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Research Article

PHYLOGENETIC ANALYSIS, OPTIMIZATION OF NUTRITIVE MEDIA COMPOSITION AND IMMOBILIZATION OF XYLANASE ENZYME PRODUCED BY *BACILLUS LICHENIFORMIS* STRAIN SC

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ABSTRACT
Enzyme productions by microorganisms are increasing with rising cost and finite supply of fossil energy. Microorganisms required for the conversion of lignocellulosic materials into fermentable sugars. A newly *Bacillus* was isolated from decaying wood and identified for high xylanase activity. It was identified as *Bacillus licheniformis* strain (KJ842626) by 16S rRNA gene sequencing and by different biochemical tests. The phylogenetic relations to other xylanolytic bacteria were also analyzed. The immobilization of enzyme had been done by gel entrapment process with final concentration of 4 % agar and 1 % agarose solution. The immobilized enzyme might be advantageous for industrial applications therefore the immobilization of cells for higher xylanase production. From the above investigations it was concluded that xylanase produced by *Bacillus* has industrially application in different industry and the cell entrapment composed of agar and agarose exhibited higher activity and better operational stability.
Keywords: Xylanases, lignocellulosic materials, *Bacillus licheniformis.*

INTRODUCTION

Xylan is the second most abundant renewable polysaccharide after cellulose occurring 20-40 % of plant dry weight. The complete degradation of xylan require a group of xylanolytic enzymes such as endo-1, 4-β-xylanase, β-xylosidase and various de branching enzymes ά-glucuronidase, ά-arabinofuranosidase and acetyl xylan esterase[1]. Xylanases (β -1, 4 D-xylan xylanohydrolase, EC 3.2.1.8) are endoenzymes, which release xylooligosaccharides and xylose residues from xylan. Xylanases have biotechnological applications in bio fuel, food and beverages as well as in chemical production, clarification of fruit juices, textile, detergents, paper and pulp industry, pharmaceuticals, waste treatment and recovery of fermentable sugars from hemicelluloses[2-4]. The industrial application of enzyme depends mainly on the utilization of microorganisms, their enzymes and reaction products. A group of microorganisms as bacteria (sea, soil, rumen), fungi, algae, protozoa, insect and cereals[5] are known to produce xylanase. Lignocellulosic substrates are cheap, readily available and their possible use in secondary fermentation processes. Lignocellulolytic microorganisms are ubiquitous in nature and can be isolated from plant residues such as agricultural waste products[6], or from hot spring environments where organic carbon is available[7,8]. For industrial applications, use of immobilized of enzymes is advantageous. As it is easy to separate the enzymes from reaction solution, stop the reaction rapidly, minimize the effluent problem as well as reduce the cost involved in enzyme production as these can be reused and applied in continuous reaction. It also improves enzyme stability during the operations as well as multi enzyme system reaction could also be developed using immobilized enzymes[9]. Immobilization means restraining the enzyme molecule in a different phase. It may be achieved by fixing enzyme to a solid suitable support or entrapping it in a gel and membrane confinement in which the enzyme molecule freely move within their phase (susbtrate or products). Therefore, for the stable and reusable enzyme preparation alternative approach is immobilization[10,11]. Different materials such as agar, agarose, sodium alginate, etc have been used for immobilization of enzyme. Agar is a polysaccharide consisting of agarose which has a strong ability of gelling. Agarose is a neutral linear polysaccharide made up of basic repeat unit agarobiose. Agarose has a strong capacity to form very strong gels even at low concentration. The cost of these materials is low than other materials commonly used for immobilization. Considering the industrial importance of xylanase and cost involved in its production, the aim of present study was to evaluate xylanase production from bacteria using various cost-effective lignocellulosic substrates as xylan substitute, optimization of cultural conditions, identification of bacteria, phylogenetic analysis and immobilization of xylanase.

MATERIALS AND METHODS

Screening of Microbial Strains

The bacteria are isolated from decaying wood, alkaline soil, dry humus, paper industry wastes. Enrichment was done using oat spelt xylan, yeast extract, peptone, xylan and agar slants (0.5: 2:1:0.5:3 %w/v) for 24-48 h at 45^0C. Colonies developed were assayed by xylanolytic activity (on the xylan agar plate). A total of 18 xylanolytic bacterial strain were isolated and screened further for their xylanolytic activity.

