Template/Pro forma for Submission

NMHS-Himalayan Institutional Project Grant

NMHS-FINAL TECHNICAL REPORT (FTR)

Demand-Driven Action Research and Demonstrations

NMHS Reference No.:	NMHS/2017-18/SG22/03	Date of Submission:	d	d	m	m	y	y	y	y

BIOCONVERSION OF PINE NEEDLES: A CHALLENGING WASTE OF HIMALAYAN FOREST TO SECOND GENERATION BIOFUEL

Project Duration: *from* (23rd **February, 2018** *to* (**31st August, 2021**)

***Submitted to*:**
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NMHS-Final Technical Report (FTR) *template*

Demand-Driven Action Research Project

DSL: Date of Sanction Letter
Project Completion

2	3	0	2	**2**	**0**	**1**	**8**
d	d	m	m	y	y	y	y

DPC: Date of

3	1	0	8	**2**	**0**	**2**	**1**
d	d	m	m	y	y	y	y

Part A: Project Summary Report

1. Project Description

i.	Project Reference No.	**NMHS/2017-18/SG22/03**					
ii.	Type of Project	Small Grant		Medium Grant	√	Large Grant	
iii.	Project Title	**Bioconversion of pine needles: a challenging waste of Himalayan forest to second generation biofuel**					
iv.	State under which Project is Sanctioned	**Himachal Pradesh**					

v.	Project Sites (IHR States covered) (Maps to be attached)	Himachal Pradesh and Uttarakhand					
vi.	Scale of Project Operation	Local		Regional		Pan-Himalayan	
vii.	Total Budget/ Outlay of the Project	50.65 lakhs					
viii.	Lead Agency	Dr Y S Parmar University of Horticulture & Forestry, Nauni, Solan, H.P., India.					
	Principal Investigator (PI)	Dr Nivedita Sharma					
	Co-Principal Investigator (Co-PI)	None					
ix.	Project Implementing Partners	-					
	Key Persons / Point of Contacts with Contact Details, Ph. No, E-mail						

2. Project Outcomes

2.1. Abstract

In the present investigation, an attempt was made to develop a microbial technology for bioethanol production from pine needles biomass from a baseline model. The major activities successfully accomplished in the project were saccharification of pine needles biomass using purified hydrolytic enzymes i.e. cellulase and xylanase. Two hyper hydrolytic enzyme producer strains i.e. *B. stratosphericus* N12 (M) and *B. altitudinis* Kd1 (M) have been used for inhouse enzyme production. Optimization of process parameters using classical one factor at a time (OFAT) and statistical model- RSM to enhance the yield of sugars production has been done. Different process parameters in OFAT had enhanced saccharification of pine needles and maximum reducing sugars yield achieved was 28.05 mg/g of biomass. The optimized conditions of OFAT were further subjected to optimization using statistical approach i.e. Response surface Methodology (RSM). Further an appreciable increase in reducing sugars i.e. 33.21 mg/g with overall 453.50 % through RSM was achieved. Quantitative analysis of sugars obtained during saccharification of biomass by crude, partially purified and purified enzymes by using HPLC technique has also been done. Fermentation process was devised by using a co-culture combination of Ethanologens (*Saccharomyces cerevisiae* and *Pichia stipitis*) with maximum ethanol and fermentation efficiency of 69.47 %. The optimized conditions were further subjected to the scale up process in a stirred tank bioreactor (7.5 litres). Standardization of scale up process parameters i.e. fermentation time, temperature and agitation rate have been accomplished in a bioreactor to maximize the bioethanol production. Saccharification of 1 kg pine needles biomass using hydrolytic enzymes i.e. cellulase and xylanase. Scale up process was transferred to the bioreactor and after optimizing process parameters, maximum of ethanol 18.96 g/l with fermentation efficiency of 72.54% was achieved. Increased saccharification and ethanol yield, higher fermentation efficiency and considerable reduction in fermentation time are the main highlighting features of the present study.

2.2. Objective-wise Major Achievements

S. No.	Objectives	Major achievements (in bullets points)
1.	Bioconversion of pine needles into ethanol by using suitable microbial technology	• The potential of pine needles waste as a substrate for biofuel i.e. bioethanol production has been successfully evaluated at small scale (1kg) in 7.5 litre capacity bioreactor with working capacity of 3.0 litre in our Microbiology Laboratory, UHF, Nauni, Solan reflecting the strong possibility of transforming it further as an innovative technology for biorefineries. • Small scale model for bioconversion of pine needles into ethanol has been developed.

2.	Scale up of the process for commercialization	• Scale up process was done using 1 KG biomass for enzymatic saccharification and further fermentation in 7.5 litre capacity bioreactor with working capacity of 3.0 litre and after optimizing process parameters, maximum of ethanol 18.96 g/l with fermentation efficiency of 72.54% was achieved. • The same technology will be shifted to scale up and validation process from 1 kg to 100 kg pine needles biomass thus it will be bear a high scope for biorefineries at commercial scale

2.3. Outputs in terms of Quantifiable Deliverables*

S. No.	Quantifiable Deliverables*	Monitoring Indicators*	Quantified Output/ Outcome achieved	Deviations made, if any, & Reason thereof:
1.	Development of the pilot model on Bioconversion of pine needles into ethanol as 2nd generation biofuel	• No. of new database/datasets generated on the identified dynamics (No.)	04 No. Genomic Sequences of *B. stratosphericus* N12 (W), *B. stratosphericus* N12 (M), *B. altitudinis* Kd1 (W) and *B. altitudinis* Kd1 (M) have been submitted to NCBI, US with their respective accession numbers. (i) *B. stratosphericus* N12 (W) Acc. no. [KC995116] *(ii) B. stratosphericus* N12 (M) Acc. no. [KC995118] (iii) *B. altitudinis* Kd1 (W) Acc. no. [KC995115] (iv) *B. altitudinis* Kd1 (M) Acc. no. [KC995117] The potential of pine needles waste as a substrate for biofuel i.e. bioethanol production has been successfully evaluated at small scale (1kg) in 7.5 litre capacity bioreactor with working capacity of 3.0	No deviations

2.	Scale up of the process through bio-digester for commercialization of green fuel for mitigation of pollution	• Periodic updates on region-specific best practice/ model developed (no.)along with the supporting manual (no.) published • No. of stakeholders benefitted (no. of rural youth, no. of women and total no. of beneficiaries	06 publications • Scale up process was done using 1 KG biomass for enzymatic saccharification and further fermentation in 7.5 litre capacity bioreactor with working capacity of 3.0 litre and after optimizing process parameters, maximum of ethanol 18.96 g/l with fermentation efficiency of 72.54% was achieved.	
3.	Manual on bioconversion of pine needles into ethanol	• Policy framework/ draft (no.) for assisting in scaling up the process for commercialization • Other publications and knowledge products (no.)	• The developed technology will be shifted to scale up and validation process from 1 kg to 100 kg pine needles biomass thus it will be bear a high scope for biorefineries at commercial scale	Scale up and validation of the process for commercialization from 1 Kg biomass to 50 and 100 Kg biomass.

(*) As stated in the Sanction Letter issued by the NMHS-PMU.

2.4. Strategic Steps with respect to Outcomes (in bullets)

S. No.	Particulars	Number/ Brief Details	Remarks/ Attachment
1.	New Methodology developed	04 No. 1. Enzyme production 2. Saccharification of pine needles by optimization of process parameters 3. Fermentation of reducing sugars by optimization of different parameters 4. Scale up of the process in a stirred tank bioreactor under optimized conditions	
2.	New Models/ Process/ Strategy developed	01 A suitable microbial technology for bioconversion of pine needles into bioethanol has been developed	
3.	New Species identified	07	
4.	New Database established	07	
5.	New Patent, if any	Nil	
	I. Filed (Indian/ International)	Nil	
	II. Granted (Indian/ International)	Nil	
	III. Technology Transfer(if any)	Nil	
6.	Others (if any)		

3. Technological Intervention

S. No.	Type of Intervention	Brief Narration on the interventions	Unit Details (No. of villagers benefited / Area Developed)
1.	Development and deployment of indigenous technology	Nil	
2.	Diffusion of High-end Technology in the region	Nil	
3.	Induction of New Technology in the region	02 1. A suitable microbial technology for bioconversion of pine needles into bioethanol has been developed 2. Scale up of the process in a stirred tank bioreactor	

4.	Publication of Technological / Process Manuals		
	Others (if any)		

4. New Data Generated over the Baseline Data

S. No.	New Data Details	Status of Existing Baseline	Additionality and Utilisation New data
1.	Hydrolytic enzyme production from potential microbial strains	Five bacterial isolates and two fungal strains were screened out depending upon their hyper enzyme production from several hundreds of microorganisms isolated from different sources in our research laboratory. These strains were used for the hydrolytic enzyme production under the present project.	
2.	A microbial technology has been developed for 2nd generation biofuel i.e. bioethanol production from pine needles biomass.	A baseline model for further scale up process of bioethanol production.	
3.	Scale up of the process in a stirred tank bioreactor	Already optimized shake flask conditions were transferred to the bioreactor for scale up the process	

5. Demonstrative Skill Development and Capacity Building/ Manpower Trained

S. No.	Type of Activities	Details with number	Activity Intended for	Participants/Trained			
				SC	**ST**	**Woman**	**Total**
1.	Workshops	**NIL**					
2.	On Field Trainings	**NIL**					
3.	Skill Development	**NIL**					
4.	Academic Supports	**-**					
	Others (if any)						

6. Linkages with Regional & National Priorities (SDGs, INDC, etc)/ Collaborations

S. No.	Linkages /collaborations	Details	No. of Publications/ Events Held	Beneficiaries
1.	Sustainable Development Goal (SDG)	Nil		
2.	Climate Change/INDC targets	Nil		
3.	International Commitments	Nil		
4.	Bilateral engagements	Nil		
5.	National Policies	Nil		
6.	Others collaborations	Nil		

7. Project Stakeholders/ Beneficiaries and Impacts

S. No.	Stakeholders	Support Activities	Impacts
1.	Gram Panchayats	NA	
2.	Govt Departments (Agriculture/ Forest)	NA	
3.	Villagers	NA	
4.	SC Community	NA	
5.	ST Community	NA	
6.	Women Group	NA	
	Others (if any)	-	

8. Financial Summary (Cumulative) April, 2018- March, 2021

S. No.	Financial Position/Budget Head	Funds Received	Expenditure/ Utilized	% of Total cost
I.	Salaries/Manpower cost	16,11,052	1356516	
II.	Travel	65781	7903	
III.	Expendables &Consumables	1124680	1105504	
IV.	Contingencies	240442	168901	
V.	Activities & Other Project cost	-	-	
VI.	Institutional Charges	468500	-	
VII.	Equipments	1300000	1225000	
	Total	**48,10,455**	**38, 63, 824**	
	Interest earned	81592		
	Grand Total	**48,92,047**		

