Int. Res. Adv. 2018, 5(1), 26-29 Article

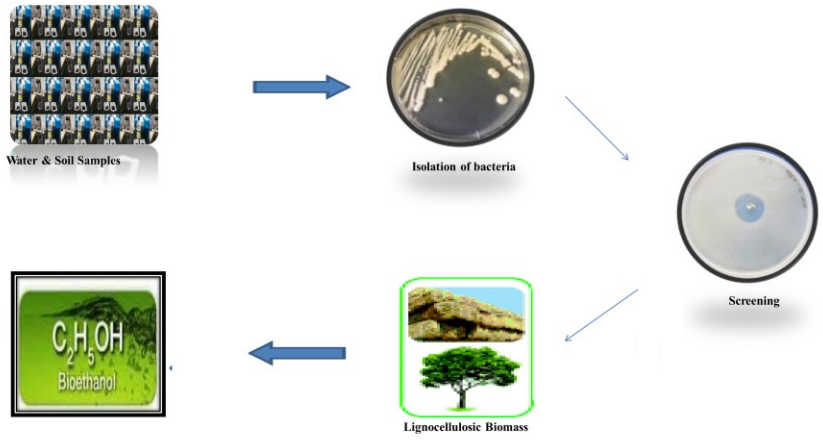
INTEGRATED
RESEARCH ADVANCES

Isolation and Screening of Thermophilic Lignolytic Bacterial Strains for Lignin Degrading Enzymes

Pankaj Sharma, Sunita Kumari, Monika Bishnoi, Sohan Lal & Narsi R Bishnoi

Department of Environmental Science & Engineering, Guru Jambheshwar University of Science & Technology, Hisar, Haryana (India)-125001

Received on: 13-Aug-2017, Revised on: 10-Dec-2017, Accepted and Published on: 03-Feb-2018



ABSTRACT

For producing bioethanol from lignocellulosic biomass, some lignocellulolytic enzymes are needed. So, in this study, bacterial strains were isolated from various agro-industrial sites for obtaining these enzymes. A total of twenty two thermophilic lignolytic bacterial strains were isolated from various water and soil samples using Nutrient Agar (NA) and Minimal Salt Medium (MSM) medium with Lignin Alkali (1.0 g/L) by incubating at pH 7 and 50°C for 48 hrs. These isolates were characterized by special reference to their ability towards wide temperature, pH resilience and generation of thermophilic enzymes. For the screening and studying lignin degrading potential, these microbes were grown on MSM agar plates with Methylene Blue dye (0.25g/L). After the incubation time of 48 hrs, these plates were observed for clear (halo) zones form around the streaked strain. Only 5 strains were showing such clear (halo) zones which indicate their potential for degrading lignin. These screened isolates were then used for further experimental work

Keywords: Thermophiles, Lignolytic, Enzymes, Lignin, Methylene Blue, etc.

INTRODUCTION

Due to changing climate and loss of fossil fuels, research in the field of Environmental biotechnology has been geared up to provide a system feasible for producing biofuels from various renewable resources. Bioethanol is considered to be viable, biodegradable, renewable and environment friendly fuel. It is produced by fermenting sugars, starches or cellulosic biomass.[1] So, in this continuation, Lignocellulosic biomass has received more attention as it is the most abundant biological resource in the nature and does not compete with the food system too.[2]

Biomass materials like coniferous and deciduous wood, corn fiber, corn stover, sugarcane bagasse, rice hulls, forest residues, industrial waste, municipal solid waste and paper mill sludge and other such sources could provide a source of lignocellulosic biomass which gives rise to fermentable sugars for ethanol production. These lignocellulosic wastes are being converted in biofuel by using various microorganisms. Among these all, bacteria are cosmopolitan and highly diverse especially thermophilic microorganisms have gained worldwide attention

Prof. Narsi R Bishnoi, GJUS & T, Hisar, Haryana (India)-125001
Tel: +91-1662-263321
Email: nrbishnoi@gmail.com