Media

Isolated xylanolytic bacteria producing under submerged fermentation at 45^0C in 50 ml production media (basal salt solution BSS) containing 1 g of wheat bran/corn cob and of BSS g/l: $MgCl_2.6H_2O$, 6.6; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $(NH_4)_2S0_4$, 2.0 g; pH 6.7 were autoclaved, inoculated with 10 %(v/w) of inocula (24 h old). At the desired intervals, the flasks were removed and the contents extracted were with 50 ml of 0.02 M phosphate buffer (pH 7.0).

DNA Extraction

Culture was harvested by centrifugation at 10,000 rpm for 20 min, at 4^oC[12]. The resulting pellet was used for DNA extraction.

Amplification of the 16S rDNA gene

The 16S rDNA was amplified using primers forward: 27f(5'-AGAGTTTGATCATGGCTCAG-3') and reverse: 1100r (5'-GGGTTGCGCTCGTTG 3'). 16SrRNA was amplified from the DNA isolated from a bacteria and mixture for PCR was as follows: 10XTaq buffer, 1.25 U Ampli Taq Gold DNA Polymerase, 2 mM dNTP mixture, 25 mM $MgCl_2$, 0.7 µg DNA, double-distilled water mixed in a final volume of 50 µl. The program for PCR was as follows: 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and extension at 72^0C for 7 min. The amplified DNA fragment was separated on 1 % agarose gel, eluted from the gel and purified using a QIA quick gel extraction kit (Qiagen).

Sequencing of the 16S rDNA

Sequencing of the purified PCR product was carried out using three forward and three reverse primers, namely
27f(5'-AGAGTTTGATCATGGCTCAG-3'),
530f(5'-GTGCCAGCAGCCGCGG-3'),
1114f (5'-GCAACGAGCGCAACC-3'),
519r (5'-GTATTACCGCGGCTGCTG -3'),
1110r (5'- GGGTTGCGCTCGTTG -3') and
1492r (5'- TACGGTTACCTTGTTACGACTT-3').
Amplified product was sequenced by dideoxy chain terminator method using the Big Dye terminator kit followed by capillary electrophoresis on an ABI 310 genetic analyzer (Applied Bio systems, USA).

Phylogenetic analysis

The sequence obtained was used to identify the isolate using BLAST (Basic Local Alignment Search Tool) program (www.ncbi.nlm.nih. gov/blast) for similar sequence. The 16S rDNA sequence obtained was added to publicly available bacterial 16S rRNA sequences. The sequences were integrated into the database with the automatic alignment tool. Phylogenetic tree was generated by multiple alignment tool Clustal Omega.

Nucleotide sequence accession number

The 16S rDNA sequence (Accession No. KJ842626) was deposited in the NCBI Gene Bankit nucleotide sequence database.

Fermentation Media

Four different media were used for the production of xylanase in submerged fermentation by *Bacillus licheniformis* strain SC. The composition of the production media used was as follows:

Media1 (g/L)

Corncob/ Rice bran/ Sugarcane bagasse/Wheat bran 10, peptone 10, yeast extract 0.02, KH_2PO_4 15, NaCl 0.5, $MgSO_4.7H_2O$ 1 and Glucose 30

Media2 (g/L)

Corncob/ Rice bran/ Sugarcane bagasse/Wheat bran 20, peptone 10, yeast extract 0.02, KH_2PO_4 0.5, NaCl 0.5, $MgSO_4.7H_2O$ 0.002 and Glucose 30

Media3 (g/L)

Corncob/ Rice bran/ Sugarcane bagasse/Wheat bran 30, peptone 10, yeast extract 10, KH_2PO_4 0, NaCl 0.5, $MgSO_4.7H_2O$ 0.002 and Glucose 5

Media 4 (g/L)