* Please attach the consolidated and audited Utilization Certificate (UC) and Year wise Statement of Expenditure (SE) separately, *ref*. **Annexure I.**

9. Major Equipment/ Peripherals Procured under the Project** (if any)

S. No.	Name of Equipments	Cost (INR)	Utilisation of the Equipment after project
1.	Spectrophotometer-20D	48,000.00 rupees	
2.	Digital autoclave	2,92,000.00 rupees	
3.	Deep freezer	1,50,000.00 rupees	
4.	High speed Centrifuge	7,35,000.00 rupees	

Details should be provided in details **(*ref* **Annexure III &IV).**

10. Quantification of Overall Project Progress

S. No.	Parameters	Total (Numeric)	Remarks/ Attachments/ Soft copies of documents
1.	IHR States Covered	*Himachal*	
2.	Project Site/ Field Stations Developed	*Himachal Pradesh and Uttarakhand*	
3.	New Methods/ Modeling Developed	*04*	
4.	No. of Trainings arranged	-	
5.	No of beneficiaries attended trainings	-	
6.	Scientific Manpower Developed (PhD/ M.Sc./JRF/SRF/ RA):	*01 (RA)* *01 (M.sc.)* *01 (PhD)*	
7.	SC stakeholders benefited	*NIL*	
8.	ST stakeholders benefited	*NIL*	
9.	Women Empowered	*NIL*	
10.	No of Workshops Arranged along with level of participation	*NIL*	
11.	On field Demonstration Models initiated	*NIL*	
12.	Livelihood Options promoted	*NIL*	
13.	Technical/ Training Manuals prepared	*NIL*	
14.	Processing Units established	*NIL*	
15.	No of Species Collected	*More than hundred*	
16.	New Species identified	*07*	
17.	New Database generated (Types):	*07*	
	Others (if any)		

11. Knowledge Products and Publications:

S. No.	Publication/ Knowledge Products	*Number*		*Total Impact Factor*	*Remarks/* Enclosures
		National	International		
1.	Journal Research Articles/ Special Issue:	06			
2.	Book Chapter(s)/ Books:	Nil			
3.	Technical Reports	Nil			
4.	Training Manual (Skill Development/ Capacity Building)	Nil			
5.	Papers presented in Conferences/Seminars	04			
6.	Policy Drafts/Papers	Nil			
7.	Others:	Nil			

* Please append the list of KPs/ publications (with impact factor and further details) with due Acknowledgement to NMHS.

12. Recommendation on Utility of Project Findings, Replicability and Exit Strategy

Particulars	Recommendations
Utility of the Project Findings	❖ The outcome of the present work will result not only in providing a safe and environment friendly source of energy, but also in the conservation of environment and biodiversity due to reduction of frequent forest fires. ❖ Thus the development of a technology for effectively converting a challenging waste of Himalayan forest to simple sugars by potential inhouse enzymes produced from isolated microorganisms and intern fermenting them to appreciable concentration of ethanol fulfils the main aim of our study, thus envisaging sustainable energy production and improved environmental quality.
Replicability of Project	Validation of developed microbial technology and Scale up of pilot plant model for commercialization

(**PROJECT PROPONENT/ COORDINATOR**)

(Signed and Stamped)

(**HEAD OF THE INSTITUTION**)

(Signed and Stamped)

Place: ……..
Date: …../……/……

PART B: PROJECT DETAILED REPORT

1 EXECUTIVE SUMMARY

In the present investigation, an attempt was made to develop a suitable microbial technology for the conversion of pine needle biomass into 2nd generation biofuel: bioethanol from baseline model through scale up of the process. Five bacterial isolates and two fungal strains were screened out depending upon their hyper enzyme production from several hundreds of microorganisms isolated from different sources in our research laboratory. All these selected potential microbial strains were identified on the basis of phenotypic as well as genotypic characterization and had been identified as *B. stratosphericus* N12 (M), *B. simplex* SD5, *B. altitudinis* Kd1 (M), *B. tequilensis* SH0, *B. subtilis* SD8, *R. oryzae* RF1 and *R. delemar* F2. Two potential hyper enzyme producer bacterial strains i.e. *B. stratosphericus* and *B. altitudinis* had been modified by inducing mutation and were found stable for 8 generations. Optimization of different process parameters was done under two different modes i.e. submerged fermentation for bacteria and solid state fermentation for fungal strains by using OFAT approach and achieved approximately 80.0- 200.00 percent increase in enzymes production. Under SmF, purified cellulase of *B. stratosphericus* N12 (M) and *B. simplex* SD5 has expressed 78.20 U/mg and 6.50 U/mg specific activities respectively. In case of purified xylanase *B. altitudinis* Kd1 (M), *B. subtilis* SD8 and *B. tequilensis* SH0 had depicted 481.14 U/mg, 266.29 U/mg and 108.80 U/mg respectively. On the other hand under SSF, cellulase (1.125 U/mg and 1.00 U/mg specific activities) and xylanase (20.10 U/mg and 19.65 U/mg) were obtained from fungal isolates *Rhizopus oryzae* RF1 and *R. delemar* F2 respectively.

Saccharification of pine needles biomass using purified hydrolytic enzymes i.e. cellulase and xylanase. Two hyper hydrolytic enzyme producer strains i.e. *B. stratosphericus* N12 (M) and *B. altitudinis* Kd1 (M) have been used for inhouse enzyme production. Optimization of process parameters using classical one factor at a time (OFAT) and statistical model- RSM to enhance the yield of sugars production has been done. Different process parameters in OFAT had enhanced saccharification of pine needles and maximum reducing sugars yield achieved was 28.05 mg/g of biomass at enzyme dosage of 12.5 ml/g in the ratio of 7.75 : 4.75 (cellulase: xylanase) after 72 h of enzymatic hydrolysis at 45°C temperature with purified enzymes. The optimized conditions of OFAT were further subjected to optimization using statistical approach i.e. Response surface Methodology (RSM). Further an appreciable increase in reducing sugars i.e. 33.21 mg/g with overall 453.50 % through RSM was achieved. Quantitative analysis of sugars obtained during saccharification of biomass by crude, partially purified and purified enzymes by using HPLC

technique has also been done. Further fermentation of reducing sugars into bioethanol was done by using monoculture of *Saccharomyces cerevisiae* and *Pichia stipitis* as well as co-culture combinations of ethanologens (*Saccharomyces cerevisiae* + *Pichia stipitis*) in shake flask at 25 ◦C for 72 h and the maximum ethanol i.e. 11.06 g/l was observed in co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis* which was selected for further studies. Fermentation process was devised by using a co-culture combination of Ethanologens (*Saccharomyces cerevisiae* and *Pichia stipitis*) with maximum ethanol (16.44 g/l) and fermentation efficiency of 69.47 %. The optimized conditions were further subjected to the scale up process in a stirred tank bioreactor (7.5 litres). Standardization of scale up process parameters i.e. fermentation time, temperature and agitation rate have been accomplished in a bioreactor to maximize the bioethanol production. The best conditions for scale up of bioethanol production in stirred tank bioreactor were 30 h of fermentation time, 25◦C temperature and 200 rpm at which the maximum of bioethanol i.e. 18.96 g/l with fermentation efficiency of 72.54 %. Increased saccharification and ethanol yield, higher fermentation efficiency and considerable reduction in fermentation time are the main highlighting features of the present study.

Saccharification of 1 kg pine needles biomass using hydrolytic enzymes i.e. cellulase and xylanase. Two hyper hydrolytic enzyme producer strains i.e. *B. stratosphericus* N12 (M) and *B. altitudinis* Kd1 (M) have been used for inhouse enzyme production. During Scale up of the process upto 1 kg biomass in flask, maximum ethanol of 16.44g/l with fermentation efficiency of 69.93 % was achieved. Scale up process was transferred to the bioreactor and after optimizing process parameters, maximum of ethanol 18.96 g/l with fermentation efficiency of 72.54% was achieved.

2. INTRODUCTION

2.1 Background of the Project

The world is facing a reduction of global fossil fuels resources, like petroleum, natural gas, or charcoal, while energy requirements are progressively growing up. Fossil fuels should be replaced, at least partially by biofuels once the current fuel supply is suspected to be unsustainable in the foreseen future. In fact, the search for sustainable alternatives to produce fuel and chemicals from non-fossil feedstocks has attracted considerable interest around the world, to face the needs of energy supply and to response to climate change issues. Alternative resources of energy are being explored in order to reduce oil dependence and increase energy production by exploring of solar, wind, hydraulic and other natural phenomena. Besides these sources of energy, also biomass possesses a potential target for

fuel and power production. Thus biomass can efficiently replace petroleum-based fuels for a long term. Bioethanol is one of the products that can be obtained via biorefinery using bio-based resources. It is one of the most attractive biofuels, since it can be easily produced in large amounts and used as a “green” fuel. Gasohol with a blending of ethanol up to 20% in petrol by 2025 is a mandate of National Fuel policy. A huge demand of ethanol thus arising laid pressure on food items like sugarcane, rice and maize (1^{st} generation biofuel). 2^{nd} generation biofuels have an edge over 1^{st} generation biofuels being produced from non-food chain items viz. woody waste- pine needles. This woody waste is rich in complex carbohydrates- cellulose and hemicellulose upto 60- 80% apart from lignin. These complex carbohydrates can be broken down to simple monomeric sugars (glucose and xylose) with the help of hydrolytic enzymes- cellulase and xylanase synthesized by specialized microorganisms.

2.2 Overview of the Major Issues to be Addressed

Himachal Pradesh has reported extent of recorded forest area (RFA) 37,033 sq km which is 66.52% of its geographical area. The reserved, protected and unclassed forests are 5.13%, 89.46% and 5.41% of the recorded forest area in the State respectively. The total forest area of Himachal Pradesh is 37, 033 km2 out of which 1, 46,000 ha is sensitive to fire. Mid-hills zone of Himachal Pradesh encompasses ten per cent of total geographical area of state and is highly prone to forest fire. Forest fires have caused extensive destruction in recent years leading to loss of wildlife habitat, biodiversity, change in micro-climate, adverse effect on livelihood of people, addition of green-house gases etc. Average estimated loss due to forest fire in Himachal Pradesh is INR 113 million per annum. Mid-hills zone of Himachal Pradesh is highly prone to forest fires during summer due to the presence of conifer species like pine due to shedding of highly inflammable chir pine needles. According to the Himachal forest department, forest fires cause an estimated loss of several crore rupees each year. From 2016-17 to 2019-20, the annual loss to the state due to forest fires has ranged from Rs 1.7 crore to Rs 3.5 crore. The main reasons for the enhanced development of bioethanol are its use as a favorable and near carbon-neutral renewable fuel, thus reducing CO_2 emissions, enhance octane in unleaded gasoline, reducing tailpipe pollutant emissions and improving the ambient air quality.