Cite as: *Int. Res. Adv., 2018, 5(1), 26-29.*

 ISSN 2456-334X http://pubs.iscience.in/ira

due to their incredible potential to produce thermostable enzymes that show wide applications in various fields.[3] Discovery and identification of such novel thermophiles and their unique properties have attracted much importance to study their roles in diverse environment.[4] So many thermostable enzymes such as polymerase, amylase, protease, lipase, cellulase, ligninase etc. have been proven to be of great exercise in the contemporary fields of biological sciences and which certainly opened new promises for industrial processes too.[2]

The major advantage of using these thermophilic microbes and their enzymes for ethanol production is their capability to degrade various organic compounds present in lignocellulosic wastes. However, none of these microbial strains can compete with *Saccharomyces cerevisiae* or *Zymomonas mobilis* as far as the conversion of hexoses sugars into various products and ethanol tolerance is concerned. Thermophilic anaerobic bacteria could be proficient candidates for conversion of the hemicellulose fraction (viz. xylose, arabinose, mannose and galactose) also.[5] Naturally, there are some major enzymes which are critically involved in ethanol production from lignocellulosic biomass such as endoglucanase, 1,4-β-cellobiosidase, β-glucosidase, Xylanase, lignin peroxidase, manganese peroxidase and laccase. The microorganisms showing excellent production of these enzymes have the potential to produce the reducing sugars for ethanol production.[6]

Thermostable ligninases are of greater applicability as they do not only denature at high temperatures, but they also linger active at such temperatures. Several workers have reported thermophiles from diverse environmental habitats such as geothermal sites, bio-compost piles, paper pulp mills and waste water from breweries.[7]

Materials and Methods

Site Selection & collection of samples: Various waste water samples were collected from various selected sites viz. food industry, bio-compost, sugar mill, pulp and paper industry, distillery and soil which were contaminated with hydrocarbons. Samples from different sites were collected in sterile sample bottle and immediately brought into the laboratory. Soil samples were also brought into the laboratory and were kept in refrigerator at a temperature of 40 ºC for further study.

Media preparation: Nutrient broth (NB) and Minimal Salt medium (MSM) were used in this study. Nutrient broth media was prepared for the enrichment of the bacterial growth and used to observe the colony characteristics of the isolates while Minimal Salt Medium was prepared by adding the following ingredients; K_2HPO_4 (7.0 g/L), KH_2PO_4 (3.0 g/L), $(NH_4)2.SO_4$ (1.0 g/L), $MgSO_4.7H_2O$ (2.0 g/L) and used for secondary screening. For observing Lignin degrading potential (screening) of isolates, Methylene Blue dye (0.25 mg/L) was added in media.

Isolation of bacteria:

For the isolation of the bacterial strains from soil and waste water samples, enrichment method was used in which the soil samples were enriched with the Nutrient broth. Nutrient broth medium was prepared by adding Peptone (5g/L), Beef extract (1.5g/L), Yeast extract (1.5g/L) and Sodium chloride (5g/L). Each sample was enriched in a 500 ml conical flask containing 100 ml broth medium. One gram of each sample was weighed and added into the broth in flask. The flasks containing media and samples were then placed in incubator cum shaking orbital for 72 hrs at 50˚C. After 72 hrs, one ml of each sample was transferred into already prepared Minimum Salt Medium (MSM). The samples were then placed in incubator shaker again for one week. After the incubation period of 7 days, serial dilution of each sample was done. During serial dilution, sterile water was used for the dilution. Now, from each serial diluted sample, one ml of the aliquot was taken out and was spread on to the Nutrient Agar plate and incubated for 72 hrs at 50˚C. After 72 hrs, bacterial colonies were observed onto the plates. The grown bacterial colonies were again streaked on to new NA plates for further 2-3 times. Pure culture of bacterial stains was then cultured into Luria Bertani (LB) broth as stock culture in refrigerator.

