UGC Reference No. & Date : **F.No. 43-62/2014 (SR) 17-08-2015**
MRP-MAJOR-BIOT-2013-13382

सत्यमेव जयते

University Grants Commission
(Ministry of Human Resource Development, Govt. of India)
Bahadurshah Zafar Marg, New Delhi – 110002

ज्ञान-विज्ञान विमुक्तये

FINAL REPORT ON THE MAJOR RESEARCH PROJECT

DEVELOPMENT OF ENZYME BASED ECOFRIENDLY BIOCONVERSION PROCESS FOR THE PREPARATION OF XYLITOL

DURATION: 01-07-2015 TO 30-06-2018

Submitted to:

UNIVERSITY GRANTS COMMISSION (UGC), NEW DELHI

by

Dr. Balwinder Singh Sooch
Assistant Professor

Department of Biotechnology,
Punjabi University, Patiala

April 2019

Annexure -VIII

FINAL REPORT ON THE PROJECT

TITLE: DEVELOPMENT OF ENZYME BASED ECOFRIENDLY BIOCONVERSION PROCESS FOR THE PREPARATION OF XYLITOL

1.	Project report No. 1st /2nd /3rd/ Final	Final
2.	**UGC Reference No.**	**F.No. 43-62/2014 (SR)**
3.	Period of report:	01-07-2015 to 30-06-2018
4.	Title of research project	Development of enzyme based ecofriendly bioconversion process for the preparation of xylitol
5.	**(a) Name of the Principal Investigator** (b) Department (c) University/College where work has progressed (d) Co-Investigator	**Dr. Balwinder Singh Sooch** Department of Biotechnology, Punjabi University, Patiala-147002, India Punjabi University, Patiala None
6.	Effective date of starting of the project	01-07-2015
7.	Grant approved and expenditure incurred during the period of the report: **a.** Total amount approved **b.** Total expenditure **c.** Report of the work done: **i.** Brief objective of the project	 Rs. **11,60,000/-** (Rupees Eleven Lakh and Sixty Thousand only) Rs. **10,59,402/-** (Rupees Ten Lakh Fifty Nine Thousand Four Hundred and Two only) **Objectives:** 1. Isolation and screening of xylose reductase producing microorganisms from natural sources. 2. Enhancement in the production of xylose reductase from selected microbial strain by employing various fermentation/process engineering strategies. 3. Development of bench scale technology for the production of xylose reductase. 4. Purification, characterization and immobilization of xylose reductase. 5. Production of xylitol using free and immobilized enzyme in batch and continuous system.

7c	**ii.** Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication	• Please Refer Appendix- A (for summary of work done and results achieved and Publications) • Appendix-B (Detailed project report).
	iii. Has the progress been according to original plan of work and towards achieving the objective? if not, state reasons	Yes, the progress of the project is as per plan.
	iv. Please indicate the difficulties if any, experience in implementing the project	None
	v. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet.	Not Applicable, as the project has been completed.
	vi. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission.	**Yes,** **Summary of the findings:** (Please refer Appendix-A) & Appendix-B (Detailed project report).
	vii. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any	(a)**MANPOWER TRAINED:** Total Six Master of Science (Three M.Sc. Microbial and food technology and three M.Sc. Biotechnology) students have worked for their dissertation in the area under the scope of the project. (b) **PH. D. AWARDED:** One research scholar has completed her Ph.D. degree, on the topic closely related to the project. No project Fellow was sanctioned and worked under the project.

		(c) **PUBLICATIONS:** 1. One Indian Patent application has been filed on basis of research outcome of this project. 2. One Book Chapter (Springer Publs.) published and One Book Chapter (De Gruyter Publs) is in press and Two Research Papers in peer reviewed journals have been published related to the research work of the project. 3. One 18S RNA sequence of the new isolated strain i.e. *Candida tropicalis* BSS-XR15 isolated under the present project was submitted with GENBANK database of NCBI under accession number KY619688.

Signature
Principal Investigator

Signature 10.7.19
Registrar
Registrar.
Punjabi University.
Patiala

Annexure -IX

INFORMATION ON THE MAJOR RESEARCH PROJECT

Development of enzyme based ecofriendly bioconversion process for the preparation of xylitol

1.	Title of the Project	Development of enzyme based ecofriendly bioconversion process for the preparation of xylitol
2.	Name and address of the Principal Investigator	Dr. Balwinder Singh Sooch Assistant Professor Department of Biotechnology, Punjabi University, Patiala-147002, India
3.	Name and address of the Institution	Department of Biotechnology, Punjabi University, Patiala-147002, Punjab
4.	UGC Approval Letter no. and Date	F.No. 43-62/2014 (SR)
5.	Date of Implementation	01-07-2015
6.	Tenure of the Project	3 years, from 01-07-2015 to 30-06-2018
7.	Total Grant Allocated	Rs. 11;60,000/- (Rupees Eleven Lakh and Sixty Thousand only)
8.	Total Grant Received	Rs. 10,95,000/- (Rupees Ten Lakh and Ninety Five Thousand only)
9.	Final Expenditure	Rs.10,59,402/- (Rupees Ten Lakh Fifty Nine Thousand Four Hundred and Two only)
10.	Title of the Project	Development of enzyme based ecofriendly bioconversion process for the preparation of xylitol
11.	Objectives of the Project	**Objectives:** 1. Isolation and screening of xylose reductase producing microorganisms from natural sources. 2. Enhancement in the production of xylose reductase from selected microbial strain by employing various fermentation/process engineering strategies. 3. Development of bench scale technology for the production of xylose reductase. 4. Purification, characterization and immobilization of xylose reductase. 5. Production of xylitol using free and immobilized enzyme in batch and continuous system.
12.	Whether objectives were achieved	Yes All objectives were achieved. (Please refer Appendix-B for details)