2.3 Baseline Data and Project Scope

In the present investigation, an attempt was made to develop a baseline model for further scale up process of bioethanol production from pine needles. Five bacterial isolates and two fungal strains were screened out depending upon their hyper enzyme production from several hundreds of microorganisms isolated from different sources in our research laboratory. All these selected potential microbial strains were identified on the basis of phenotypic as well as genotypic characterization and had been identified as *B. stratosphericus* N12 (M), *B. simplex* SD5, *B. altitudinis* Kd1 (M), *B. tequilensis* SH0, *B. subtilis* SD8, *R. oryzae* RF1 and *R. delemar* F2. Two potential hyper enzyme producer bacterial strains i.e. *B. stratosphericus* and *B. altitudinis* had been modified by inducing mutation and were found stable for 8 generations. Optimization of different process parameters was done under two different modes i.e. submerged fermentation for bacteria and solid state fermentation for fungal strains by using OFAT approach and achieved approximately 80.0- 200.00 percent increase in enzymes production. Under SmF, purified cellulase of *B. stratosphericus* N12 (M) and *B. simplex* SD5 has expressed 78.20 U/mg and 6.50 U/mg specific activities respectively. In case of purified xylanase *B. altitudinis* Kd1 (M), *B. subtilis* SD8 and *B. tequilensis* SH0 had depicted 481.14 U/mg, 266.29 U/mg and 108.80 U/mg respectively. On the other hand under SSF, cellulase (1.125 U/mg and 1.00 U/mg specific activities) and xylanase (20.10 U/mg and 19.65 U/mg) were obtained from fungal isolates *Rhizopus oryzae* RF1 and *R. delemar* F2 respectively.

2.4 Project Objectives and Target Deliverables (as per the NMHS Sanction Order)

Project Objectives	Target Deliverables
• Bioconversion of pine needles into ethanol by using suitable microbial technology • Scale up of the process for commercialization	• Development of the pilot model on Bioconversion of pine needles into ethanol as 2nd generation biofuel • Scale up of the process through bio-digester for commercialization of green fuel for mitigation of pollution • Manual on bioconversion of pine needles into ethanol

3. METHODOLOGIES, STARTEGY AND APPROACH

3.1 Methodologies used for the study

1. Collection of Biomass: Pine needles were collected from the forests of adjoining Himalayas and brought to the laboratory. Biomass was washed with tap water and dried at 60^0 C temperature in the oven. Dried biomass was chopped into small pieces and then grinded into 2 mm sieve size and stored for the further experiments.

2. Microbial strains used for hydrolytic enzymes production: (a) Bacterial strains

Sr No.	Name	Accession No.	Source
1.	*Bacillus stratosphericus* N12 (M)	KC995118 9 [NCBI, US]	soil
2.	*Bacillus simplex* SD5	KF844070 [NCBI, US]	soil
3.	*Bacillus subtilis* SD8	KF844068 [NCBI, US]	soil
4.	*Bacillus altitudinis* Kd1 (M)	KC995117 [NCBI, US]	soil
5.	*Bacillus tequilensis* SH0	. JX129359.1 [NCBI, US]	compost

(a) Fungal strains

Sr No.	Name	Accession No.	Source
1.	*Rhizopus oryzae* RF1	KJ192199	soil
2.	*Rhizopus oryzae* F2	KX5123312	soil

3. Production of hydrolytic enzymes from potential bacterial strains under submerged fermentation (SmF)

3.1 Inoculum Preparation: Each bacterial strain was grown in 100 ml of nutrient broth at 35±2°C for 24 h. As soon as the substantial growth was observed in the broth, the optical density was set to 1.0 using autoclaved distilled water.

3.2 Enzyme production: 5 ml of inoculum was added to each 45 ml of specific broth media in 250 ml of Erlenmeyer flasks and the flasks were incubated their optimized incubation days at 35±2°C. After incubation, the culture contents were centrifuged at 10,000 rpm for 15 min (4°C). The supernatant was collected and enzyme assays and protein estimation was done.

3.3 Enzyme Assays:

(i) CMCase assay (Reese & Mandel, 1963)

(ii) FPase assay (Reese & Mandel, 1963)

(iii) ß-Glucosidase assay (Berghem and Petterson, 1973)

3.4 Xylanase Assay: Dinitrosalicylic acid (DNSA) method (Miller, 1959)

3.5 Protein Assays: Lowry's method (Lowry *et al*., 1951)

4. Production of hydrolytic enzymes (cellulase and xylanase) from potential fungal strains under solid state fermentation (SSF)

4.1 Fungi used: *Rhizopus oryzae* RF1 [Accession No. KJ192199] and *R. delemar* F2 [Accession No. KX5123312] fungi were used as a source of cellulase and xylanase production in the present study.

4.2 Cellulase and xylanase production by *Rhizopus oryzae* RF1 and *R. delemar* F2

To 5 g of each untreated and microwave pretreated biomass, 10 ml of moistening agent *viz.* Basal salt medium was added (in the ratio of 1:6 i.e., substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 1×10^7 spores/ml of *Rhizopus oryzae* RF1 and *R. delemar* F2 and incubated at 35°C for 7 days, 15 days, 21 days and 28 days in static phase.

4.3 Extraction of cellulase and xylanase enzyme by Repeated Extraction method (Bollag & Edelstein, 1993)

To 5 g of each untreated and microwave pretreated biomass, 50 ml of phosphate buffer (0.1M, pH 6.9) was added and the contents were kept in the shaker for 1 h at 120 rpm and then filtered through muslin cloth. The process was repeated twice with 50 ml of phosphate buffer. After filteration, contents were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and enzyme assays were performed.

(a) Enzyme Assays:

(iv) CMCase assay (Reese & Mandel, 1963)

(v) FPase assay (Reese & Mandel, 1963)

(vi) ß-Glucosidase assay (Berghem and Petterson, 1973)

(b) **Xylanase Assay:** Dinitrosalicylic acid (DNSA) method (Miller, 1959)

(c) **Protein Assays:** Lowry's method (Lowry *et al*., 1951)

5. Partial Purification by Ammonium sulphate precipitation

Different concentrations of ammonium sulphate i.e. 0-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90% were evaluated to attain saturation point for each of cellulase subunits i.e. CMCase, FPase and β-glucosidase. The preparations

were kept at 4°C for overnight and centrifuged which resulted in separation of pellets and supernatants. CMCase and FPase were precipitated at 30-60%, β-glucosidase at 0-30% and xylanase at 0-70 % level of saturation of ammonium sulfate. Precipitates of each subunit so obtained were dissolved in phosphate buffer (0.1 M, pH 6.9) separately.

6. Purification of enzymes by Gel Filtration Column chromatography (Bollag, 1991)

Sephadex G-100 (5g) was suspended in 500 ml of distilled water for 24 h. It was packed into the glass column having dimensions of (31x2.5 cm). It was equilibrated with three bed volumes of 0.1M Phosphate buffer (pH 6.9). Partially purified protein sample (2 ml) was loaded on the Sephadex G-100 column. It was eluted with three bed volumes of 0.1M phosphate buffer (pH 6.9) and 3 ml fractions were collected. A flow rate of 3 ml in 7 min was maintained. The protein content of collected fractions was measured at 280 nm and fractions showing maximum absorbance were analyzed for enzyme activity. The most active fractions were pooled and stored at 4°C.

7. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

7.1 Equipment

Minigel apparatus, Power supply (200 V, 500mA), Boiling water bath, eppendorf centrifuge (Sigma), Hamilton syringes (50 µl and 100 µl capacity), Small glass or plastic container with lid Eppendorf tubes, Rocking or rotary shaker

7.2 Working Solution

Solution A (Acrylamide stock solution). 100 ml 30 % w/v acrylamide, 0.8 % (w/v) bisacrylamide

Solution B (4 x separating gel buffer), 100 ml, 75 ml 2 M Tris HCl pH 8.8, 4 ml 10 % SDS and 21 ml H_2O.

Solution C (4 x stacking gel buffer: 50 ml 1 M Tris HCl (pH 6.8), 4 ml 10 % SDS , 46 ml H_2O, 10 % ammonium persulfate, 5 ml, 0.5 g ammonium persulfate, 5 ml H_2O

Electrophoresis buffer

3 g Tris, 14.4 g Glycine, 1g SDS, H_2O to make 1 litre and pH should be approximately 8.3

5 x sample buffer: 0.6 ml 1 M Tris -HCl pH 6.8, 50 % glycerol, 10 % SDS, mercaptoethanol, 1% bromophenol blue, H_2O

7.3 Assembling of Gel Sandwich

For minigel, bottom of both gel plates and spacer were perfectly flushed against a flat surface before tightening lamp assembly. Solutions A and B and water was combined in a small Erlenmeyer flask or a test tube. Ammonium persulfate and TEMED were added and mixed by swirling or inverting container gently. Gel solution was introduced into gel sandwich using a pipette. When appropriate amount of separating gel solution had been added, 1 cm of water was gently layered on top of separating gel solution to keep the gel surface flat. Gel was allowed to polymerize (30 – 60 min). When the gel has polymerized, a distinct interface appeared between the separating gel.

7.4 Pouring of Stacking Gel

Water covering the separating gel was poured off. Solution A, C and water were combined in a test tube. Ammonium persulfate and TEMED was added and mixed by gently swirling or inverting the container. Stacking gel solution was pipette onto separating gel until solution reached top of front plate. The comb was inserted into gel sandwich until bottom of teeth reached top of front plate. Care was taken in order to avoid making of any bubbles. Staking gel was allowed to polymerize (about 30 minutes). After staking gel polymerized, the comb was removed carefully. The gel was placed into electrophoresis chamber. Electrophoresis buffer was added into the buffer tank making sure that both top and bottom of gel were immersed in buffer. Air bubbles clinging to bottom of gel were removed to ensure even current flow.

7.5 Preparation and Loading of Samples

Protein sample and 5x sample buffer (20µl + 5µl) were combined in an eppendorf tube and heated at 100^{o}C for 2-10 min. This sample solution was spinned for 1 sec in microfuge. Sample solution was introduced into well using Hamilton syringe. Molecular weight standards were run in one side of the well.

7.6 Running of Gel

Electrode plugs were attached to proper electrodes. Current was allowed to flow towards anode. Power supply was turned on to 200 V. The dye front was allowed to migrate to 1 cm from the bottom of the gel in 30 - 40 min for two 0.75 mm gels. Power supply was turned off. Electrode plugs were removed from electrodes. Gel plates were removed from electrode assembly. A spacer was removed carefully and the space inserted in one corner between the plates gently used to apart the gel plates. The gel sticked to one of the plate.

