (for cellulase), Starch (for amylase), xylan (for xylanase) and LBG (for mannanase) were added at a concentration of 0.5% w/v to nutrient agar before autoclave. For laccase, 2 mM guaiacol was added after autoclave.

Collection of Soil samples

Following samples were collected:

Rhizospheric Sample: A small plant was taken off from soil layer. The soil which was sticking immediately next to roots was taken off and used as a rhizospheric soil.

Non-Rhizospheric Soil: Soil sample was taken where no plant growth was present.

Landfill Soil (depth 6 inches): Soil sample was taken from landfill area in Punjab and Chandigarh.

Wet soil samples were collected in plastic bags. The samples were taken to the lab and processed immediately. Appropriate dilution of the samples were plated on nutrient agar plate in duplicate. All the plates were incubated at 37 ^{O}C for 48 hrs.

After 24 h the plates were analyzed in terms of:

a) Number of colony forming unit (CFU).
b) Colony morphology.
c) Gram staining.

Observation

Bacteria take up primary stain is Gram positive bacteria
Bacteria take up counter stain is Gram negative bacteria

Screenig of Organisms for Enzymes

Twenty Colonies of different characteristics were picked from the plates and they were replicated on nutrient agar + Xylan, nutrient agar + casein, nutrient agar + starch, nutrient agar + locust bean gum, nutrient agar + cmc and Nurient agar + guaiacol. One copy was kept of nutrient agar also to be used as master plate. Then plates were incubated at 37 ˚C for 24 hours. The enzyme production (Cellulase, amylase, protease, xylanase, mannanase, laccase) was checked by staining the plates with different reagents.

Identification of Isolated microorganism: With the help of Gram staining method given by Hans Christian Gram in 1882. Biochemical Characterization by Catalase test, Oxidase test, Indole test, citrate test Urease test, Methyl red Test and Voges proskauer test.

From all the mentioned enzymes mannanase is selected for further process.

Mannanase production in liquid medium

20 ml broth of MM pH 8.0 was taken in 100 ml flask. It was inoculated with 1% inoculum of log phase grown cells of isolate no 11 and incubated in a shaker (150 rpm) at 37 ˚C for 72 hrs. The culture was centrifuged at 10000 rpm for 10 min. Mannanase activity was assayed in cell free supernatant.

Assay of mannanse enzyme

Mannanase activity was measured with DNSA (Miller, 1959) [18] by measuring the amount of reducing sugars released.

Enzyme Unit

One unit of enzyme activity is defined as micromoles of reducing sugars (mannose) released per min per ml of crude enzyme used under standard assay conditions.

Formula

$$\frac{X*D.F}{M.M*T*V}$$

X = microgram of mannose/ml which is calculated from standard curve using O.D value.
M.W = molecular weight
D.F = dilution factor
T = incubation time
V = volume of enzyme used

Characterization of mannanase

The mannanase of the isolate no. RN11 was produced under optimum conditions. This enzyme was used to study the effect of various parameters on enzyme activity.

Temperature optima

The optimal temperature for enzyme activity was determined by incubating the assay mixture in the temperature range of 40-85 °C for 5 min.

pH optima

The optimal pH of the enzyme was determined by preparing the substrate (0.5% LBG) as well as suitable enzyme dilutions in the buffers of different pH values i.e. pH 7.2, pH 8, pH 8.8 and pH 9.2 and performing the assay under standard conditions.

Application in Reduction of Kappa number

Pulp sample

Unbleached kraft pulp was used for the present study. Kraft pulp was kindly provided by Ballarpur Industries Limited, Yamunanagar.

Pretreatment of pulp

Pulp was initially pretreated with distilled water and filtered. The process was repeated until clear water elutes from the pulp and the pH of the pulp turns neutral. This pulp was then dried in oven at 55 °C overnight. This oven dried pulp was then treated with mannanase enzyme. During the treatment different parameters which are effecting the enzyme action on pulp were optimized. This included enzyme dose, optimum time of action of enzyme, temperature optimum and pH optimum.

Treatment of pulp with enzyme
For the enzyme treatment of the pulp 1g of pulp was used and consistency was set at 5% i.e total reaction volume was taken to be 20 ml.

C= (w/v) *100

where; C- consistency
w - weight of dried pulp,
v - volume of the reaction mixture
As c - 5% and w - 1g, the volume of reaction mixture (v) came out to be 20 ml.

1g of pulp was put in the polybag. Calculated volume of enzyme was taken in a test tube and total volume was made to 20 ml of the buffer of required pH value. This diluted enzyme was then added in the poly-bag with the pulp. This reaction mixture was incubated at set temperature for different time interval. The sample was taken out of the polybag and filtered by using muslin cloth. Suitable enzyme and pulp control was prepared by adding only enzyme and buffer in one and only pulp along with buffer in the other and they were processed similar to the test.