13.	Achievements from the Project	An enzyme based ecofriendly bioconversion process for the preparation of xylitol has been developed under the project. A novel efficient XR producing yeast strain designated as *Candida tropicalis* BSS-XR-15 has been isolated and thee 18S RNA sequence has been submitted to Genbank NCBI database. The increase in production of XR from the isolate was achieved by tailoring various nutritional and process parameters. The immobilization of purified xylose reductase enzyme obtained after gel filteration chromatography was carried out on sodium alginate beads. The stability of the xylose reductase enzyme immobilized in sodium alginate beads modified by glutaraldehyde treatment was improved. A packed bed reactor packed with sodium alginate beads containing xylose reductase immobilized enzyme was used for the production of xylitol in continuous system and good conversion of xylose to xylitol (more than 85%) was obtained till 5th day of operation. The process developed under the present study can be exploited in future for production of xylitol at industrial level after scale up studies.
14.	Summary of the Findings	**Summary of the findings:** (Please refer Appendix-A)
15.	Contribution to the Society	An enzyme based ecofriendly bioconversion process for the preparation of xylitol developed under the project would be beneficial to the society in multifaceted manner. The primary aim of this project proposal was to develop bioconversion process for the xylitol which has applications in food, pharmaceutical and chemical industries. This ecofriendly technology in combination with other enzymes like xylanase will be beneficial because it will utilize various agrowaste, which are available in large amount in India in the form of sugarcane bagasse, wheat straw, rice straw, corn waste, rice bran, etc. The production cost and pollution problems for the production of xylitol would be significantly reduced by the use of this bioconversion technology as compared to chemical process. The process developed under the present study can be exploited in future for production of xylitol at industrial level after scale up studies.
16.	Whether any Ph.D. enrolled/produced out of the Project	One research scholar has completed her Ph.D degree at Department of Biotechnology, Punjabi University, Patiala in the area closely related to the project
17.	No. of Publications out of the Project (please attach)	06 (Appendix C)

Signature
Principal Investigator

Signature
Registrar 10.7.15
Registrar
Punjabi University,
Patiala

Appendix- A

(Website copy)

SUMMARY OF THE WORK DONE AND RESULTS ACHIEVED, AND PUBLICATIONS RESULTING FROM THE PROJECT

➢ Executive Summary of the Work Done and Results Achieved

The present study was carried out to develop an enzyme based ecofriendly bioconversion process for the preparation of xylitol. To achieve the proposed objectives, soil samples were collected from various localities to isolate xylose reductase (XR) producing microorganisms through specific isolation techniques. The morphological characteristics of various isolates, isolated obtained from different samples were recorded and screened for XR production to find the efficient strain. Out of the various isolates isolated from different samples, only seventeen isolates were found xylose reductase positive. Amongst these isolates, the isolate designated as BSS-XR-15 has been found most efficient for intracellular xylose reductase production. The efficient selected strain BSS-XR-15 was further identified and characterized through polyphasic approach using phenotypic, biochemical and genotypic methods. The 18S RNA sequencing has also established its identity as *Candida tropicalis*. The 18S RNA sequence has been submitted to Genbank_NCBI.

The growth of isolated strain in relation to xylose reductase production was studied by cultivating the yeast in fermentation medium of pH 5.5 containing xylose, peptone and yeast extract at 30°C for 60 h under shaking conditions. It has been observed from the growth curve that selected yeast strain reaches the stationary phase after 24 h. It has also been noticed that the xylose reductase production progressively increased during log phase of growth and reaches at maximum level (after 24h) before the microorganism is about to reach the stationary phase of growth.

Different parameters like carbon and nitrogen sources, growth promoters and salts were optimized to fulfill the nutritional requirements of yeast strain to obtain maximum production of XR at flask level from the selected strain by various experimental sets. Similarly, different

variables of process parameters like pH, temperature, age and size of inoculum etc. were used in separate experiments for maximum production of xylose reductase at flask level. The maximum xylose reductase production was recorded at pH of 5.0 and 30 °C temperatures. The use of 10% (v/v) of 12 h old inoculum under agitation mode (100rpm) was found optimum for maximum production of XR. The media and process parameters optimized at flask level were tested for the production of xylose reductase at Laboratory scale (1.5 L) fermenter. The aeration, agitation, foam control was optimized at bioreactor scale to enhance the xylose reductase.

The enzyme was purification of 1.95 fold was achieved with specific activity of 882.4 IU/mg through ammonium sulphate precipitation, dialysis, ultrafiltration and gel filtration chromatography. The immobilization of purified enzyme obtained after gel filtration chromatography was carried out through different strategies on different supports. The results obtained in terms of immobilization yield of immobilized xylose reductase obtained after the process of immobilization on different matrices clearly depicts that amongst all the immobilization supports, the maximum immobilization yield for xylose reductase was achieved with sodium alginate beads. After optimizing immobilization parameters, the maximum immobilization yield was obtained with sodium alginate beads with glutaraldehyde treatment.

The purified free and immobilized enzyme was characterized for various parameters. The enzyme was observed to be active over a wide range of pH (5.5-7.0), however, pH optima of xylose reductase has been shifted after immobilization of enzyme from pH 5.5 to 6.5. The temperature optima of free and immobilized xylose reductase were found at 25°C and 30°C, respectively. The K_m value for immobilized xylose reductase was higher than that of free enzyme which indicated a significant decrease in the substrate affinity of the immobilized enzyme after immobilization in glutaraldehyde treated sodium alginate beads. The V_{max} of immobilized xylose reductase was also found to be lower than that of free enzyme. The effect of different salts as inhibitors or inducers was tested on enzyme activity at different concentrations and only $ZnCl_2$, $CaCl_2$ and $MgSO_4$ were able to induce the enzyme activity. In terms of pH stability, the enzyme has retained more than 95% activity at pH range of 5.5 to 6.5. The thermal stability of the immobilized enzyme was tested and it has been observed that enzyme possesses very good stability at 25°C and 30°C and has retained more than 95% relative activity, whereas, relative enzyme activity was found to be decreased at higher temperatures after certain intervals of time.