7.7 Staining of a Gel with Coomassie Blue

Method of staining was used to detect as little as 0.1 µg of protein in a single band. Gel was picked up and transferred to a small container containing coomassie staining (approx. 20 ml). It was agitated for 5-10 min on slow rotatory shaker. Stain was poured out. Coomassie destain was added about 50 ml. To destain completely, destain solution was changed 3 times after 20-20 min and agitated overnight. After destaining clear bands of both the isolates appeared on the gel indicating homogeneity of protein.

7.8 Molecular Weight Determination

The molecular weight of partially purified xylanase was determined with the help of molecular marker ranging between 14.3 kDa- 97.4 kDa.

8. Optimization of process parameters for enzymatic hydrolysis of biomass

The optimization of enzymatic hydrolysis of biomass was carried out for microwave irradiation dose, incubation period, enzyme dosage, enzymatic ratio and temperature by one factor at a time approach (OFAT).

8.1 Inhouse enzyme cocktail preparation

The inhouse enzymes which were prepared had been mixed in the ratio of (3:2) i.e. 3.0 ml of cellulase from cellulase from *B. stratosphericus* N_{12} (M) (CMCase: 1.706 IU, FPase: 2.008 IU and β-glucosidase: 0.196 IU) and 2.0 ml of xylanase from *B. altitudinis* Kd_1 (M) (41.86 IU) and enzymatic dose was adjusted @ 1ml/g of biomass for hydrolysis.

8.2 Optimization of microwave irradiation dose

1 g untreated dried lignocellulosic biomass was taken in different petriplates and subjected to different doses of microwave irradiation i.e. 100, 300, 600 and 900 W for different time intervals of 4 min and 5 min. Sodium citrate buffer (0.05 M, pH 5.5) was added as moistening agent in 1:4 ratio. Purified enzymatic mixture of different inhouse hydrolytic enzymes (cellulase: xylanase) in 3:2 @ 5ml/g dose was employed for biomass hydrolysis at 50◦C temperature for 72 h of incubation period. After incubation reducing sugars were estimated (Miller, 1959).

8.3 Optimization of incubation period

To each 1 g untreated and microwave (600 W, 4 min) pretreated biomass was taken and to these sodium citrate buffer (0.05 M, pH 5.5) was added and autoclaved. Inhouse enzymes in the ratio of 3:2 (cellulase: xylanase) @ 5.0 ml/g dose was added to each flask

under sterile conditions and incubated at optimum temperature, 50°C. The hydrolysis period was varied from 24 h, 48 h, 72 h, 96 h and 120 h for enzymatic hydrolysis. After incubation period, biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars.

8.4 Optimization of enzymatic dose

To each 1 g untreated and pretreated biomass sodium citrate buffer (1:4 ratio) was added and autoclaved. Inhouse enzymes in the ratio of 3:2 (cellulase: xylanase) was added in different doses i.e. 5.0 ml/g, 7.5 ml/g, 10.0 ml/g, 12.5 ml/g and 15.0 ml/g were added to each flask under sterile conditions and incubated at temperature, 50°C for 72 h. After 72 h, saccharified biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars.

8.5 Optimization of enzymatic ratio

To each 1 g untreated and pretreated biomass sodium citrate buffer (0.05M, pH 5.5) was added and autoclaved. Then enzymatic mixture of inhouse enzymes in different ratio i.e. 6.25: 6.25, 6.75: 5.75, 7.25: 5.25, 7.75: 4.75 and 8.25: 4.25 @ 12.5 ml/g doses were added for hydrolysis and the flasks were incubated at 45°C for 72 h to undergo enzymatic hydrolysis. After 72 h, biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars.

8.6 Optimization of temperature

To each 1 g untreated and microwave pretreated biomass sodium citrate buffer (0.05 M, pH 5.5) and autoclaved. The best selected enzymatic ratio of 7.75: 4.75 (cellulase: xylanase) with optimum enzyme dose @ 12.5 ml/g was added to each flask under sterile conditions. The flasks were incubated at different temperatures i.e. 35°C, 40°C, 45°C, 50°C and 55°C for 72 h to undergo enzymatic saccharification. After 72 h, saccharified biomass was filtered and centrifuged at 10,000 rpm for 10 min. The supernatant was used for estimation of reducing sugars.

9. Enzymatic saccharification of pine needles biomass

Enzymatic saccharification of untreated and pre treated pine needles biomass using crude, partially purified and purified inhouse enzymes cocktail by applying previously optimized conditions i.e. enzyme dosage of 12.5 ml/g in the ratio of 7.75:4.75 (cellulase: xylanase) for 72 h of enzymatic hydrolysis at 45◦C has been done and reducing sugars were estimated (Miller, 1959).

10. Optimization of process parameters for saccharification of pine needles biomass using Response Surface Methodology (RSM) approach

In this method, prior knowledge of significant conditions obtained from previous One Variable at a Time (OVAT) approach experiment had been necessary for achieving a more realistic model. RSM based on Central Composite Design was used for the optimization of independent variables for reducing sugar production in untreated and pretreated pine needles biomass. Following parameters were optimized by using a statistical approach i.e. Response Surface Methodology (RSM)

- Incubation time
- Enzyme dose
- Temperature

➢ Three independent variables were chosen for optimization studies by employing Central Composite Design (CCD) of Response Surface Methodology (RSM). The experiment contained 20 runs. The design involved 6 centre points, 14 non centre points. The mathematical relationship of response (reducing sugars) and variables i.e. A, B and C was approximated by a quadratic model equation. The optimization of enzymatic hydrolysis of biomass was carried out for three independent variables (A) incubation time (low-36, high-60 hours), (B) enzymatic dose (low-10, high-15 ml/g) and (C) temperature (low- 40, high-50◦C) following the CCD of Response Surface Methodology (RSM) experimental design.

11. Quantitative analysis of sugars to estimate 5 and 6 C sugars by High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography was performed for the estimation of 6 C (glucose) and 5 C (xylose) sugars produced in saccharified solution during enzymatic saccharification of pine needles biomass. Saccharification of biomass was done by applying crude, partially purified and purified enzymes and sugars were quantified through HPLC under the following conditions.

Formula to calculate the concentration of 5-HMF

$$\text{Concentration of 5-HMF} = \frac{\text{Area of Sample}}{\text{Area of Standard}} \times \text{Standard dilution (100 ppm)}$$

12. Fermentation of reducing sugars into bioethanol

12.1 **Ethanologens used:** co-culture combination of *Saccharomyces cerevisiae*(MTCC 3089) + *Pichia stipitis* (NCIM 3498)

12.2 Fermentation conditions: 72 h of fermentation time at 25◦C temperature

12.3 Ethanol estimation:

34.0 g of potassium dichromate was dissolved in 500ml of distilled water. To this 375 ml of concentrated sulphuric acid was added, mixed thoroughly and allowed to cool. Final volume was made 1000ml by adding distilled water. To the distillation flask 29 ml of distilled water and 1 ml of sample was added. On the other side to the 50 ml volumetric flask 25 ml of potassium dichromate was added. Distillation was set at 60^0C and tap water was turned ON. To the 25 ml of potassium dichromate, 20 ml of distilled sample was collected and it became total 45 ml. To this 45 ml solution 5 ml of distilled water was added and total volume became 50 ml and was incubated at 60^oC for 20 min. After that O.D. was measure at 600 nm against blank (Caputi *et al.,* 1969).

Bioethanol was estimated in terms of g/l of fermented liquor and g/g of biomass on dry weight basis. Fermentation efficiency was calculated using the following formula:

$$\text{Fermentation efficiency} = \frac{\text{ethanol produced (g/g)}}{\text{theoretical yield of ethanol}} \times 100$$

Theoretical yield was referred as standard value of 0.511 g/g of sugars.

13. Optimization of process parameters for scale up of bioethanol production in stirred tank bioreactor

Fermentation process for bioethanol production was subjected to scale up process in a 7.5 litre capacity bioreactor from shake flask experiment. Different process parameters viz. fermentation time, temperature and agitation rate were optimized to maximize the bioethanol production in the bioreactor.

13.1 **Ethanologens used**

Co-culture combination of *Saccharomyces cerevisiae* + *Pichia stipitis*

13.2 Batch Fermentation

Batch cultivation for the production of ethanol was carried out in a 7.5 L stirred tank bioreactor (New Brunswick Scientific, New Jersey USA) at temperature 25◦C and pH 5.5. 3.0 litre sugary syrup prepared after saccharification of pine needles biomass was subjected to the bioreactor and to this yeast extract (0.5%) and peptone (0.5%) were added. Foaming was controlled with addition of 2-3 drops of polypropylene glycol. The medium was sterilized in situ for 20 min at 121^oC. The bioreactor was inoculated with 10% inoculum of *S. cerevisiae* II + *P. Stipitis*. The fermentation was carried out under anaerobic conditions. The agitation

speed 100 rpm and agitation rate 1.0 vvm respectively used for batch cultivation in bioreactor. The sample was withdrawn regularly at different intervals ranging from 6, 12……..48 h and ethanol estimation was done.

13.3 Optimization of Fermentation time

Fermentation time was standardized by observing bioethanol production at different time intervals i.e. 6, 12, 18, 24, 30, 36 and 42 hours. Sampling was done at mentioned different time interval.

13.4 Optimization of Temperature

Different temperatures i.e. 20 ^{0}C, 25 ^{0}C and 30 ^{0}C were used for the fermentation.

13.5 Optimization of Agitation rate

Different agitation rates i.e. 100 rpm, 200 rpm and 300 rpm were used for maximum ethanol production

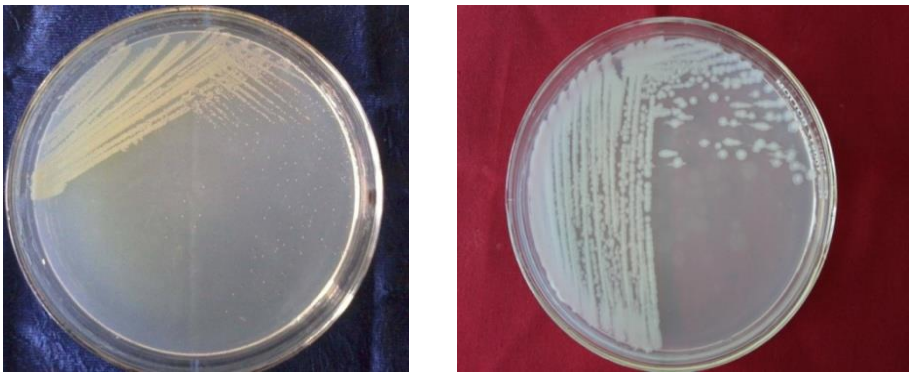
3.2 Details of Scientific data collected and Equipments Used

S. No.	Equipment Name (Qty)	Details (Make/ Model)	Cost	Date of Installation	Photographs of Equipment
1.	Spectrophotometer-20D	Spectrometer 106/ Systronics	48,000.00 rupees	24/01/2019	
2.	Digital autoclave	SLEFA-SS7441/Equitron	2,92,000.00 rupees	29/03/2019	
3.	Deep freezer	Celfrost/GN650	1,50,000.00 rupees	24/04/2019	
4.	High speed Centrifuge	Eppendorf/5810R	7,35,000.00 rupees	03/04/2019	

4. KEY FINDINGS AND RESULTS

4.1 Major Research Findings

- 5 bacterial isolates and 2 fungal strains were screened out depending upon their hyper enzyme production from several hundreds of microorganisms isolated from different sources in our research laboratory.