Determination for thermo-tolerance:

Pure cultures of the bacterial isolates were checked for their thermophilic characteristics. Each bacterial isolates were inoculated first into Nutrient Agar broth medium in culture tubes. The tubes were incubated at different temperature ranging from 37-80 ºC for 24-72 h.[7] After specified incubation period, each broth culture of bacteria was streaked onto freshly prepared Nutrient Agar plates. Bacterial colonies growing onto the plates were selected and again tested for their thermo-tolerance at higher temperature. Finally a bacterium that could tolerate temperature of 50°C was selected for further study.

Screening (Secondary) of the isolates:

Screening of the isolates was done by using Methylene Blue decolorization method to detect the lignin degrading potential of the isolates. The experiment was done in 2 ways i.e. plate as well as liquid assay. For plate method assay, MSM agar medium containing 0.25% Methylene Blue (MB) dye was used to culture the isolates. All the isolates were streaked on MSM plated containing MB dye and incubated for one week at 50˚C. The appearance of halo zones around the colonies was observed consequently for 10 days.

In case of liquid method assay, MSM broth medium containing the same dye (MB) was used. So isolates were inoculated into the medium containing the MB and kept in incubator at 120 rpm at 50˚C for a period of 7 days. After 7 days, the optical density of each sample was taken using UV-Vis Spectrophotometer at a wavelength of 520 nm. For doing so, about 2 ml of aliquot from each sample was taken into 5 ml capacity centrifuge tube and got the sample centrifuged for 5 min. at 10000 rpm. The supernatant obtained was taken into cuvette and observed its optical density at 520 nm. The percentage of decolorization efficiency of bacterial isolate was calculated as;

$$\text{Decolorization (\%)} = \frac{(\text{Initial OD} - \text{Final OD}) * 100}{(\text{Initial OD})}$$

Result & Discussion

Sample collection: Waste water samples from various sources were collected in 500 mL HDPE (high density

polyethylene) wide mouth sterilized plastic containers. Temperature and pH parameter of the samples were recorded at that moment with the help of portable thermometer and pH meter respectively. Samples were then directly carried to the laboratory and stored in the refrigerator at 4 °C. For the collection of contaminated soil sample, zip-lock plastic bag were used. In the case of compost pile, temperature was measured to make sure that it was in thermophilic heating stage. Like waste water samples, soil samples were also brought to the laboratory and stored in re refrigerator at 4 °C.

Isolation of bacteria:

Enriched water samples were made diluted till 10^{-6} times in autoclaved distilled water and were spread on lignin Minimal Salt Medium agar plates. Plates were then incubated in incubator at 50 °C for 3-4 days. Phenotypically different bacterial colonies were selected and purified by repeated sub-culturing. Pure cultures were inoculated in LB broth media to achieve maximum growth rate and subsequently used for selection of potential ligninolytic bacterial isolates.

Altogether 22 bacterial isolates were obtained from water and soil samples collected from hot spring, food industry, bio-compost, sugar mill, pulp and paper industry, distillery and the soil which was contaminated with hydrocarbons. The bacterial isolates were screened for their thermo-tolerance property in different temperature starting from 50°C to 80°C. Isolated bacterial strains were then screened to check the presence of lignocellulosic compound degrading enzymes in them.

Maintenance of isolated pure cultures

In order to ensure the availability of pure microorganisms and their initial metabolic activities, the isolated cultures were sub-cultured periodically. Finally, the isolated pure cultures were stored. Generally, two storage methods viz., short term and long term storage methods have been used.

Temperature tolerance profile:

Screening of all the isolates for temperature tolerance revealed that these isolates were found as obligate, facultative and thermophiles too. Owing to their modest thermophillic nature, facultative thermophiles may be secondarily adapted to hot environments which may be one of the reasons for getting higher percentage of facultative thermophiles. Isolated microbes were found growing best at 50 °C.

Screening of Isolated Bacteria:

Spread plate assay: After serial dilution, bacterial colonies from last three dilution factors of each samples and spread on the plate containing Methylene Blue dye. The plates were then incubated at 50 °C for 3-4 days. Out of 22, only five isolates showed clear halo zones around streaked area which shows the degradation potential of alkali lignin.