To test the stability of xylose reductase enzyme immobilized in sodium alginate beads modified by glutaraldehyde treatment for long term operations, the immobilized enzyme was recycled in batches for xylitol production. The stability of the xylose reductase enzyme immobilized in sodium alginate beads modified by glutaraldehyde treatment was found to be improved as compared to the beads prepared without glutaraldehyde treatment. The xylitol production of 10.5 g/L and 8.1 g/L was obtained in 1st and 8th cycle, respectively. However, the beads prepared without glutaraldehyde treatment were disintegrated completely during 5th cycle.

A continuous system employing packed bed reactor packed with sodium alginate beads containing XR enzyme was used for the production of xylitol. The flow rate of feed solution was optimized to obtain maximum xylitol production. The maximum xylitol production of ninty gram per gram of xylose with xylose conversion of ninty percent was obtained with 1 mL/min flow rate. The continuous system fed with feed solution was operated for 8 days to test its stability for xylitol production for long term. It has been recorded during experimentation that good conversion of xylose to xylitol was obtained till 5th day of operation, whereas, a sharp decrease in xylitol production (45%) was obtained on 6th day and it become stagnant after 8th day.

A laboratory scale XR enzyme based bioconversion process for preparation of xylitol was successfully developed under the present research project

Future Recommendations:

The stability of the xylose reductase enzyme immobilized in modified sodium alginate beads to be utilized for long term operations was improved. However, the major economical problem associated with this enzyme based immobilization system was the consumption of coenzyme i.e. NADPH, used in each cycle for xylitol production. The development of coenzyme regeneration and recovery system is required for economical and viable industrial project for production of xylitol. The added advantage of this system is the easy availability of xylose containing hemicellulosic materials as raw material, which can make this process economical at industrial level. This ecofriendly technology in combination with other enzymes like xylanase will be more beneficial because it will utilize various agrowaste, which are available in large amount in India in the form of sugarcane bagasse, wheat straw, rice straw, corn waste, rice bran, etc. The process developed under the present study can be exploited in future for production of xylitol at industrial level after scale up studies.

➢ DETAILS OF PUBLICATIONS RESULTING FROM THE PROJECT WORK

PATENTS

- Sooch, B.S., Lugani, Y. (2018). A Process for Biotechnological Production of Xylitol. Punjabi University, Patiala (Assignee). Indian Patent Application (Application no. 201811031769).

SEQUENCE SUBMITTED

- Sooch, B.S. (2018). 18S RNA sequence of the new isolated strain i.e. *Candida tropicalis* BSS-XR15 isolated under the present project was submitted with GENBANK database of NCBI under accession number KY619688.

BOOK CHAPTERS

- Lugani, Y., Sooch, B.S. (2018). Book Chapter 24: Insights into Fungal Xylose Reductases and its Applications in Xylitol Production. In: Kumar, S., Dheeran, P., Taherzadeh, M., Khanal, S. (Eds.). Fungal Biorefineries. Springer. 121-144.

- Sooch, B.S., Lugani, Y. Singh, R.S. (2019). Agro-industrial lignocellulosic residues for the production of industrial enzymes. In: Yadav, M., Kumar, V., Sehrawat, N. (Eds.). Industrial Biotechnology-Plant systems, Resources and Products. Walter de Gruyter Publs, GmbH, Deutschland: 31-50. (In press)

RESEARCH PAPERS

- Lugani, Y., Sooch, B.S. (2017). Development of Cell Disruption Strategy for Enhanced Release of Intracellular Xylose Reductase from *Pseudomonas putida* BSX-46. International Journal of Current Microbiology and Applied Sciences. 6(8): 3682-3697.

- Lugani, Y., Sooch, B.S. (2017). *In Silico* Characterization of Xylose Reductase using Computational Tools. International Journal of Advances in Science, Engineering and Technology. 5(2): 59-66.

RELATED REVIEW ARTICLES

- Lugani, Y., Sooch, B.S. (2017). Xylitol, An Emerging Prebiotic: A Review. International Journal of Applied Pharmaceutical and Biological Research. 2(2): 67-73.

- Lugani. Y., Oberoi, S., Sooch, B.S. (2017). Xylitol: A Sugar Substitute for Patients of Diabetes Mellitus. World Journal of Pharmacy and Pharmaceutical Sciences. 6(4): 741-749.

➢ PAPERS PRESENTATION IN INTERNATIONAL/ NATIONAL CONFERENCES/ SEMINARS

- 'Production of Xylose Reductase from *Candida* sp.' presented in International Symposium on 'Emerging Discoveries in Microbiology' & 56th Annual Conference of Association of Microbiologists of India at School of Life Sciences, Jawaharlal Nehru University, New Delhi on December 7-10, 2015

- 'Production of Xylose Reductase for Conversion of Xylose into Xylitol' presented in International Conference on 'Recent Advances in Emerging Technologies' at Sri Guru Granth Sahib World University, Fatehgarh Sahib, Punjab on February 23 -24, 2016

- 'Current Status in Xylitol Production and Future Prospects.' presented in UGC sponsored National Conference on 'Emerging Trends in Biotechnology: A Paradigm Shift to Cleaner and Greener India' at GSSDGS Khalsa College, Patiala, Punjab on October 8, 2016.

- Enhanced Cell disruption Strategy for release of Xylose Reductase from Novel *Pseudomonas putida* BSX-46 and its Application in Xylitol Production' presented in National conference on 'Recent Advances in Biomedical Science: Diagnostics & Research' by 'The Society of Biomedical Laboratory Scientists, India'at Gandhi Peace Foundation, New Delhi on December16, 2016.

- '*In Silico* Characterization of Xylose Reductase using Computational Tools' presented in International Conference on 'Advance in Science, Engineering and Technology' at ICASER, Pune on February 19, 2017.

Appendix-B

(Website copy)

DETAILS OF THE OBJECTIVES ACHIEVED

➢ Objectives of the Project

1. Isolation and screening of xylose reductase producing microorganisms from natural sources.
2. Enhancement in the production of xylose reductase from selected microbial strain by employing various fermentation/process engineering strategies.
3. Development of bench scale technology for the production of xylose reductase.
4. Purification, characterization and immobilization of xylose reductase.
5. Production of xylitol using free and immobilized enzyme in batch and continuous system.