(a) ***B. stratosphericus*** **N12 (M)** **(b)** ***B. simplex***

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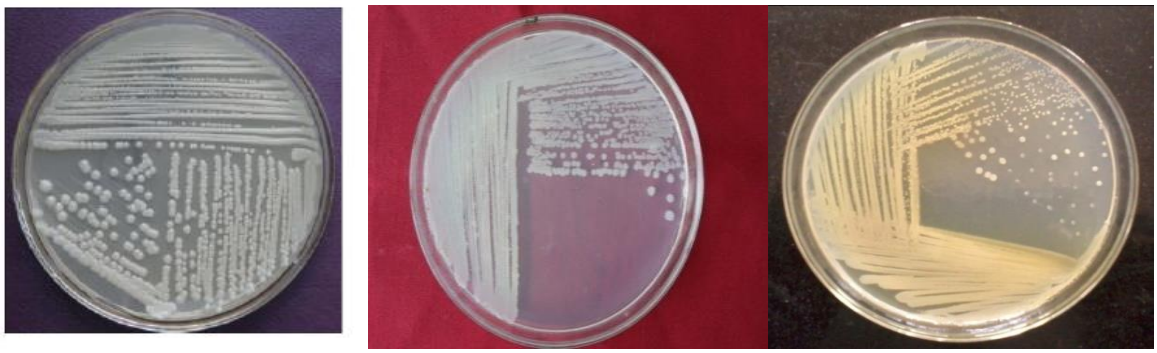
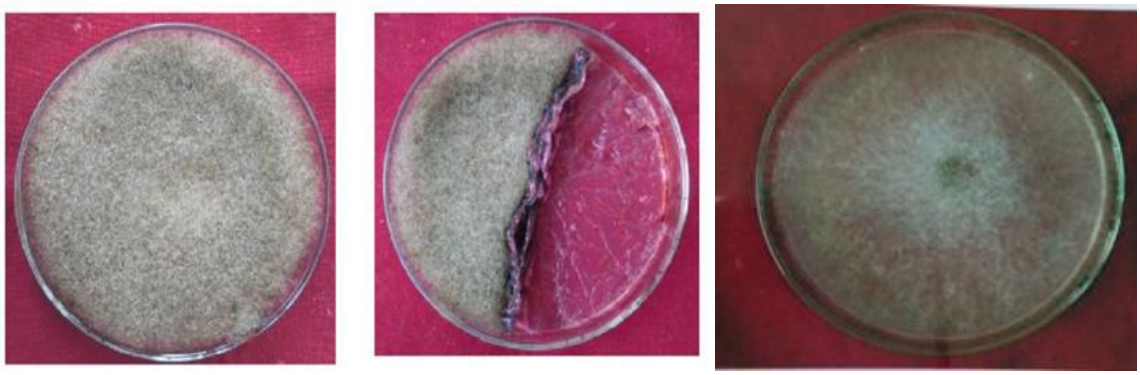


(a) B. subtilis **SD8** ***(b) B. tequilensis*** **SH0** ***(c) B. altitudinis*** **Kd1(M)**

Morpl



(a) **(b)**

Fig 3 Morphology of hyperenzyme producing fungi (a) ***Rhizopus delemar*** **F2 (b) and** ***R. oryzae*** **RF1**

- All these selected potential microbial strains were identified on the basis of phenotypic as well as genotypic (16S rRNA and 18S r RNA) characterization and had been identified as *B. stratosphericus* N12 (M), *B. simplex* SD5, *B. altitudinis* Kd1 (M), *B. tequilensis* SH0, *B. subtilis* SD8, *R. oryzae* RF1 and *R. delemar* F2. These identified isolates were registered with NCBI, US by submitting their respective gene sequences.
- 2 potential hyper enzyme producer bacterial strains i.e. *B. stratosphericus* and *B. altitudinis* had been modified by inducing mutation and were found stable for 8 generations.
- Optimization of different process parameters was done under two different modes i.e. submerged fermentation (SmF) for bacteria and solid state fermentation (SSF) for fungal strains by using OFAT approach and achieved approximately 80.0- 200.00 percent increase in enzymes production.
- Under SmF, purified cellulase of *B. stratosphericus* N12 (M) and *B. simplex* SD5 has expressed 78.20 U/mg and 6.50 U/mg specific activities with molecular weight 36.0KDa and 34 KDa respectively.
- In case of purified xylanase *B. altitudinis* Kd1 (M), *B. subtilis* SD8 and *B. tequilensis* SH0 had depicted 481.14 U/mg, 266.29 U/mg and 108.80 U/mg with molecular weight of 58.0 KDa, 56.0KDa and 56.0 KDa respectively.
- On the other hand under SSF, cellulase (1.125 U/mg and 1.00 U/mg specific activities) and xylanase (20.10 U/mg and 19.65 U/mg) were obtained from fungal isolates *Rhizopus oryzae* RF1 and *R. delemar* F2 respectively. The purified cellulase showed 8.58 and 3.68 fold increases in cellulase activity with the specific activity of 78.20 %.The purified xylanase showed 4.83 fold increases in xylanase activity with the specific activity of 481.14 % yield.

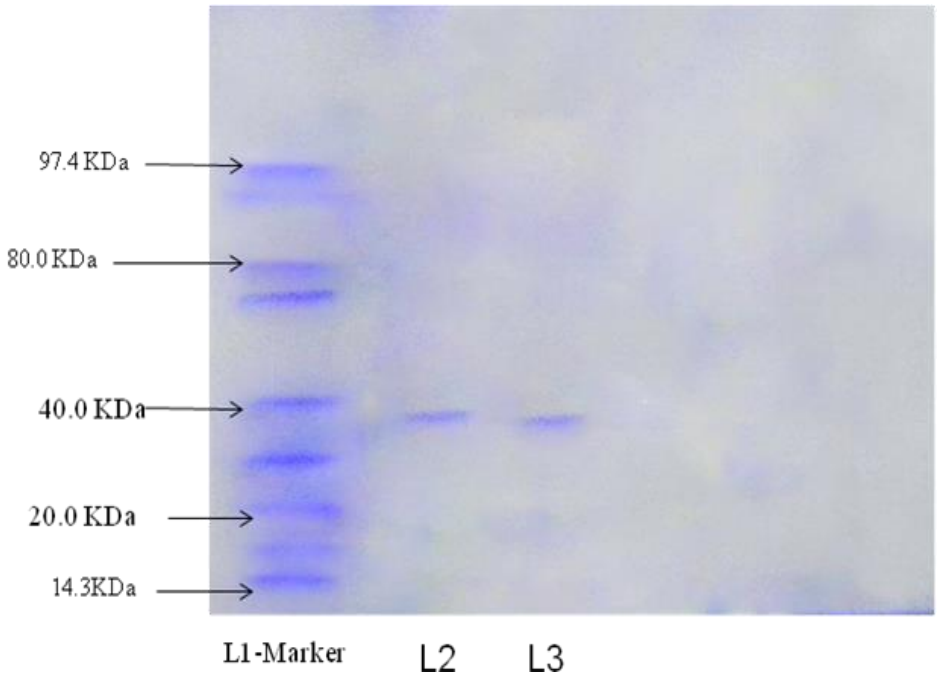


Fig. Purification of cellulase from *B. stratosphericus* N12 (M) (L2) and *B. simplex SD5* (L3) by SDS-PAGE

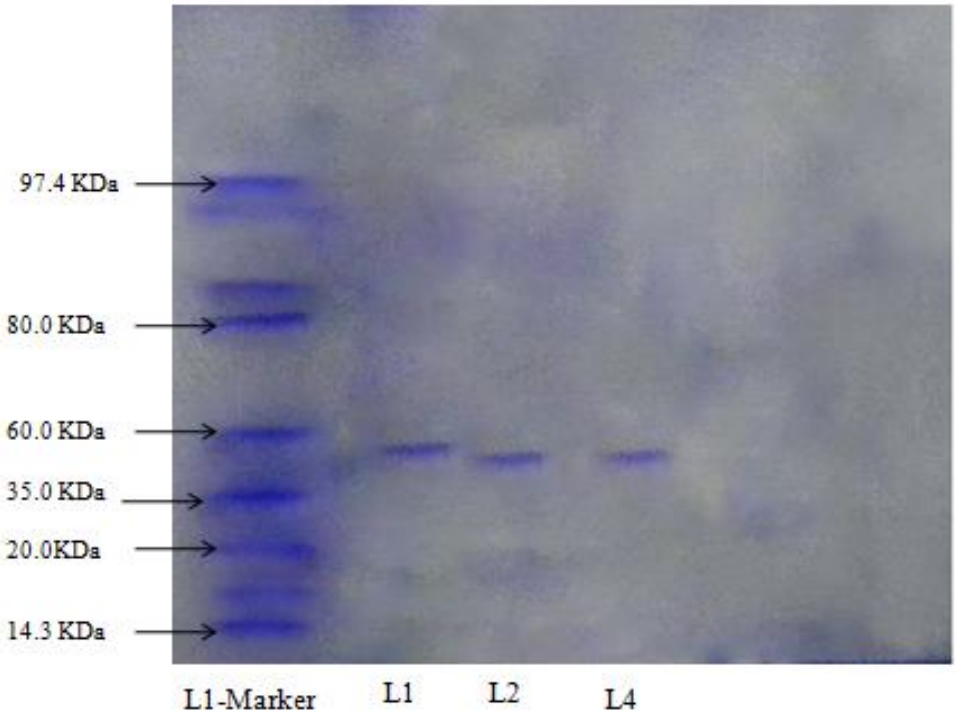


Fig . Purification of xylanase from *B. altitudinis* Kd1 (M) (L2), *B. subtilis* SD8 (L3) and *B. tequilensis* SH0 (L4) by SDS-PAGE