Analytical method for determining kappa number.
Kappa number
Kappa number is the volume (in ml) of 0.1 N potassium permanganate solution consumed by one gram of moisture free pulp under the conditions specified in the method (Tappi method T236).

Results and Discussion
Enumeration of number of microorganisms in diifferent soil samples

Colony forming unit (CFU) count for different soil samples.
A) Rhizospheric soil
B) Non-rhizospheric soil C) Landfill soil

CFU in rhizospheric surface soil detected was 18 x 10^6 = 1.8 x10^7cfu/g.
CFU in rhizospheric soil 6 inches depth detected by was 7.7 x10^5 cfug^{-1}
CFU in landfill area sample detected was 36.7 x10^6ml^{-1} = 3.6x10^7 ml^{-1}.

It is well established that the number of microorganisms in rhizosphere is higher than nonrhizospheric soil. (Brimecombe *et al.*, 2001) [19]. This is because of the reason that rhizospheric microflora has continous access to a flow of low and high molecular weight organic substrate derived from roots. This flow, together with specific physical, chemical and biological factors, can markedly affect microbial activity and community structure of the rhizosphere soil (Brimecombe *et al.*, 2001).

Colony morphology and Gram staining characterstic of different microorganisms obtained from different soils.
Twenty colonies were selected showing different morphology from different samples and Gram staining was done. Some of them were Gram positive and some were Gram negative. Colony from Non rhizospheric soil was selected which is morphologically punctiform and Gram Positive Bacilli.

Screening of the organisms for production of different enzymes
The type and kind of enzyme particular microorganism secrete specific property of the microorganism. Environmental and nutritional factors influence the type of enzymes secreted by particular microorganism. In this regard the isolated microorganisms from different soils were screened for the presence of different enzymes including Laccase, mannanase, xylanase, cellulase, amylase, and protease. From twenty different colonies gram positive colony isolated from non rhizospheric soil which produce mannanase was selected.

Mannanase

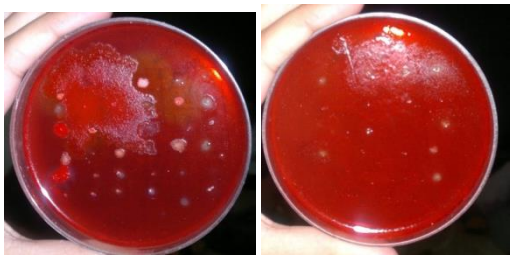


Plate 1: Showing zone of clearance on LBG plate stained with Congo red

Laccase, mannanase, xylanase, cellulase, protease and amylase are indutrially important enzyme. They have find uses in pulp and paper industry, detergent industry detoxification of xenobiotics, leather industry, biofuel production etc.The estimated value of world market is presently about 2.7 $ and is estimated to increase by 4% annually 2016. Detergents (37%), textile(12%), starch (11%), baking (8%) and animal feed (6%) are the main indutries, which use about 75% of industrially produced enzymes, the use of thermoalkali stable enzyne are gaining wide indutrial and biotechnological interest that these enzymes are better suited for harsh industrial processes.
Mannanase is one of the important enzyme useful in pulp and paper industry. In the enzymatic treatment for pulp bleaching, β- mannanase and its accessory enzymes are able to cleave the mannan portion in pulps selectively without affecting cellulose (Filho, 1998) [20]. The extraction of lignin from wood fibers is an essential step in bleaching of dissolving pulps. Pulp pretreatment under alkaline conditions hydrolyzes hemicelluloses covalently bound to lignin and thus facilitates subsequent removal of lignin. There is a drawback to alkaline treatment of wood pulps. However, in that it creates an environmental pollution problem due to release of chlorinated compound (Hongpattarakere, 2002) [21]. The pretreatment of softwood kraft pulp with β-mannanase (Man5D) of *P. chrysosporium* indicated that Man5D exhibited a positive effect as a prebleaching agent with a reduced loss of fiber yield (Benech *et al.*, 2007) [22].
Mannanases having substrate specificities for galactomannan constituents would make excellent candidates for use in enzymatic bleaching of softwood pulps (Gubitz *et al.*, 1997) [23].
Mannanase is useful in chlorine-free bleaching processes for paper pulp (chemical pulps, semichemical pulps, mechanical pulps or kraft pulps) in order to increase the brightness, thus decreasing or eliminating the need for hydrogen peroxide in the bleaching process (Tenkanen *et al.*, 1997) [24]. Isolate number 11 was choosen based on its high zone of clearance.

Identification of Selected Isolate

Selected isolate was designated as isolate RN.11and was identified based on morphological microscopic and biochemical characterstics. The results obtained were shown in table 1.