➢ **The objectives of present research project have been achieved and have been discussed as under:** A laboratory scale XR enzyme based bioconversion process for preparation of xylitol was successfully developed under the present research project through the following objectives.

1. **Objective 1: Isolation, screening and identification of xylose reductase producing microorganisms from natural sources:** Different soil samples were collected from various localities to isolate xylose reductase (XR) producing microorganisms through specific isolation techniques. The morphological characteristics of various isolates, isolated obtained from different samples were recorded and screened for XR production to find the efficient strain. Out of the fifty six isolates isolated from different samples, only seventeen isolates were found xylose reductase positive. Amongst these isolates, the isolate designated as BSS-XR-15 has been found most efficient for intracellular xylose reductase production. The efficient selected strain BSS-XR-15 was further identified and characterized through polyphasic approach using phenotypic, biochemical and genotypic methods. The 18S RNA sequencing has also established

its identity as *Candida tropicalis*. The 18S RNA sequence has been submitted to Genbank_NCBI.

Table 1: Summary of samples collection.

Field	Location	Number of samples collected
Sugarcane	Nabha, Rajpura. Bhadson, Patiala	12
Rice	Sangrur, Zirakhpur, Chhajli, Patiala	9
Wheat	Chhajli, Nabha, Samana, Patiala	10
Baggase	Fatehgarh Sahib, Rajpura, Chhajli, Banur	11
Pup and paper industry	GT road	2
Corn	Chhajli, Nabha, Sunam,	6

Various Soil samples collected from different locations were incubated in YPAX and PDAX for 48h at 28°C

Different dilutions spreader on YPAX and PDAX and incubated for 48h at 28°C and 37 °C

Isolated colonies were streaked further on YPAX and PDAX and incubated for 48h at 28°C and 37 °C

Morphological identification of isolated colonies
Cell and colonial characteristics

Flow Chart 1: Isolation from soil samples

2. <u>Objectives 2:</u> **Enhancement in the production of xylose reductase from selected microbial strain by employing various fermentation/process engineering strategies:**_The sonication technique for disruption of cells for extraction of intracellular xylose reductase was selected after testing different methods of cell disruption. The enzyme assay method was also standardized for estimation of xylose reductase activity. Xylose reductase activity was estimated

with different coenzyme specificity (NADPH) by the method of Yokoyama et al. (1995). One unit of xylose reductase is defined as the amount of enzyme required to catalyze the reduction of 1 µmol of NADPH per minute under standard assay conditions

Table 2: XR activity with different cell disruption methods.

METHOD	XR ACTIVITY AFTER CELL DISRUPTION (IU/g of cells)
Acidified river sand Method	56
Freeze Thawing and Maceration Method	79
Sonication method	230
Freeze Thawing and Sonication	84
Yeast Protein Extraction Lysis buffer (PE LB) containing zymolyase	198

The growth of isolated strain in relation to xylose reductase production was studied by cultivating the yeast in fermentation medium of pH 5.5 containing xylose, peptone and yeast extract at 30°C for 60 h under shaking conditions.

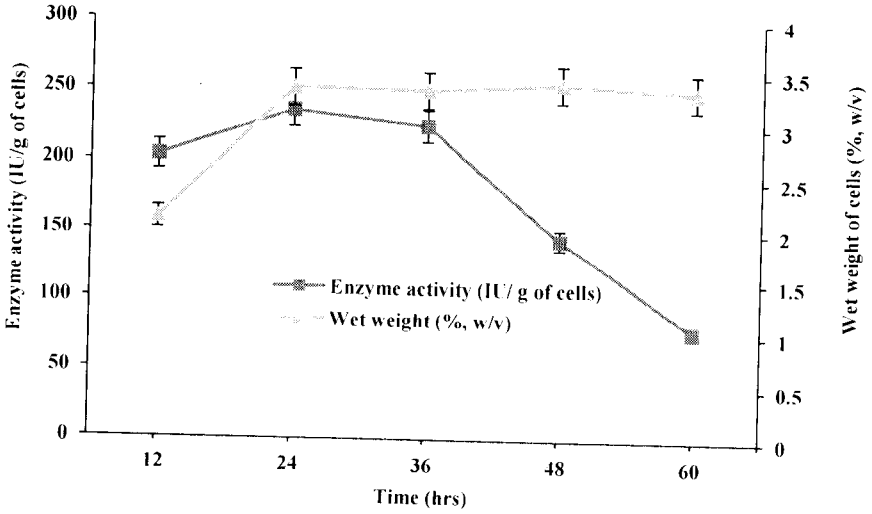

Fig. 1: XR production and growth of selected yeast.

It has been observed from the growth curve that selected yeast strain reaches the stationary phase after 24 h. It has also been noticed that the xylose reductase production progressively increased during log phase of growth and reaches at maximum level (after 24h) before the microorganism is about to reach the stationary phase of growth.

Different parameters like carbon and nitrogen sources, growth promoters and salts were optimized to fulfill the nutritional requirements of yeast strain to obtain maximum production of XR at flask level from the selected strain by various experimental sets. Similarly, different variables of process parameters like pH, temperature, age and size of inoculum etc. were used in separate experiments for maximum production of xylose reductase at flask level. The maximum xylose reductase production was recorded at pH of 5.0 and 30 °C temperatures. The use of 10% (v/v) of 12 h old inoculum under agitation mode (100rpm) was found optimum for maximum production of XR.