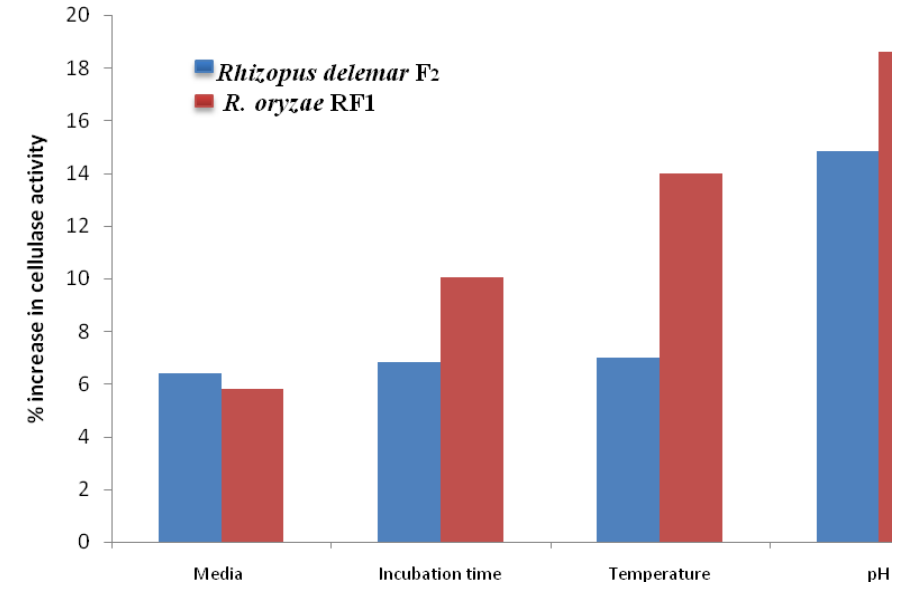


Fig. Overall % increase in cellulase from *Rhizopus delemar* F2 and *R. oryzae* RF1 after optimization

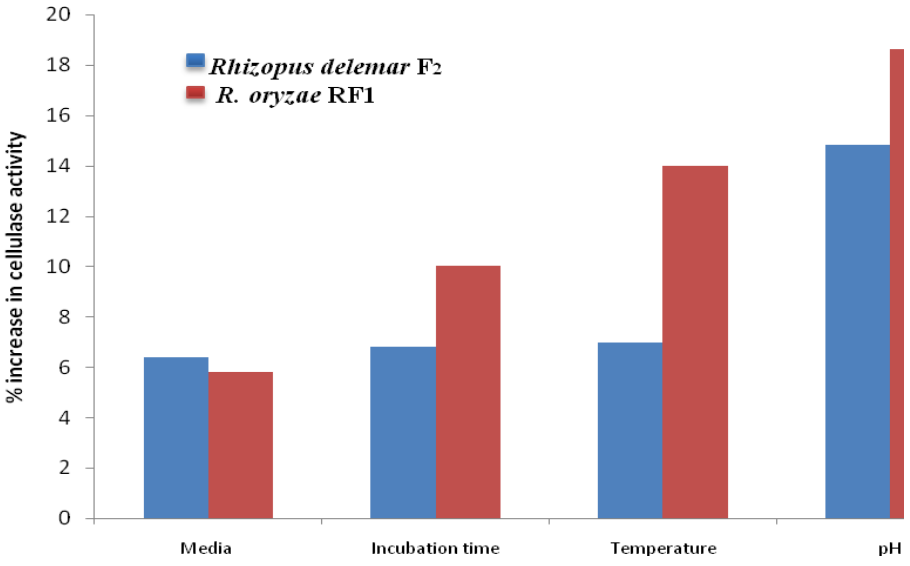


Fig. Overall % increase in xylanase (b) from *Rhizopus delemar* F2 and *R. oryzae* RF1 after optimization

- After the optimization of process parameters viz. microwave dose, incubation period, enzyme dose, enzyme ratio and temperature a good appreciable increase was observed in reducing sugars with overall maximum of percent increase i.e. 272.50 from microwave pretreated biomass over the untreated biomass by optimizing process parameters by one factor at a time approach.

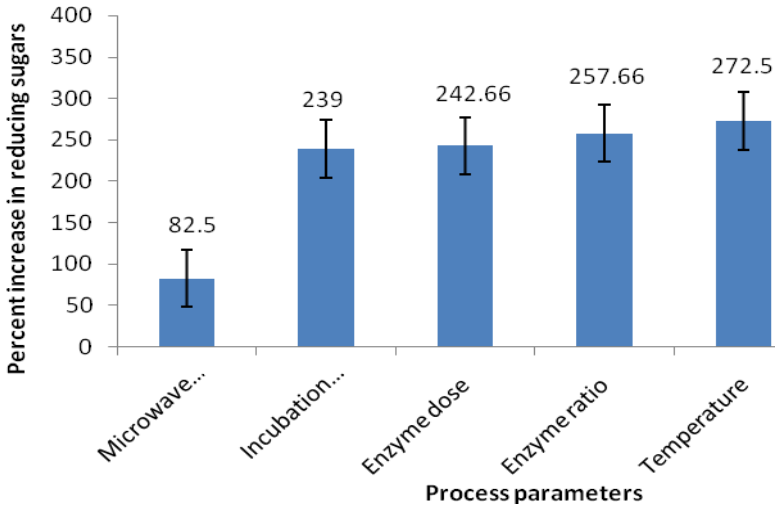


Fig Overall percent increase in reducing sugars in pretreated biomass over untreated biomass after optimization of process parameters

- Maximum reducing sugar yield 22.35 mg/g of biomass was observed at enzyme dosage of 12.5 ml/g in the ratio of 7.75:4.75 (cellulase: xylanase) using pine needles as substrate after 72 h of enzymatic hydrolysis at 45◦c after optimization of process parameters by one factor at a time approach.
- Enzymatic saccharification of untreated and pre treated pine needles biomass using crude, partially purified and purified inhouse enzymes cocktail by applying previously optimized conditions i.e. enzyme dosage of 16.70 ml/g for 72 h of enzymatic hydrolysis at 45◦C has been done and achieved 28.05 mg/g reducing sugars from purified enzymes.
- Maximum reducing sugars of were obtained from pretreated pine needles biomass. Overall percent increase i.e. 453.50 % in reducing sugars production was achieved after optimization of process parameters during RSM approach.

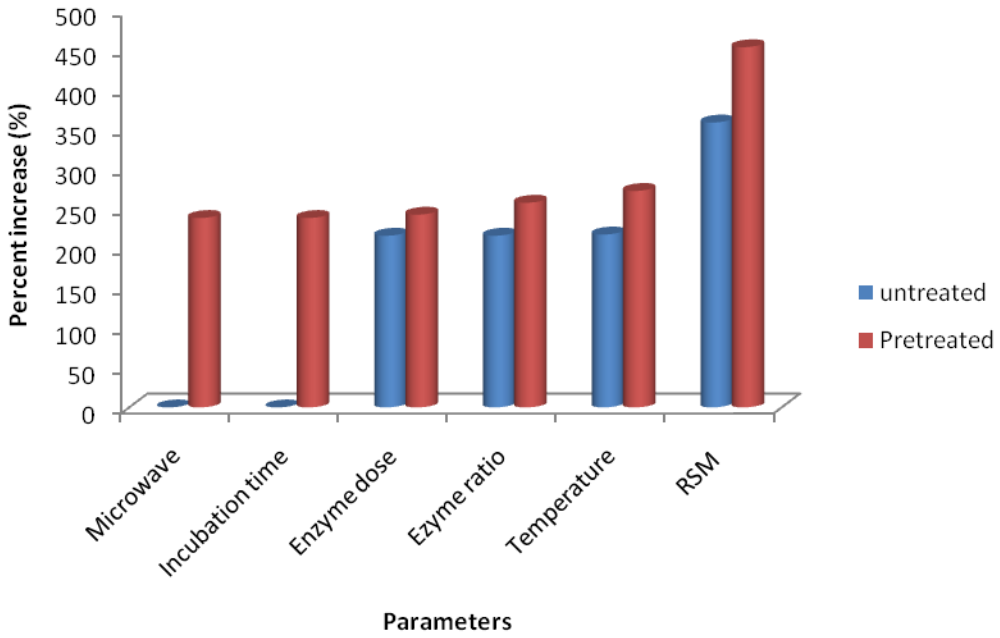


Fig. Step wise increase in reducing sugars after optimization of process parameters by OFAT and RSM

- Quantitative analysis of sugars obtained during saccharification of biomass by crude, partially purified and purified enzymes by using HPLC technique has been done and found that the biomass broken down to form 6 C (29.96 mg/g) sugars (glucose) and 5 C (1.50 mg/g xylose).
- Fermentation of reducing sugars into bioethanol by using co-culture combination of *Saccharomyces cerevisiae* + *Pichia stipitis* has been done and maximum ethanol production of with fermentation efficiency of 69.47 % was observed under pretreated pine needle biomass in shake flask.

- **Fig Bioethanol production from lignocellulosic waste – pine needles**

- Fermentation process for bioethanol production was subjected to scale up process in a 7.5 litre capacity bioreactor from shake flask experiment. Different process parameters viz. fermentation time, temperature and agitation rate were optimized to maximize the bioethanol production in the bioreactor. After optimization of process parameters in a stirred tank bioreactor maximum of ethanol with fermentation efficiency of 72.54% was observed.

Fig Bioconversion process in a stirred tank bioreactor

4.2 Key Results

- 5 bacterial isolates and 2 fungal strains were screened out depending upon their hyper enzyme production from several hundreds of microorganisms isolated from different sources in our research laboratory.
- 2 potential hyper enzyme producer bacterial strains i.e. *B. stratosphericus* and *B. altitudinis* had been modified by inducing mutation and were found stable for 8 generations.
- Optimization of different process parameters was done under two different modes i.e. submerged fermentation (SmF) for bacteria and solid state fermentation (SSF) for fungal strains by using OFAT approach and achieved approximately 80.0- 200.00 percent increase in enzymes production.
- Enzymatic saccharification of untreated and pre treated pine needles biomass using crude, partially purified and purified inhouse enzymes cocktail by

applying previously optimized conditions i.e. enzyme dosage of 16.70 ml/g for 72 h of enzymatic hydrolysis at 45◦C has been done and achieved 28.05 mg/g reducing sugars from purified enzymes.

- ❖ Maximum reducing sugars of 33.21 mg/g were obtained from pretreated pine needles biomass. Overall percent increase i.e. 453.50 % in reducing sugars production was achieved after optimization of process parameters during RSM approach.
- ❖ Fermentation of reducing sugars into bioethanol by using co-culture combination of *Saccharomyces cerevisiae* + *Pichia stipitis* has been done and maximum ethanol production of 16.44 g/l with fermentation efficiency of 69.47 % was observed under pretreated pine needle biomass in shake flask.
- ❖ Upon transfer of process in to stirred tank bioreactor and optimization of process parameters in a stirred tank bioreactor maximum of ethanol 18.96 g/l with fermentation efficiency of 72.54% was achieved.