Dye Decolorization assay: In this assay, isolates were inoculated into the flasks having medium containing the MB. The flasks were kept in orbital shaker cum incubator at 120 rpm at temperature 50˚C for a period of 7 days. After completion of the incubation period, Optical Densities (OD) was scanned using UV-Vis Spectrophotometer at a wavelength of 520 nm.

Table 1 Optical Densities (OD) of the samples and % of dye decolorization

Sr. No	Name of Sample	OD (Average)	Decolorization (%)
1.	Blank	0.957	0
2.	CK	0.488	44.7
3.	SM	0.535	39.7
4.	SK	0.506	42.8
5.	GS	0.518	41.5
6.	PI	0.416	52.2
7.	T-1-A	0.452	48.4

Table 1 shows the percentage of removal of MB dye after 7 days. According to the table 1, the maximum decolorization efficiency after 7 days, is observed to be 52.2% and 48.4% in isolates namely PI and T-1-A respectively. The bacterial pellet was not colored, which shows the biosorption. The optical density was recorded at 520 nm. The bacterium PI and T-1-A was inoculated in flask containing Methylene Blue separately. The flasks were incubated in incubator shaker at 120rpm and 50°C. The samples were collected after 7 days and reduction was observed. Reduction in the OD readings shows that bacteria consumed and used dye as sole carbon source. Bacteria take energy from dye on its consumption due to which the reduction in the medium color is observed.

CONCLUSION

Soil and water samples were collected from industrial premises and some other sources containing the micro flora which were having the potential to degrade the lignocellulosic compounds. These bacteria also have potential to degrade lignocellulosic compounds. The decolorization capability and degradation of kraft lignin was investigated in these bacteria. The bacteria screened to check the dye decolorization activity. Followed by the experiment to check the lignin degrading enzyme in the two bacteria and it was found that both the strains were showing the lignin degrading activity which is responsible for dye decolorization and kraft lignin degradation. After the complete experiment the results showed that PI and T-1-A were selected for further studies. The strains selected for this study were CK, SM, SK, GS, PI and T-1-A. These bacteria were isolated from the industrial premises and after several sets of experiments it was found that these strains were having the lignocellulose degrading capability. Therefore, they can be use for degradation of lignin and ethanol production from lignocellulosic biomass.

ACKNOWLEDGEMENT

This experimental work was supported by major research project granted by University Grants Commission, New Delhi and authors are gratefully acknowledged for the same.

REFERENCES

1. C.C. Dai, J. Tao, Y. Wang, Y. Hao. Research advance and superiority of microdiesel production with biowastes. *African Journal of Microbiology Research*. 2010, 4(10): 977-983.
2. K. Min, G.G. Gyeongtaek, H.M. Woo, Y. Kim, Y. Um. A dye-decolorizing peroxidase from *Bacillus subtilis* exhibiting substrate-dependent optimum temperature for dyes and b-ether lignin dimer. *Scientific Reports*. 2014, 5:8245.
3. M.K. Panda, M.K. Sahu1, K. Tayung. Isolation and characterization of a thermophilic *Bacillus sp.* with protease activity isolated from hot spring of Tarabalo, Odisha, India. *Iranian Journal of Microbiology*. 2013, 5:159-165.
4. P. Shirkot A. Verma. Assessment of thermophilic bacterial diversity of thermal springs of Himachal Pradesh. *ENVIS Bulletin Himalyan Ecology*. 2015, 23: 27-34.
5. T. Sommer, T. Georgieva, B.K. Ahring. Potential for using thermophilic anaerobic bacteria for bioethanol production from Hemicelluloses. *Biochemical Society Transaction*. 2004, 32: 283-289.
6. R. Hooda, N.K. Bhardwaj, P. Singh. Screening and identification of ligninolytic bacteria for the treatment of pulp and paper mill effluent. *Water Air Soil Pollution.* 2015, 226: 305.
7. S. Devi, S.S. Kanwar. Deciphering the diversity of aerobic culturable thermophiles in hot springs of Manikaran, Himachal Pradesh. *International Journal of Farm Sciences*. 2016, 6(1): 156-162.