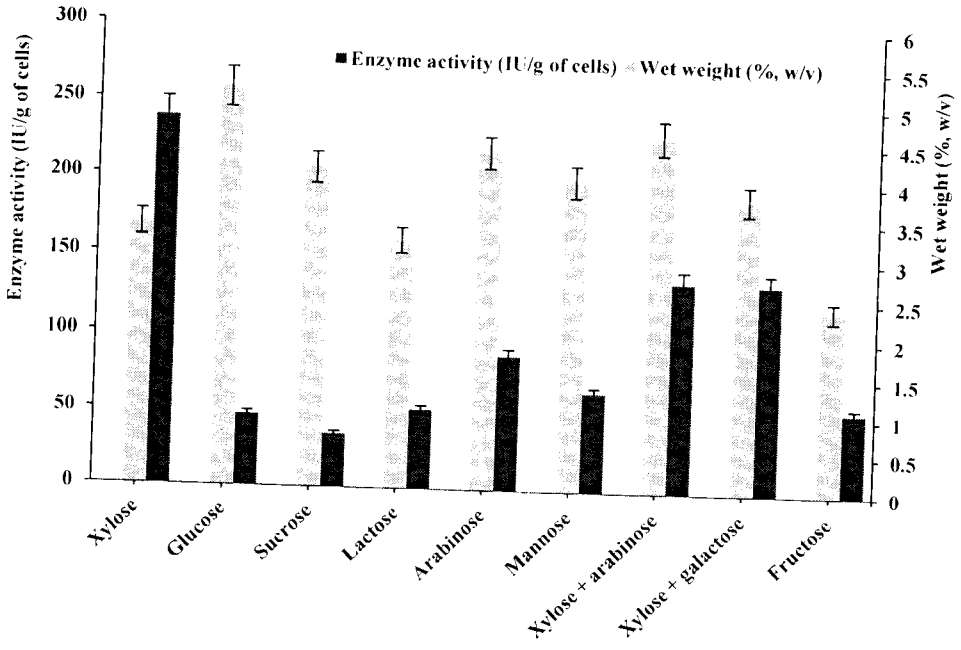

Fig. 2: XR production with different carbon sources.

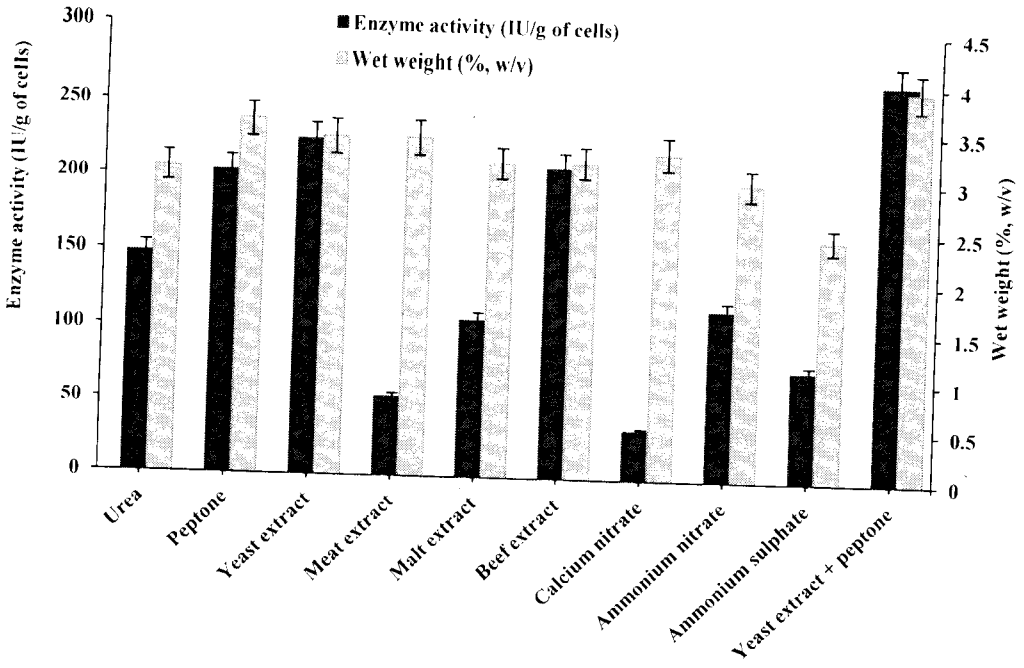

Fig. 3: XR production with different nitrogen sources.

3. <u>Objective 3:</u> **Development of bench scale technology for the production of xylose reductase:** The media and process parameters optimized at flask level were tested for the production of xylose reductase at Laboratory scale (1.5 L) fermenter. The aeration, agitation, foam control and other factors were studied at bioreactor scale to develop bench scale bioreactor level technology for the production of xylose reductase

4. <u>Objective 4:</u> **Purification, characterization and immobilization of xylose reductase:**

<u>Purification and immobilization of xylose reductase:</u> The enzyme was purified by using ammonium sulphate precipitation, dialysis and ultrafiltration, and purification of 1.95 fold was achieved with specific activity of 882.4 IU/mg. The immobilization of purified enzyme obtained after gel filtration chromatography was carried out through different strategies on different supports. The results obtained in terms of immobilization yield of immobilized xylose reductase obtained after the process of immobilization on different matrices clearly depicts that amongst all the immobilization supports, the maximum immobilization yield for xylose reductase was achieved with sodium alginate beads. After optimizing immobilization parameters, the maximum

immobilization yield was obtained with sodium alginate beads with glutaraldehyde treatment. The following studies were conducted for optimizing the immobilization process:

a. Immobilization of xylose reductase for xylitol production on various supports.
b. Optimization of immobilization process of xylose reductase through gel entrapment method.
    i. Effect of glutaraldehyde concentration.
    ii. Effect of treatment time

Characterization of purified free and immobilized xylose reductase enzyme: The purified free and immobilized xylose reductase (in sodium alginate beads) was characterized for the pH, temperature, pH stability and thermal stability. The effect of inducer and inhibitors was also determined on XR activity. The enzyme was observed to be active over a wide range of pH, however, pH optima of xylose reductase has been shifted after immobilization of enzyme to higher side. The temperature optima of free and immobilized xylose reductase were found at 25°C and 30°C, respectively. In terms of pH stability, the enzyme has retained more than 95% activity activity at pH range of 5.5 to 6.5. The thermal stability of the immobilized enzyme was tested and it has been observed that enzyme possesses very good stability at 25°C and 30°C and has retained more than 95% relative activity, whereas, relative enzyme activity was found to be decreased at higher temperatures after certain intervals of time. The effect of different salts as inhibitors or inducers was tested on enzyme activity at different concentrations and only $ZnCl_2$, $CaCl_2$ and $MgSO_4$ were able to induce the enzyme activity.