Corncob/ Rice bran/ Sugarcane bagasse/Wheat bran 40, peptone 2, yeast extract 0.02, KH_2PO_4 0, NaCl 0.5, $MgSO_4.7H_2O$ 0.002 and Glucose 5

Xylanase Production and Assay

The active culture of bacteria was inoculated in 100 ml of basal salt medium contained in Erlenmeyer flasks (250 ml) and was incubated at 45^0C for 48 h. The bacterial culture was centrifuged at 36,000 g for 55 sec and used as crude enzyme. Xylanase activity was measured by incubating 0.5 ml of 0.4 % (w/v) oat spelt xylan in 0.02 M phosphate buffer (pH 7.0) and 0.5 ml of suitably diluted enzyme extract at 45°C for 30 min. The release of reducing sugar was measured as xylose by dinitro salicylic acid method[13]. One unit (U) of xylanase is defined as the amount of enzyme that releases 1 µmol xylose/ml/min under the assay conditions.

Immobilization of Bacterial Cells and Xylanase Enzyme - Gel Entrapment Approach

Bacillus licheniformis strain SC was grown for 24 h at 45^0C under shaking condition (10,000 rpm). After 24 h bacterial culture was centrifuged at 10000 rpm for 15 min at 4^oC. Cell pellet was washed and suspended in 5 ml 50 mM sodium acetate buffer, pH 5.5 containing 1.8 % NaCl and stored at 4^oC. It was used for immobilization in agar and agarose. The cell suspension was immobilized by gel entrapment using agar and agarose solution. Cell suspension was mixed with 5 ml of molten agar and agarose solution (45^0C) to get a final concentration of 4 % agar and 1 % agarose solution. After cooling down to room temperature, 1 ml of enzyme was mixed with 9 ml of both agarose and agar solution (making a total volume of 10 ml of matrix and enzyme mixture) and immediately casted on preassembled glass plates. After solidification at room temperature both the gels were cut into small beads of 5 X 5 mm size. Beads were washed several times to remove any enzyme attached to the gel surface before use. Beads were stored into 50 mM sodium acetate buffer (pH5.5) at 4^0C.

Xylanase Assay for immobilized enzyme

Immobilized xylanase agarose or agar beads suspended in 0.5 ml sodium acetate buffer pH5.5 were incubated with 0.5 ml of 0.5 % xylan solution at 37^0C for 3 min. The reaction was stopped with 1 ml of 3, 5 dinitrosalicylic acid followed by heating in a boiling water bath for 5 min and then cooling down to room temperature. After addition of 10 ml of distilled water the amount of reducing sugar produced was determined spectrophotometrically at 540 nm[13]. The percentage immobilization was calculated as (total activity in immobilized gel/total activity of the soluble enzyme loaded) X 100.

RESULTS AND DISCUSSION

Isolation, Morphological and Biochemical Characterization

Bacterial strains were isolated from the soil of different region at 45^0C after 48 h. A total of 18 xylanase producing microorganisms were isolated from different sources. These microorganisms were isolated on solid medium containing

oat spelt xylan as sole carbon source along with other components. Xylanolytic bacteria were selected on the basis of clear zone of xylan hydrolysis after 48 h. Bacterial cultures were maintained by repeated culturing on medium containing yeast extract, peptone, xylan and agar (2:1:0.5:2 %w/v) after every fifteen days. The strain showing the maximum activity (12.6 U/ml) in liquid medium with oat spelt xylan was selected for further investigation. The potential strain *Bacillus licheniformis* strain SC was having cocci, pinkish color, regular margin and motile. Biochemical characterization was found to be the isolate Gram positive, oxidase positive, catalase positive, casein and starch hydrolysis positive with nitrate reducing capacity. Highest xylanase producing strain was characterized as *Bacillus licheniformis* Acc. No. KJ842626 on the basis of morphological biochemical and molecular characterization (16sRNA).

