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Research paper

# Bioethanol production potential of a novel thermophilic isolate *Thermoanaerobacter* sp. DBT-IOC-X2 isolated from Chumathang hot spring

Nisha Singh[a,b], Munish Puri[b,c,**], Deepak K. Tuli[a], Ravi P. Gupta[a], Colin J. Barrow[b], Anshu S. Mathur[a,*]

[a] DBT-IOC Centre for Advance Bioenergy Research, Research & Development Centre, Indian Oil Corporation Limited, Sector-13, Faridabad, 121007, India
[b] Centre for Chemistry and Biotechnology, Waurn Ponds, Deakin University, Victoria, 3217, Australia
[c] Centre for Marine Bioproducts Development, College of Medicine and Public Health, Flinders University, Bedford Park 5042, Adelaide, Australia

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ABSTRACT

Dilute acid pretreatment of biomass generates enormous amount of hydrolysate (rich in inhibitors and pentose sugars), that remains unutilized for bioethanol production due to inadequacy of efficient $C_5$-fermenting organisms. In this study, a predominantly pentose fermenting extremely thermophilic bacterium strain DBT-IOC-X2, pertaining to the genus *Thermoanaerobacter* was isolated from Himalayan hot spring. Batch experiments indicated substantial inhibitor resistance ($2\,g\,dm^{-3}$ for furfural, 5-HMF, and acetic acid), substrate tolerance ($\sim 15\,g\,dm^{-3}$), co-sugar fermentation ability (co-production ethanol yield of 0.29 g/g), and high ethanol yield (83.57% and 91.12% of the theoretical maximum from $5\,g\,dm^{-3}$ glucose and xylose, respectively) by the bacterium at 70 °C and pH 8.0. Here, bioethanol production process was developed using pre-treated rice straw hydrolysate (PRSH) as low-cost agro-waste and 83.47% of the total sugar conversion was obtained. This study shows that *Thermoanaerobacter* sp. DBT-IOC-X2 could utilize diluted PRSH efficiently to improve the overall cost-effectiveness of biomass processing to bioethanol.

## 1. Introduction

To overcome the economic and environmental issues caused by the use of petroleum-based products, development of sustainable sources of energy is necessary. Bioethanol is sought as one of the major alternatives to conventional liquid transportation fuels since it is renewable and has the potential to mitigate green house gas emissions [1]. First generation bioethanol production from sugar-rich food materials e.g. maize, sugar beet and sugarcane is not widely accepted due to its food vs. fuel conflict [2]. However, lignocellulosic biomass feedstocks such as rice straw, wheat straw, sugarcane bagasse/straw, corn stover/cob, cotton stalk etc. could serve as a promising resource, as they are generated in huge amount, doesn't compete with food security, and has worldwide acceptance [3]. Currently, enzyme-microbe based fermentation of non-food lignocellulosic biomass emerged as an attractive route and termed as second-generation bioethanol production.

Second generation bioethanol production process mainly involved 4 steps: pretreatment, hydrolysis, fermentation, and distillation [4]. Biomass pretreatment is a key step essential to disintegrate complex lignocellulose matrix and release carbohydrate polymers which undergo hydrolysis by a mixture of cellulolytic enzymes to yield fermentable sugars. Thereafter, the released sugar is fermented to ethanol by robust fermenting microorganism having high volumetric productivity. The commercial-scale development of a cost-effective bioethanol production process faces some major bottlenecks which owes in part to biomass recalcitrance, higher cost of cellulolytic enzymes, and inhibition of both enzymes and fermenting organisms by the molecules released or produced during pretreatment [1,4]. Therefore, there is a constant search for better microorganisms having efficient cellulase production and fermentation capability. So far, filamentous fungi (for cellulases) and yeast (for $C_6$ fermentation) have been predominantly exploited for second generation bioethanol production. Most of the current research has been focussed on; metabolic engineering of these microbes, process consolidation, and conditions optimization to achieve desired production level of bioethanol.

Alternatively, thermophilic anaerobic bacteria can also be explored for bioethanol production. A microbial fermentation process employing thermophilic anaerobes is often preferred over mesophilic microorganisms due to their inherent ability to utilize both pentose and hexose sugars, natural resistance to fermentation inhibitors, and several

* Corresponding author.
** Corresponding author. Centre for Marine Bioproducts Development, College of Medicine and Public Health, Flinders University, Bedford Park 5042, Adelaide, Australia.
*E-mail addresses:* munish.puri@deakin.edu.au (M. Puri), mathuras@indianoil.in (A.S. Mathur).

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associated process benefits offered by high temperature conditions (> 70 °C) [5]. Previously, high ethanol yield on both simple sugars and complex biomass have been reported by several thermophilic anaerobic bacteria belonging to the genus *Clostridium*, *Thermoanaerobacter*, and *Thermoanaerobacterium* [6]. In addition to above mentioned sources, waste effluents derived from various pretreatment processes could be a source for bioethanol production, where waste valorization to fuel would be an advantage [7–9]. The slurry obtained after dilute-acid pretreatment of biomass contains a cellulose rich solid fraction and liquid hydrolysate rich in $C_5$ sugars and inhibitors. This liquid hydrolysate is usually separated or detoxified to prevent the inhibition of enzymes and fermenting organisms in subsequent steps, which leads to sugar loss and cost escalation. Thus, the effective utilization of this sugar-rich stream by efficient thermophilic anaerobic bacteria can improve the economic value of the whole process. Anderson et al., recently reviewed and reported most efficient ethanolic fermentation (> 90% of the theoretical maximum) of such $C_5$-rich liquid fraction (comprising glucose, xylose, arabinose, and furfural) derived from dilute acid pre-treatment of wheat straw using genetically engineered *Thermoanaerobcater italicus* Pentocrobe 411X [7].