Further, the kinetic characterization of purified XR was also performed. The Michaelis-Menten constant (K_m) and maximal reaction velocity (V_{max}) for purified free and immobilized enzyme was determined experimentally from Lineweaver-Burk plot by conducting a series of tests with varying xylose concentrations. K_m and V_{max} values was calculated from Lineweaver-Burk plots are shown in Fig. 4 and Fig. 5. The K_m value for immobilized xylose reductase was higher than that of free enzyme which indicated a significant decrease in the substrate affinity of the immobilized enzyme after immobilization in glutaraldehyde treated sodium alginate beads. The V_{max} of immobilized xylose reductase was also found to be lower than that of free enzyme.

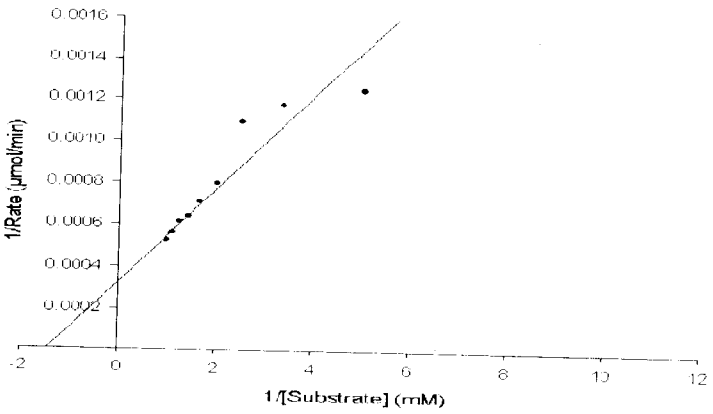

Fig. 4: Kinetic constants (K_m and V_{max}) of free XR.

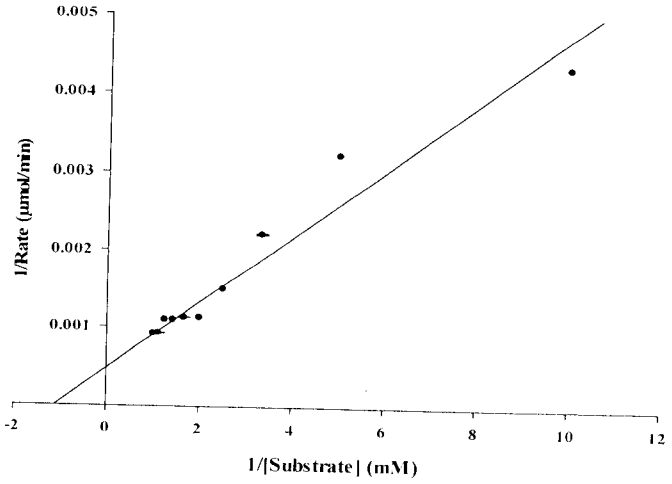

Fig. 5: Kinetic constants (K_m and V_{max}) of immobilized XR.

5. <u>Objective 5:</u> **Production of xylitol using free and immobilized enzyme in batch and continuous system:** The following studies were conducted to achieve objective 5.

a. Studies on xylitol production by immobilized XR in batch system
b. Studies on operational stability of immobilized XR in batch system
c. Studies on xylitol production by immobilized XR in continuous system
d. Studies on operational stability of immobilized XR based continuous system.

To test the stability of xylose reductase enzyme immobilized in sodium alginate beads modified by glutaraldehyde treatment for long term operations, the immobilized enzyme was recycled in batches for xylitol production. The stability of the xylose reductase enzyme immobilized in sodium alginate beads modified by glutaraldehyde treatment was found to be improved as compared to the beads prepared without glutaraldehyde treatment. The xylitol production of 10.5 g/L and 8.1 g/L was obtained in 1st and 8th cycle, respectively. However, the beads prepared without glutaraldehyde treatment were disintegrated completely during 5th cycle.

A continuous system employing packed bed column based reactor packed with sodium alginate beads containing XR enzyme was used for the production of xylitol. The flow rate of feed solution was optimized to obtain maximum xylitol production and maximum xylitol production of 0.90 g/g of xylose with xylose conversion of 90% was obtained with 1 mL/min flow rate. The continuous system fed with feed solution was operated for 8 days to test its stability for xylitol production for long term. It has been recorded during experimentation that good conversion of xylose to xylitol (more than 85%) was obtained till 5th day of operation, whereas, a sharp decrease in xylitol production (45%) was obtained on 6th day and it become stagnant after 8th day.

It has been concluded from the present study that the stability of the xylose reductase enzyme immobilized in sodium alginate beads modified by glutaraldehyde treatment was improved. A laboratory scale XR enzyme based bioconversion process was successfully developed under the present research project for production of xylitol. This process can be exploited in future for production of xylitol at industrial level after scale up studies due to abundance availability of agro waste based raw material.

Appendix-C

- **ITEM 7 (C) (ii) of Annexure VIII**
- **ITEM 17 of Annexure IX**

DETAILS OF PUBLICATIONS RESULTING FROM THE PROJECT WORK

PATENTS

- Sooch, B.S., Lugani, Y. (2018). A Process for Biotechnological Production of Xylitol. Punjabi University, Patiala (Assignee). Indian Patent Application (Application no. 201811031769).

SEQUENCE SUBMITTED

- Sooch, B.S. (2018). 18S RNA sequence of the new isolated strain i.e. *Candida tropicalis* BSS-XR15 isolated under the present project was submitted with GENBANK database of NCBI under accession number KY619688.

BOOK CHAPTERS

- Lugani, Y., Sooch, B.S. (2018). Book Chapter 24: Insights into Fungal Xylose Reductases and its Applications in Xylitol Production. In: Kumar, S., Dheeran, P., Taherzadeh, M., Khanal, S. (Eds.). Fungal Biorefineries. Springer; 121-144.