4.3 Conclusion of the study

- Revalidation of bacterial and fungal strains isolated from different sources in our research laboratory has been done. Production and purification of hydrolytic enzymes (cellulase and xylanase) have been done under submerged (SmF) and solid sate fermentation (SSF) which will be further applied for saccharification of lignocellulosic biomass. A Baseline model has been developed for bioconversion of pine needles into bioethanol.
- Saccharification of pine needles biomass using purified hydrolytic enzymes i.e. cellulase and xylanase has been done successfully. The process parameters were optimized using OFAT approach and statistical model- RSM. Maximum reducing sugars were obtained from pretreated pine needles biomass with 453.50 % overall increase during optimization.
- Different process parameters viz. fermentation time, temperature and agitation rate were optimized to maximize the bioethanol production in the bioreactor. The best optimized conditions were 30h of fermentation time, 25 ◦C temperature and 200rpm of agitation rate. After optimization of process parameters in a stirred tank bioreactor maximum of ethanol with fermentation efficiency of 72.54% has been obtained.

5. OVERALL ACHIEVEMENTS

5.1 Achievement on Project Objectives

In the present project, hydrolytic potential bacteria and fungi have been selected on high titers enzymes production basis for efficient hydrolysis of pine needles. Indigenous enzymes had made the process cost effective as compared to commercial enzymes with a very high cost. Pine needles were made more accessible to enzymatic hydrolysis by standardized pretreatment. Saccharification of pine needles biomass using hydrolytic enzymes i.e. cellulase and xylanase has been done successfully. Sugars produced after enzymatic hydrolysis had been subjected to fermentation with yeast combination for complete sugar conversion to ethanol. Fermentation of sugars into bioethanol using co- culture combination of ethanologens (*S. cerevisiae* and *P. stipitis*) has been done. After optimization of process parameters in a stirred tank bioreactor maximum of ethanol with fermentation efficiency of 72.54% was achieved. A suitable microbial technology has been developed for bioconversion of pine needles into 2nd generation biofuel i.e. bioethanol. The same technology can be shifted to large scale pilot plant process, thus it will be bear a high scope for biorefineries at commercial scale.

5.1 Establishing New Database/Appending new data over the Baseline Data

- Revalidation of bacterial and fungal strains isolated from different sources in our research laboratory has been done. Production and purification of hydrolytic enzymes (cellulase and xylanase) have been done under submerged (SmF) and solid sate fermentation (SSF) which will be further applied for saccharification of lignocellulosic biomass. A Baseline model has been developed for bioconversion of pine needles into bioethanol.
- Saccharification of pine needles biomass using purified hydrolytic enzymes i.e. cellulase and xylanase has been done successfully.
- The process parameters were optimized using OFAT approach and statistical model- RSM. Maximum reducing sugars of 33.21 mg/g were obtained from pretreated pine needles biomass with 453.50 % overall increase during optimization. Quantitative analysis of sugars i.e. 6 C (glucose) and 5 C (xylose) has been done by applying High Performance Liquid Chromatography (HPLC).

- Fermentation of sugars into bioethanol using co- culture combination of ethanologens (*S. cerevisiae* and *P. stipitis*) has been done. Different process parameters viz. fermentation time, temperature and agitation rate were optimized to maximize the bioethanol production in the bioreactor. The best optimized conditions were 30h of fermentation time, 25 ◦C temperature and 200rpm of agitation rate.
- After optimization of process parameters in a stirred tank bioreactor maximum of ethanol 18.96 g/l with fermentation efficiency of 72.54% has been obtained. Fermentation of sugars into bioethanol using co- culture combination of ethanologens (*S. cerevisiae* and *P. stipitis*) has been achieved.

5.2 Technological Intervention

- ❖ Production and purification of hydrolytic enzymes (cellulase and xylanase) have been done under submerged (SmF) and solid sate fermentation (SSF) which will be further applied for saccharification of lignocellulosic biomass.
- ❖ Saccharification of pine needles biomass using purified hydrolytic enzymes i.e. cellulase and xylanase has been done successfully.
- ❖ The process parameters were optimized using OFAT approach and statistical model- RSM. Maximum reducing sugars were obtained from pretreated pine needles biomass with 453.50 % overall increase during optimization.
- ❖ Fermentation of sugars into bioethanol using co- culture combination of ethanologens (*S. cerevisiae* and *P. stipitis*) has been done.
- ❖ Different process parameters viz. fermentation time, temperature and agitation rate were optimized to maximize the bioethanol production in the bioreactor. After optimization of process parameters in a stirred tank bioreactor maximum of ethanol with fermentation efficiency of 72.54% has been obtained.

5.3 On field Demonstration and Value-addition of Products

Pine needles are the major lignocellulosic biomass which is a promising renewable resource for attaining value added products. Pine needles are major recalcitrant lignocellulosic softwood biomass rich in cellulose and hemicelluloses along with lignin that cannot serve as fodder. These do not even decay like any other biomass and piled up pine needles are a major cause of wild forest fires and adversely affecting biodiversity as well as soil fertility. Forest fires deteriorate the

fertility of soil and top layer of soil left with pine needle litter prevents absorption of rain water by soil thus resulting in depletion of ground water and thus demolishing livestock of important food. Furthermore, dry pine needles fallen from the tree act as a barrier between the sunlight and the ground, thus slopping the growth of grasses.

Cellulolytic enzymes play an important role in natural biodegradation processes in which pine needles materials are efficiently degraded by cellulolytic fungi, bacteria and actinomycetes. Generally, complete cellulose hydrolysis is responded by three main types of cellulases combinations: endoglucanases, exoglucanases, and β-glucosidase. These enzymes are widely used in numerous application areas including beverage, agriculture, paper, textiles, detergent, animal feed as well as an alternative for generating green energy. Being rich in cellulase, xylanase and laccase, lignocellulolytic microorganisms serve as a source for biodegradation of pine needles for the production of biofuels besides its safe disposal helping to solve the burning issue of global warming. Since pine needles act as a potential source of carbohydrate polymers for fermentation into simple sugars and subsequently to biofuels by saccharification of its structural polysaccharides into simple sugars.

The main reasons for the enhanced development of bioethanol are its use as a favourable and near carbon-neutral renewable fuel, thus reducing CO_2 emissions, enhance octane in unleaded gasoline, reducing tailpipe pollutant emissions and improving the ambient air quality. The conversion of biomass into bioethanol not only can accomplish the demand for energy resources but all have positive effects on the environmental and socioeconomic position of the world.

6. PROJECT'S IMPACTS IN IHR

6.1 Scientific Management of Natural Resources In IHR

- Gasohol with a blending of ethanol upto 20% in petrol by 2023 is a mandate of National Fuel policy. A huge demand of ethanol thus arising laid pressure on food items like sugarcane, rice and maize (1^{st} generation biofuel). 2^{nd} generation biofuels have an edge over 1^{st} generation biofuels being produced from non-food chain items viz. woody waste- pine needles.
- These do not even decay like any other biomass and piled up pine needles are a major cause of wild forest fires and adversely affecting biodiversity as well as soil fertility. Forest fires deteriorate the fertility of soil and top layer of soil left with pine needle litter prevents absorption of rain water by soil thus

resulting in depletion of ground water and thus demolishing livestock of important food. Furthermore, dry pine needles fallen from the tree act as a barrier between the sunlight and the ground, thus slopping the growth of grasses. Forest fires have caused extensive destruction in recent years leading to loss of wildlife habitat, biodiversity, change in micro-climate, adverse effect on livelihood of people, addition of green-house gases etc.

- This woody waste is rich in complex carbohydrates- cellulose and hemicellulose upto 60- 80% apart from lignin. These complex carbohydrates can be broken down to simple monomeric sugars (glucose and xylose) with the help of hydrolytic enzymes- cellulase and xylanase synthesized by specialized microorganisms.
- In the present innovation, hydrolytic potential bacteria *Bacillus altitudinis* [KC995117] and *Bacillus stratosphericus* [KC995118] have been selected on high titers enzymes production basis for efficient hydrolysis of pine needles. Indigenous enzymes had made the process cost effective as compared to commercial enzymes with a very high cost.
- Pine needles were made more accessible to enzymatic hydrolysis by standardized pretreatment i.e. grinding and microwave irradiation. C6 and C5 sugars produced after enzymatic hydrolysis had been subjected to fermentation with a co-culture yeast combination for complete sugar conversion to ethanol. Ethanol so obtained was further refined to fuel grade level by distillation process. The same technology can be shifted to utilize other similar wastes like agricultural residue, other hardwood and softwood waste generated in nature thus bearing a high scope for biorefineries at commercial scale.

6.2 Conservation of Biodiversity in IHR

Pine needles are a major cause of wild forest fires and adversely affecting biodiversity as well as soil fertility. Forest fires have caused extensive destruction in recent years leading to loss of wildlife habitat, biodiversity, change in micro-climate, adverse effect on livelihood of people, addition of green-house gases etc. Furthermore, dry pine needles fallen from the tree act as a barrier between the sunlight and the ground, thus slopping the growth of grasses. Pine needles are the rich source of lignocellulose and lignocellulose consists of three main components: cellulose, hemicellulose and lignin which make about 50% of the total biomass in the

world. Bioethanol is one of the products that can be obtained via biorefinery using bio-based resources. It is one of the most attractive biofuels, since it can be easily produced in large amounts and used as a “green” fuel. Enzymatic hydrolysis of lignocellulosic biomass is considered as the most efficient and least polluting methods for generating fermentable hexose and pentose sugars. Cellulolytic enzymes play an important role in natural biodegradation processes in which pine needles materials are efficiently degraded by cellulolytic fungi, bacteria and actinomycetes. Microorganisms such as fungi and bacteria are important producers of hydrolytic enzymes by using cheap biomass resources as cultivation media can help to reduce enzyme prices making it a cost effective process.