16S rDNA Amplification and Sequence Analysis

The consensus sequence (1309 bases rDNA) of the bacterial isolate was identified on the basis of 16S rDNA sequencing. A comparison of the DNA sequence with sequences in the National Center for Biotechnology Information (NCBI) database was done using BLAST software with BLAST N search tool. Bacterial isolate showed 96 % sequence identity with the 16S rDNA sequences of *Bacillus sonorensis* and *Bacillus licheniformis* available in NCBI database. The homologous 16S rRNA gene sequences of the selected strains with respect to isolate were obtained from the microbial nucleotide databases through NCBI database. Multiple alignment software, Cluster Omega was used to align homologus 16SrDNA sequence of bacterial isolate with homologus 16SrDNA sequence of the isolated strain from the microbial nucleotide database of NCBI and a phylogenetic tree was generated (Figure 1). Bacterial isolate under study showed more relatedness to *B. licheniformis, B. sonorensis* and *B. aerius*. Comparisons with *B. atrophaeus, B. amyloliquefaciens, B. tequilensis, B. subtilis sub sp subtilis, B. mojavensis, B. vallismortis* and *Brevibacterium halotolerans* showed less relation. The isolate cannot be assigned as *Bacillus sonorensis*, because results of other tests done on this strain showed that this bacterium, due to its different characteristics, could hold some of the traits found in other strains, especially that this strain is a spore forming bacteria and its known that spores here protective shields against extreme conditions. Therefore, this bacterium was assigned as *Bacillus licheniformis* belonging to Bacilllaceae family. On this basis of the selected bacterial isolate confirmed as new xylanase producing *Bacillus licheniformis* (accession no. KJ842626).

Medium optimization

The utilization of xylan as a carbon source is a costly affair for enzyme production. Various cheap and abundantly available ligocelluloses (maize straw, wheat straw, corncob, rice bran, sugarcane baggase, bajra straw, jowar straw, oat hay) have been used to replace xylan. In this study, use of lignocellulosic substrate in the production medium resulted in the production of xylanase, *Bacillus licheniformis* strain SC produced xylanase on different agricultural wastes under submerged fermentation. Different media (M-I to M-IV) with different concentrations of lignocellulosic substrates and other medium constituents were optimized for xylanase production under agitated conditions (240 rpm). Different microorganisms require different nutrition condition for the growth and other processes. Xylanase production was carried out in medium M-I for different time periods viz 24 h, 48 h, 72 h and 96 h in media-I (M-I) under continuous shaking conditions at 240 rpm (Figure 2). Results of this experiment revealed that the highest level of enzyme was detected in wheat bran (23.52 U/ml) followed by sugarcane bagasse (19.45 U/ml) and corn cob (14.56 U/ml) at 48 h. The medium M-I with rice bran produced 10.49 U/ml after 24 h of incubation. Therefore, wheat bran was the best carbon source in M-I. It is known that commercial wheat bran consists of 30 % cellulose, 27 % hemicellulose, 21 % lignin, and 8 % ash[14]. *Aspergillus niger* showed maximum enzyme (12.65 U/ml) production in wheat after an incubation period for 6 days at 28^0C[15]. Using wheat bran maximum enzyme level (7.52 U/gds) was obtained from *Acinetobacter sp*. with 75 % moisture content after 72 h under solid state fermentation[16].

Xylanase production by *Bacillus licheniformis* strain SC using media M-II with different agricultural wastes at further higher concentrations under submerged fermentation indicated that wheat bran (23.86 U/ml) acted as a best substrate for the maximum enzyme production followed by corn cob(15.87 U/ml) after 48 h. The level of enzyme was 14.57 U/ml with sugarcane bagasse and 11.24 U/ml rice bran after 24 h (Figure 3). Using wheat bran as a substrate different strains of *Bacillus* as (2 %, w/v) *Bacillus pumilus*[17], *Bacillus coagulans* BL69[18], *Bacillus megaterium*[19], *Bacillus sp*[20], *Bacillus licheniformis* A99[21] produced maximum xylanase at different time period.

In medium-III sugar cane bagasse at still higher concentration resulted in maximum level xylanase with 16.78 U/ml. Higher concentrations of wheat bran and corncob resulted in decreased production, exhibiting 14.89 U/ml and 13.45 U/ml of enzymes, respectively after 48 h (Figure 4). The medium M-III supplemented with rice bran produced 8.75U/ml of enzyme after 24 h. Therefore in medium M-III sugarcane bagasse was better inducer for xylanase production at 48 h of fermentation. Similar results were obtained *T. harzianum*[22] and *A. awamori* [23] with sugarcane bagasse as a substrate for xylanase production.