- Sooch, B.S., Lugani, Y. Singh, R.S. (2019). Agro-industrial lignocellulosic residues for the production of industrial enzymes. In: Yadav, M., Kumar, V., Sehrawat, N. (Eds.). Industrial Biotechnology-Plant systems, Resources and Products. Walter de Gruyter Publs, GmbH, Deutschland: 31-50 (In press).

RESEARCH PAPERS

- Lugani, Y., Sooch, B.S. (2017). Development of Cell Disruption Strategy for Enhanced Release of Intracellular Xylose Reductase from *Pseudomonas putida* BSX-46. International Journal of Current Microbiology and Applied Sciences. 6(8): 3682-3697.

- Lugani, Y., Sooch, B.S. (2017). *In Silico* Characterization of Xylose Reductase using Computational Tools. International Journal of Advances in Science, Engineering and Technology. 5(2): 59-66.

RELATED REVIEW ARTICLES

- Lugani, Y., Sooch, B.S. (2017). Xylitol, An Emerging Prebiotic: A Review. International Journal of Applied Pharmaceutical and Biological Research. 2(2): 67-73.
- Lugani. Y., Oberoi, S., Sooch, B.S. (2017). Xylitol: A Sugar Substitute for Patients of Diabetes Mellitus. World Journal of Pharmacy and Pharmaceutical Sciences. 6(4): 741-749.

PAPERS PRESENTATION IN INTERNATIONAL/ NATIONAL CONFERENCES/ SEMINARS

1. 'Production of Xylose Reductase from *Candida* sp.' presented in International Symposium on 'Emerging Discoveries in Microbiology' & 56th Annual Conference of Association of Microbiologists of India at School of Life Sciences, Jawaharlal Nehru University, New Delhi on December 7-10, 2015

2. 'Production of Xylose Reductase for Conversion of Xylose into Xylitol' presented in International Conference on 'Recent Advances in Emerging Technologies' at Sri Guru Granth Sahib World University, Fatehgarh Sahib, Punjab on February 23 -24, 2016

3. 'Current Status in Xylitol Production and Future Prospects.' presented in UGC sponsored National Conference on 'Emerging Trends in Biotechnology: A Paradigm Shift to Cleaner and Greener India' at GSSDGS Khalsa College, Patiala, Punjab on October 8, 2016.

4. Enhanced Cell disruption Strategy for release of Xylose Reductase from Novel *Pseudomonas putida* BSX-46 and its Application in Xylitol Production' presented in National conference on 'Recent Advances in Biomedical Science: Diagnostics & Research' by 'The Society of Biomedical Laboratory Scientists, India'at Gandhi Peace Foundation, New Delhi on December16, 2016.

5. '*In Silico* Characterization of Xylose Reductase using Computational Tools' presented in International Conference on 'Advance in Science, Engineering and Technology' at ICASER, Pune on February 19, 2017.

Publications (Reprints)

1. Patent Application Filed

PATENT OFFICE
INTELLECTUAL PROPERTY OFFICE BUILDING
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GOVERNMENT OF INDIA

Docket NO : 67597

To

DR. BALWINDER SINGH SOOCH
ASSISTANT PROFESSOR, DEPARTMENT OF BIOTECHNOLOGY, PUNJABI UNIVERSITY, PATIALA-147002, PUNJAB, INDIA.

Sr. No.	CBR Number	Reference Number /Application Type	Application Number	Title/Remarks	Amount Paid
1	22299	ORDINARY APPLICATION Pages:-7 , Claims:-0,Drawings:-0,Abstract:-0,Claims pages:-0	201811031769	A PROCESS FOR BIOTECHNOLOGICAL PRODUCTION OF XYLITOL	8900
2		E-101/61395/2018-DEL	201811031769	Correspondence	0
3		E-2/1409/2018-DEL	201811031769	Form2	0
4		E-3/33443/2018-DEL	201811031769	Form3	0
5		E-5/961/2018-DEL	201811031769	Form5	0
Total Amount					8900

Received a sum of Rs. 8900 (Rupees Eight Thousand Eight Hundred only) as under

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Draft	State Bank of India	380072	17/08/2018

Note: This is electronically generated receipt hence no signature required.

2. SEQUENCE SUBMITTED

- Sooch, B.S. (2018). 18S RNA sequence of the new isolated strain i.e. *Candida tropicalis* BSS-XR15 isolated under the present project was submitted with GENBANK database of NCBI under accession number KY619688.

Candida tropicalis isolate BSS-XR15 small subunit ribosomal RNA gene, partial sequence

GenBank: KY619688.1

LOCUS KY619688 1004 bp DNA linear PLN 15-FEB-2018
DEFINITION Candida tropicalis isolate BSS-XR15 small subunit ribosomal RNA gene, partial sequence.
ACCESSION KY619688
VERSION KY619688.1
KEYWORDS .
SOURCE Candida tropicalis
ORGANISM Candida tropicalis Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Candida/Lodderomyces clade; Candida.
REFERENCE 1 (bases 1 to 1004)
AUTHORS Sooch,B.S.
TITLE Xylose reductase producing yeast strain
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AUTHORS Sooch,B.S.
TITLE Direct Submission
JOURNAL Submitted (15-FEB-2017) Department of Biotechnology, Punjabi University, Rajpura Road, Patiala, Punjab 147002, India
COMMENT ##Assembly-Data-START##Sequencing Technology, Sanger dideoxy sequencing ##Assembly-Data-END##
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ORIGIN

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Chapter 7
Insights into Fungal Xylose Reductases and Its Application in Xylitol Production

Yogita Lugani and Balwinder Singh Sooch

Abstract Xylose reductase (EC 1.1.1.21), an aldo-keto reductase enzyme, catalyzes the conversion of xylose into xylitol. It is present in animals, plants, and many microorganisms. In microorganisms, in addition to its production by many fungal (yeasts and molds) cultures, a few members of bacteria such as *Corynebacterium* sp. and *Enterobacter* sp. have also been reported to produce NADPH-dependent xylose reductase (XR). In fungi, XR directly converts xylose into xylitol during the metabolism of xylose by using NADH and/or NADPH as coenzyme. The tetrad of amino acids (Tyr, His, Asp, and Lys) at catalytic site is responsible for XR activity. Several attempts have been made to improve XR production using recombinant DNA technology by introducing xylose reductase gene (*xyl1*) into different fungal strains from other microorganisms for efficient conversion of xylose to xylitol. Site-directed mutagenesis at the catalytic site is another approach to increase the turnover number and catalytic efficiency of XRs. Xylitol is a rare pentol sugar whose global market is increasing at a very fast pace due to its applications in food, cosmetic, odontological, pharmaceutical, and medical sector. The microbial production of xylitol is emerging as a good alternative due to abundance of agriculture waste material. The present chapter will describe the different aspects of fungal XRs including their structural characteristics, sources, production, purification and characterization, immobilization, patent status, and xylitol applications.