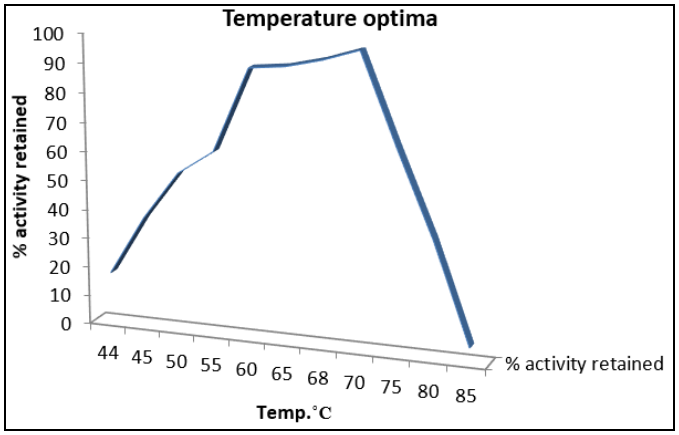
Table 1: Morphological, physiological and biochemical characteristics of *Bacillus sp.* RN11

Character	Results
Morphology	
Motility	Cells are rod shaped, gram positive and motile, spore central and oval motile
Microscopic Analysis	
Gram Reaction	+
Cell Shape	Rods
Spores	+
Position	Central
Shape	Oval
Growth parameters	
Growth temp	37°C
Growth pH	7.0
Biochemical Analysis	
Oxidase	-
Catalase	+
MR reaction	-
VP reaction	-
Citrate	-
Nitrate	+
Indole	-
Arginine	-
Ornithine	-
Lysine	-
Sucrose	+
Xylose	-
Glucose	+
Maltose	+
Mannitol	+
HL	-
PPA	-
Urease	+
TSI	K/A
Coagulase	-
Xyalanase	-
Mannanase	+
Lipase	+
Amylase	+
Protease	+

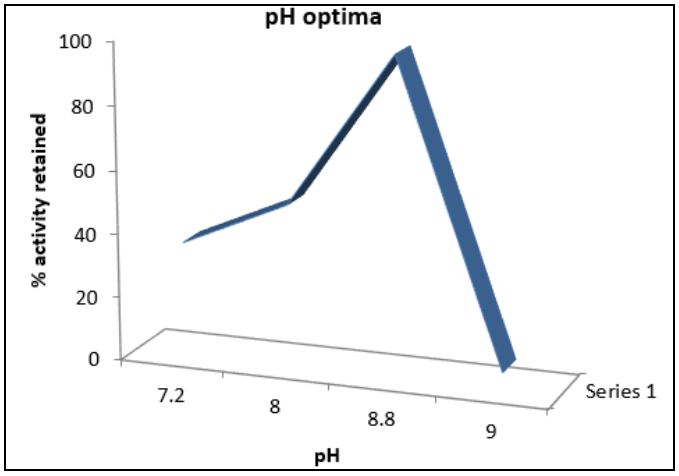
Based on the morphological and biochemical analysis the organism was idetified as *Bacillus actenomycetes* from Bacillus famiy. Baccilus are known producers of mannanase. Many species of *Bacillus* including *Bacilli sp., Cellvibrio japonicas, Pseudomonas fluorescens and Rhodothermus marinus, Bacillus circulans, Bacillus subtilus* (Chauhan *et al.*, 2012) [25].

Characterization of mannanase from isolate 11

The mannanase produced from isolate RN11 Was characterized in terms of different
temperatures and pH so as to obtain their optimum temperature and pH of activity.



Graph 1: Activity of mannanase from bacterial isolate RN-11 at different temperature



Graph 2: Activity of mannanase from bacterial isolate RN-11 at different pH

Application of enzyme for bleaching of pulp

The bleaching experiments on kraft pulp were carried out with mannanase from isolate RN 11 at temperature 70 °C and pH 8.8 for 4 hrs by using 25U of enzyme. The pre-bleaching with mannanase showed the decrease of kappa (κ) number by 14.86%.

Table 2: Data showing decrease in kappa (κ) number.

	Titration volume (ml)	p=(b-a)*2	(p_a/b)*100 (%)	f- factor	K= (p_a*f)/w	%reduction (C-T)/C*100
Blank	2.52					
C	1.63	1.78	70.63	1.044	14.8	
T	1.75	1.54	61.11	1.024	12.6	**14.86**

where,
B= blank
T= test sample (Enzyme treated pulp)
C=control (untreated pulp)
p= $KMnO_4$ consumed
p_a= actual amount of $KMnO_4$ consumed
(p_a/b)*100 = percentage of $KMnO_4$ consumed.
f-factor = factor of correction to 50% $KMnO_4$ consumed (dependanton value of p_a)
K= kappa (κ) number
w= weight of pulp used.
Thermophilic mannanase

was applied on wheat straw-rich-soda pulp to evaluate its blea ching potential. A 14.86% reduction of kappa no. was observed for enzyme treated pulp sample than the untreated pulp.

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