6.3 Protection of Environment

Forest fires deteriorate the fertility of soil and top layer of soil left with pine needle litter prevents absorption of rain water by soil thus resulting in depletion of ground water and thus demolishing livestock of important food. Furthermore, dry pine needles fallen from the tree act as a barrier between the sunlight and the ground, thus slopping the growth of grasses. Pine needles are the major lignocellulosic biomass which is a promising renewable resource for attaining value added products. Pine needles are major recalcitrant lignocellulosic softwood biomass rich in cellulose and hemicelluloses along with lignin that cannot serve as fodder. Cellulolytic enzymes play an important role in natural biodegradation processes in which pine needles materials are efficiently degraded by cellulolytic fungi, bacteria and actinomycetes. Generally, complete cellulose hydrolysis is responded by three main types of cellulases combinations: endoglucanases, exoglucanases, and β-glucosidase. These enzymes are widely used in numerous application areas including beverage, agriculture, paper, textiles, detergent, animal feed as well as an alternative for generating green energy. Being rich in cellulase, xylanase and laccase, lignocellulolytic microorganisms serve as a source for biodegradation of pine needles for the production of biofuels besides its safe disposal helping to solve the burning issue of global warming. Since pine needles act as a potential source of carbohydrate polymers for fermentation into simple sugars and subsequently to biofuels by saccharification of its structural polysaccharides into simple sugars. The main reasons for the enhanced development of bioethanol are its use as a favourable and near carbon-neutral renewable fuel, thus reducing

CO_2 emissions, enhance octane in unleaded gasoline, reducing tailpipe pollutant emissions and improving the ambient air quality.

7. EXIT STRATEGY AND SUSTAINABILITY

Major recommendations for sustaining the outcome of the projects in future

Bio-ethanol, acclaimed as a potential alternative to petroleum-based fuels, not only offers a sustainable source of energy but also helps in environment conservation by reducing greenhouse gas emission. The fermentable sugar for bioethanol production can be obtained from lignocellulosic biomass through enzymatic hydrolysis of cellulose, hemicellulose and lignin. In the present study, pine needles, which besides being one of the major causes of forest fires during summers and adversely affecting biodiversity, were used for bioethanol production. Initially, cellulose degrading microorganisms isolated from various locations of Himachal Pradesh were screened for their ability to degrade lignocellulosic material so as to select the best organism for fermentation and ethanol production. In order to enhance the cellulose degradation potential of this isolate, various culture conditions were optimized. Efforts are being made to find out a stable microbial consortium of cellulose and hemicellulose degrading microorganisms for maximum yield of fermentable sugar from pine needles for production of ethanol. The outcome of the present work will result not only in providing a safe and environment friendly source of energy, but also in the conservation of environment and biodiversity due to reduction of frequent forest fires. Thus the development of a technology for effectively converting a challenging waste of Himalayan forest to simple sugars by potential inhouse enzymes produced from isolated microorganisms and intern fermenting them to appreciable concentration of ethanol fulfills the main aim of our study, thus envisaging sustainable energy production and improved environmental quality.

8. ACKNOWLEDGEMENT

Principal Investigator gratefully acknowledge the financial support given by National Mission on Himalayan studies (NMHS), Ministry of Environment, Forest and Climate Change (MoEF&CC), Govt of India, New Delhi and G.B. Pant National Institute of Himalayan Environment and Sustainable Development (GBPNIHESD), Kosi-Kataramal, Almora, Uttarakhand.

Annexure-I

Consolidated and Audited Utilization Certificate (UC) and Statement of Expenditure (SE)

For the Period: April, 2018 to March, 2021

1.	Title of the project/Scheme/Programme:	**Bioconversion of pine needles: a challenging waste of Himalayan forest to 2nd generation biofuel**
2.	Name of the Principle Investigator & Organization:	**Dr Nivedita Sharma** **Dr Y S Parmar University of Horticulture & Forestry, Nauni, Solan, H.P., India**
3.	NMHS-PMU, G.B. Pant National Institute of Himalayan Environment, Kosi-Katarmal, Almora, Uttarakhand Letter No. and Sanction Date of the Project:	**GBPNI/NMHS-2017-18/SG-03/544, Dated:23.02.2018**
4.	Amount received from NMHS-PMU, G.B. Pant National Institute of Himalayan Environment, Kosi-Katarmal, Almora, Uttarakhand during the project period (Please give number and dates of Sanction Letter showing the amount paid):	1. 22, 98,000.00 GBPNI/NMHS-2017-18/SG-03/544, Dated:23.02.2018 2. 11,32, 815.00 GBPNI/NMHS-2017-18/SG-03/544/221 Dated:23.07.2019 3. 1,54,000.00 GBPNI/NMHS-2017-18/SG-03/544/221/367 Dated:06.12.2019 4. 12, 25,640.00 GBPNI/NMHS-2017-18/SG-03/544/221/367/131/286 Dated:14.01.2021 **Total: Rs. 48, 92, 047.00/-**
5.	Total amount that was available for expenditure (Including commitments) incurred during the project period:	**Rs. 48, 92, 047.00/-**
6.	Actual expenditure (excluding commitments) incurred during the project period:	**Rs. 38, 63, 824.00/-**
7.	Unspent Balance amount refunded, if any (Please give details of Cheque no. etc.):	**-**
8.	Balance amount available at the end of the project (on April, 2021):	**Rs. 10, 28, 223.00/-***
9.	Balance Amount (on April, 2021)::	**Rs. 10, 28, 223.00/-***
10.	Accrued bank Interest:	Included in the balance amount

*** Amount has been utilized during the extension period of the project i.e. April, 2021 to August, 2021. The statement of expenditure and utilization will be audited in the next financial year (2021-2022) after March, 2022.**

Certified that the expenditure of **Rs. 38, 63, 824/- (Rupees: Thirty eight lakhs sixty three thousands eight hundred twenty four)** mentioned against Sr. No. 6 was actually incurred on the project/scheme for the purpose it was sanctioned.

Date:

(Signature of Principal Investigator) (Signature of Registrar/ Finance Officer)

(Signature of Head of the Institution)

OUR REF. No.

ACCEPTED AND COUNTERSIGNED

Date:

COMPETENT AUTHORITY
NATIONAL MISSION ON HIMALAYAN STUDIES (GBP NIHE)

Statement of Consolidated Expenditure

[Institution Name here]

Statement showing the expenditure of the period from
Sanction No. and Date : GBPNI/NMHS-2017-18/SG-03/544,
Dated:23.02.2018

1. Total outlay of the project : 50.65 lakhs

2. Date of Start of the Project : 23.02.2018

3. Duration : 3 years & 6 months

4. Date of Completion : 31.08.2021

a) Amount received during the project period : 48, 92, 047. 00

b) Total amount available for Expenditure : **38, 63, 824. 00**

S. No.	Budget head	Amount received	Expenditure	Amount Balance/ excess expenditure
1	Salaries	16,11,052.00	13,56,516.00	2,54,536.00
2	Permanent Equipment Purchased	13,00000.00	12,25,000.00	75,000.00
3	Travel	65,781.00	7,903.00	57,878.00
4	Contingency	2,40,442.00	1,68,901.00	71,541.00
5	Consumables	11,24,680.00	11,05,504.00	19,176.00
6	Institutional charges	4,68,500.00	-	4,68,500.00
7	Accrued bank Interest	81,592.00	-	81592.00
8.	**Total**	**48,92,047.00**	**38,63,824.00**	**10,28,223.00**

Certified that the expenditure of **Rs. 38, 63, 824/- (Rupees: Thirty eight lakhs sixty three thousands eight hundred twenty four)** mentioned against Sr. No.08 was actually incurred on the project/ scheme for the purpose it was sanctioned.

Date:

(Signature of Principal Investigator) (Signature of Registrar/ Finance Officer)

(Signature of Head of the Institution)

OUR REF. No.

ACCEPTED AND COUNTERSIGNED

Date:

COMPETENT AUTHORITY
NATIONAL MISSION ON HIMALYAN STUDIES (GBP NIHE)

Annexure-II

Consolidated Interest Earned Certificate

Please provide the detailed interest earned certificate on the letterhead of the grantee/ Institution and duly signed.

Annexure-III

Consolidated Assets Certificate

Assets Acquired Wholly/ Substantially out of Government Grants

(Register to be maintained by Grantee Institution)

Name of the Sanctioning Authority: _____

1. Sl. No. _____
2. Name of Grantee Institution: _____
3. No. & Date of sanction order: _____
4. Amount of the Sanctioned Grant: _____
5. Brief Purpose of the Grant:
   _____
6. Whether any condition regarding the right of ownership of Govt. in the property or other assets acquired out of the grant was incorporated in the grant-in-aid Sanction Order:
   _____
7. Particulars of assets actually credited _____ or acquired_____
8. Value of the assets as on _____
9. Purpose for which utilised at present _____
10. Encumbered or not _____
11. Reasons, if encumbered _____
12. Disposed of or not _____
13. Reasons and authority, if any, for disposal_____
14. Amount realised on disposal _____

Any Other Remarks: _____

_____

(PROJECT INVESTIGATOR)
(Signed and Stamped)

(FINANCE OFFICER)
(Signed and Stamped)

(HEAD OF THE INSTITUTION)
(Signed and Stamped)

Annexure-IV

List or Inventory of Assets/ Equipment/ Peripherals

S. No.	Name of Equipment	Quantity	Sanctioned Cost	Actual Purchased Cost	Purchase Details

(PROJECT INVESTIGATOR)
(Signed and Stamped)

(FINANCE OFFICER)
(Signed and Stamped)

(HEAD OF THE INSTITUTION)
(Signed and Stamped)

Annexure-V

Letter of Head of Institution/Department confirming Transfer of Equipment Purchased under the Project to the Institution/Department

To,

The Convener, Mountain Division
Ministry of Environment, Forest & Climate Change (MoEF&CC)
Indira Paryavaran Bhawan
Jor Bagh, New Delhi-110003

Sub.: Transfer of Permanent Equipment purchased under Research Project titled "...." funded under the NMHS Scheme of MoEF&CC – reg.

Sir/ Madam,

This is hereby certified that the following permanent equipment purchased under the aforesaid project have been transferred to the Implementing Organization/ Nodal Institute after completion of the project:

1. ……
2. ……
3. ……
4. ……
5. ……
6. ……
7. ……

Head of Implementing Organization:
Name of the Implementing Organization:
Stamp/ Seal:
Date:

Copy to:

1. The Nodal Officer, NMHS-PMU, National Mission on Himalayan Studies (NMHS), G.B. Pant National Institute of Himalayan Environment (NIHE), Kosi-Katarmal, Almora, Uttarakhand-263643

Annexure-VI

Details, Declaration and Refund of Any Unspent Balance

Please provide the details of refund of any unspent balance and transfer the balance amount through RTGS (Real-Time Gross System) in favor of **NMHS GIA General** and declaration on the official letterhead duly signed by the Head of the Institution.

Kindly note the further Bank A/c Details as follows:

Name of NMHS A/c **:** NMHS GIA General

Bank Name & Branch: Central Bank of India (CBI), Kosi Bazar, Almora, Uttarakhand 263643

IFSC Code **:** CBIN0281528

Account No. **:** 3530505520 (Saving A/c)

In case of any queries/ clarifications, please contact the NMHS-PMU at e-mail: nmhspmu2016@gmail.com