7.1 Introduction

Xylose reductase (XR) (EC 1.1.1.21), an aldo-keto reductase (*AKR*) enzyme, catalyzes the conversion of xylose into xylitol. *AKRs* are exclusively involved in reversible reduction of aldehydes and/or ketones to their corresponding alcohols by utilizing NADPH and/or NADH as coenzyme. *AKRs* are present in animals, plants,

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2 Agro-industrial lignocellulosic residues for the production of industrial enzymes

Abstract: Recently, the utilization of agro-industrial lignocellulosic waste materials has received much attention for the production of value-added products like enzymes, organic acids and biofuels. Microbial-based environment-friendly approaches are receiving greater attention for the management of these waste materials in both developing and developed countries due to the increase in problems associated with industrialization and environmental pollution. Agro-industrial lignocellulosic biomass is renewable, inexpensive and abundant and hence can be used as a unique natural source for the cost-effective and large-scale production of these valuable products. Microbial and enzyme technology is one of the most promising approaches in the field of industrial biotechnology for the production of numerous commercial products. Currently, enzymes are extensively used in various industrial sectors like food, pharmaceutics, odontology, textile, detergent, bioremediation, paper and pulp, and wine and brewery. Many industries focused on the utilization of agro-based wastes containing lignocellulosic material as a substrate for the large-scale production of industrial enzymes through either solid-state or submerged fermentation. However, biomass transport, proper handling and pretreatment methods for delignification are some of the limitations associated with the use of lignocellulosic materials. Recently, there has been a resurgence in the utilization of various agricultural wastes like sugarcane baggase, wheat straw, wheat bran, rice straw, rice bran, corn cobs, corn stover, banana waste, hardwood, softwood, newspaper, office paper, groundnut shell, coffee by-products, sweet sorghum and grasses for the cost-effective production of enzymes with better yield and novel activity, which can help bring sustainability in the agriculture sector. Further improvements in enzyme technology can be achieved by incorporating desirable characteristics through some latest techniques like *in silico* characterization, regulation of metabolic flux, recombinant DNA technology and microarray technology. This chapter describes the biotechnological importance of the utilization of lignocellulosic biomass for the production of industrial enzymes.

Keywords: Lignocellulose, industrial enzymes, value addition, biocatalyst, agrowaste.

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Development of Cell Disruption Strategy for Enhanced Release of Intracellular Xylose Reductase from *Pseudomonas putida* BSX-46

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Xylose reductase, Xylitol, *Pseudomonas putida*, Cell disruption, Sonication.

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A B S T R A C T

Xylose reductase (XR) is responsible for biotransformation of xylose to xylitol and it has gained significant position amongst industrial enzymes due to its role in meeting the demand of xylitol by utilizing agriculture waste. Xylitol is a rare pentose sugar alcohol having a number of therapeutic and pharmaceutical applications. There is a paradigm shift for biotechnological production of xylitol over conventional chemical method. Amongst biotechnological methods, enzymatic method is efficient over whole cell method for industrial xylitol production due to increased product yield and easy recovery of purified product. But, an efficient cell disruption strategy is critically required for the efficient recovery of intracellular XR from the microbial cell. The focus of present study is to test various physical, chemical and enzymatic methods for the disruption of cells of novel isolated *Pseudomonas putida* BSX-46 for maximum release of xylose reductase. Amongst all the methods adopted, sonication treatment given to cells pretreated with EDTA and β-mercaptoethanol was found to be most effective for maximum release of XR with an activity of 48.70±0.05 IU/mg of cells. The findings from the present study can result in development of an efficient method for making use of XR for industrial production of xylitol.

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IN SILICO CHARACTERIZATION OF XYLOSE REDUCTASE USING COMPUTATIONAL TOOLS

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Abstract - Xylose reductase (EC 1.1.1.21), an industrially important enzyme is produced mainly by bacteria, yeast, mold and algae. The major application of xylose reductase is in the production of xylitol from xylose with the help of NADH and/or NADPH as coenzyme. Xylitol has wide range of applications in industrial sector like food, medical, pharmaceutical, odontological and cosmetics due to its unique properties. The present in silico study deals with the use of bioinformatics tools for the characterization of xylose reductase. Xylose reductase protein sequences from various microorganisms (Candida boidinii Q8X19; Candida tropicalis P87039; Candida parapsilosis Q6Y0Z; Aspergillus niger A2Q8B5; Aspergillus flavus B8N195; Enterobacter aerogenes A0A0H3FX88) were retrieved using Uniprot. The physicochemical properties of xylose reductase from selected microorganisms were computed using Expasy's ProtParam tool. The pI of all the organisms were predicted to be acidic in nature and this enzyme was found to be stable among all the microorganisms except Enterobacter aerogenes A0A0H3FX88. The negative value of GRAVY among all the organisms showed its better interaction with water. Protscale's Kyte and Doolittle hydropathy plots were also computed and the transmembrane region of this enzyme was found to contain more number of hydrophilic amino acids. The secondary structure was predicted using Expasy's GOR IV and SOPMA tools which showed the dominance of random coil conformation in the protein. Multiple sequence analysis was done by Clustal Omega and phylogenic tree was constructed by Mega 6.0 software using Neighbor Joining Method. The 3D structure of xylose reductases were obtained by RaptorX Structure Prediction server.

Keyword - Xylose reductase, ProtParam, Protscale, Mega 6.0, RaptorX