Different levels of xyalanse production were detected in medium (M-IV) supplemented with different agriculture wastes. The production of enzyme was maximum with corn cob (12.45 U/ml), followed by wheat bran (9.84 U/ml), sugarcane bagasse (8.46 U/ml) and rice bran (3.48 U/ml) at 48 h of fermentation period (Figure 5). The production of xylanase was observed from S*porotrichum thermophile* grown on corn cobs [2.7 % (w/v)] under submerged condition[24]. The highest yield of xylanase was observed at 72 h of fermentation in *Bacillus licheniformis* using corn cob as substrate[20].

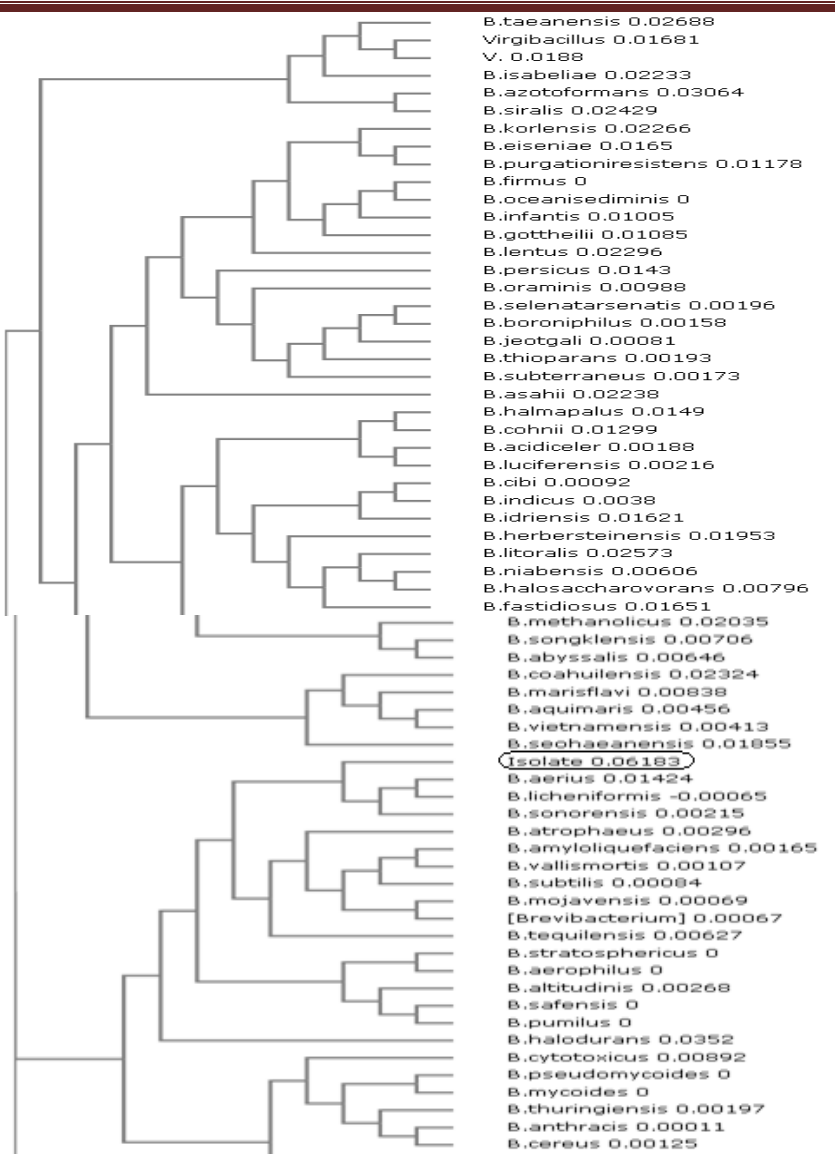

Figure 1: Phylogenetic tree representing the analysis of the 16S rRNA sequences of bacterial strain under study compared to the bacterial database

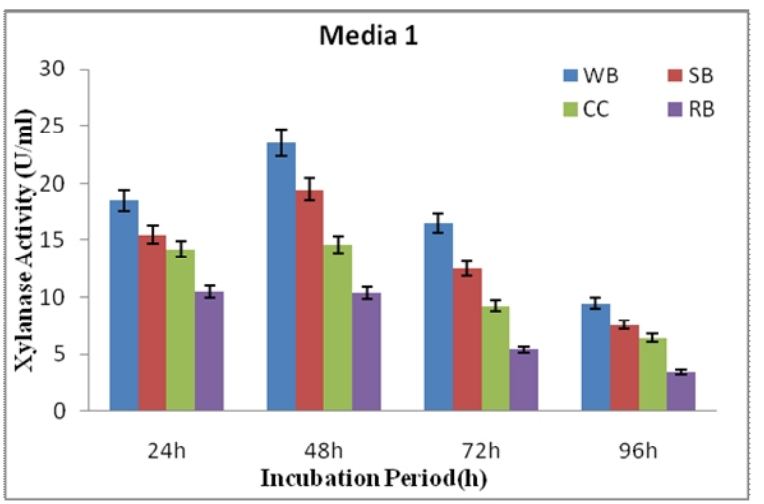

Figure 2: Production of xylanase by *Bacillus licheniformis* KJ710510 using media-I at 45°C

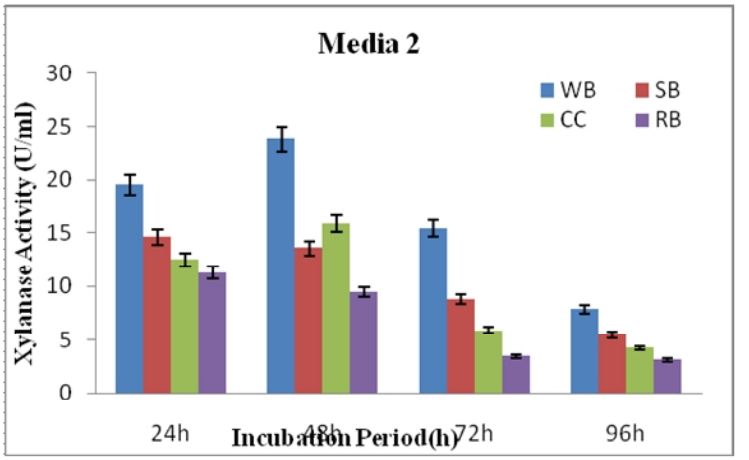

Figure 3: Production of xylanase by *Bacillus licheniformis* KJ710510 using media-II at 45°C

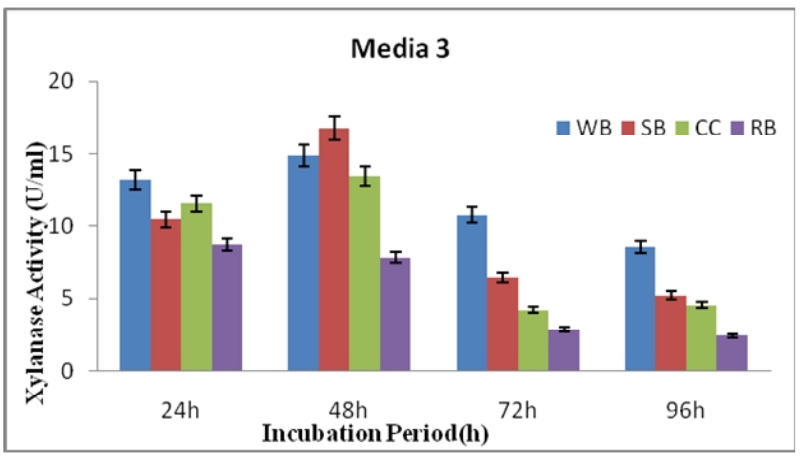

Figure 4: Production of xylanase by *Bacillus licheniformis* KJ710510 using media-III at 45°C

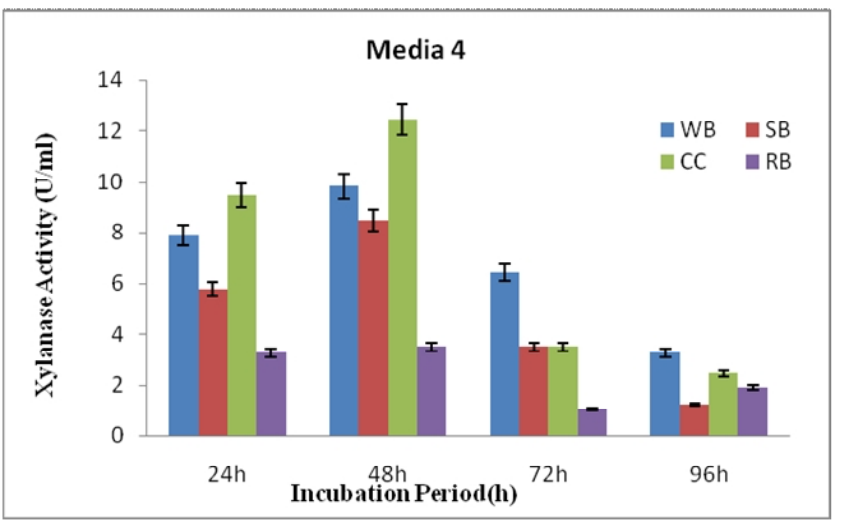

Figure 5: Production of xylanase by *Bacillus licheniformis* KJ710510 using media-IV at 45°C

Entrapment of xylanase in agarose and agar gel

Numerous methods of enzyme immobilization on a variety of different materials have been developed. Immobilized enzymes are currently the object of considerable interest. It is due to the expected benefits over soluble enzymes or alternative technologies. For enzyme entrapment in the gel, different concentration of agarose (0.5- 2 %) were used to achieve optimum immobilization. At 0.5 % agarose, only 41.2 % enzyme immobilization was achieved due to the fragile nature of the membrane and its susceptibility during handling. At 1 % agarose, the percentage immobilization was about 49.6 %. At still higher concentrations (1.5 %), stable agarose beads were obtained with the optimum immobilization of about 65.7 %. On further increasing the concentrations of agarose to 1.75 % and 2 %, decreased percentage of immobilization 42.66 % and 16.9 %, respectively was evaluated due to the solidification of gel Table 1.

Table 1: Percent immobilization with varying agarose and agar concentration

% Agarose (w/v)	% Immobilization
0.5	41.2
1	49.6
1.5	65.7
1.75	42.66
2	16.9
% Agar(w/v)	**% Immobilization**
1	38.5
2	45.6
3	73.2
4	66.2
5	42.8
6	21.3

Xylanase was also immobilized in agar gel using different concentrations of agar (1-6 % w/v). At 1 % and 2 % concentration, the beads were soft and fragile giving 38.5 and 45.6 % immobilization. However at 3 % concentration 73.2 % immobilization was obtained that means the optimum level of immobilization was achieved. On further increasing the concentration of agar (4-6 %) decreased level of immobilization was obtained. Therefore 1.5 % (w/v) agarose and 3 % (w/v) agar solution was employed for xylanase immobilization studies

CONCLUSION

The commercial scale production of xylanase using xylan is expensive. Production cost is the major limiting factor for the production of any enzyme. Therefore the use of agriculture wastes for xylanases was investigated as these are cheap and easily available. Supplementation of different nutrient components in different media affected the enzyme production. Our studies indicated that *Bacillus licheniformis* strain SC yielded maximum level of enzyme in medium M-II supplemented with wheat bran along with other medium components. Therefore, xyalanase production was maximum with wheat bran as a substrate under submerged fermentation as it contains sufficient nutrients. Therefore wheat bran could be the best option to be utilized for xylanase production at industrial level. In this study xylanase enzyme was immobilized in agar and agarose gel and from technological point of view separation of immobilized enzymes from the reaction liquid becomes very easy. Also immobilization enables repetitive use of enzymes and therefore significant cost savings.

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