Bioethanol production from $C_5$-rich liquid hydrolysate derived from pre-treated biomass has not been fully explored. In present study, a potent bacterial isolate which can produce bioethanol directly from pre-treated lignocellulosic hydrolysate was isolated and its biochemical characteristics were explored. An undisturbed thermal hot spring site situated at utmost location was chosen for isolating such bacteria. To the best of our knowledge, this is the first study presenting isolation and characterization of a predominantly pentose fermenting thermoanaerobe from Chumathang hot spring site. Rice straw biomass (RSB) was chosen for this study as one of the most abundant feedstock in the world. Around 650–975 million tons of rice straw is produced annually worldwide, which could generate around 250 billion litres of bioethanol based on its high holocellulose (32–47% cellulose and 19–27% hemicellulose) content [10]. However, the structure of RSB is severely complex due to high degree of cellulose polymerization and high silica content that slow down its biodegradation by hydrolytic organisms and enzymes [11]. Therefore, pretreatment of RSB is essential to breakdown its recalcitrant structure. Here, pre-treated rice straw hydrolysate (PRSH) was used in batch mode fermentation to evaluate the applicability of the isolate for bioethanol production from real substrates. In addition to this, fermentation performances on simple sugars and sugar mix ($C_6$ and $C_5$) were also explored. We have chosen to focus on the simultaneous co-sugar fermentation and inhibitor tolerance of the newly isolated strain, in order to acquire a basic knowledge which could be applicable for the fermentation of mixed sugars derived from real substrates. Process parameters such as pH, temperature, and initial concentration of substrates were optimized to achieve maximum production of bioethanol. Here, a comparison has been made between the fermentation performance of newly isolated hot spring strain with already established and closely related *Thermoanaerobacter ethanolicus* DSM 2246 [12].

## 2. Materials and methods

### 2.1. Growth medium and reference strain

A chemically defined minimal medium (M), used for all the enrichment, isolation and fermentation studies was in accordance to Sizova et al., [13]. M medium was prepared by boiling under a constant flow of nitrogen gas to remove dissolved oxygen. Medium was cooled and dispensed into serum bottles (125 $cm^3$, Wheaton) inside a Coy anaerobic chamber (USA) having a headspace of $N_2$:$CO_2$:$H_2$ (90:5:5). The bottles were sealed using butyl rubber stopper (Bellco, USA) and aluminium crimp to ensure anaerobic conditions. Sealed bottles were autoclaved for 15 min at 121 °C and reduced using a concentrated stock solution of L-cysteine HCl. Moreover, the carbon sources supplemented in M medium were simple sugars and complex polysaccharides, added at a concentration specified in text. Concentrated stocks of sugar solution were prepared separately and added to the autoclaved medium after filter sterilization, to avoid charring and sugar loss. Likewise, pretreated liquid hydrolysate obtained after dilute-acid pretreatment of RSB was also supplemented with M medium components (as described in section 2.4). After mixing all the solutions, desired pH of the media was adjusted just before inoculation using anaerobic stock solution of 1N HCl and 1N NaOH. Standard anaerobic culture techniques were used throughout the experimental manipulations, as previously described [14,15].

A reference culture *Thermoanaerobacter ethanolicus* DSM 2246 was procured from the DSMZ collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and revived using fresh medium 640 (composition provided by DSMZ). Pure culture was stored in 30% deoxygenated glycerol at −80 °C and revived before each experiment.

### 2.2. Isolation and identification of $C_5$-fermenting thermophilic anaerobic bacteria

Bacterial strain DBT-IOC-X2 used in this study was isolated from hot water and bacterial mat samples collected from Chumathang hot spring site (N33°21′37.11"; E78°19′24.50″, altitude 3950 m), located 150 km southeast of Leh district, North West Himalayan, India. The enrichment and isolation using hot spring samples was performed under strict anaerobic conditions, according to the procedure published earlier [14]. M medium (50 $cm^3$, pH 7.0) with 10 g $dm^{-3}$ xylose as a sole carbon source was inoculated anaerobically with hot spring samples to selectively enrich and purify $C_5$-fermenting thermophilic anaerobic bacteria. Enrichment cultures were incubated at 70 °C, as the suggestive temperature optima for most sugar-fermenting thermoanaerobes, without shaking till positive growth was evidenced in the form of turbidity, pH drop, and gas production. The stable enrichment culture responsible for maximum ethanol production was selected. Individual colonies were purified from this enrichment culture using Hungate roll tube technique of culture purification, as described previously [14]. The best $C_5$-fermenting isolate used in this study was selected on the basis of maximum ethanol production and designated as strain DBT-IOC-X2.

Total genomic DNA of the purified bacterial isolate DBT-IOC-X2 was extracted using DNeasy blood and tissue kit (Qiagen India Pvt. Ltd) following the manufacturer's instruction. The 16S rRNA was amplified by PCR using bacterial domain-specific universal set of primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) in accordance to the conditions described previously [14]. The sequencing was carried out by Institute of Microbial Technology, Chandigarh, India. Gene sequences obtained were then analyzed for evolutionary history as described previously [14] and references therein.

### 2.3. Strain characterization

The effect of temperature, initial pH, and varying concentration of substrates and inhibitors on the growth of strain DBT-IOC-X2 was studied in triplicate in serum bottles containing 50 $cm^3$ M medium and 10 g $dm^{-3}$ xylose as the sole carbon source. Each experiment set was inoculated with 5% (v/v) inoculum from a freshly grown culture ($OD_{600}$ ~ 0.8–1.0) prepared by passaging thrice on M medium (pH 7.0) containing 10 g $dm^{-3}$ xylose.

Influence of initial pH of the medium on the growth and maximum ethanol production by strain DBT-IOC-X2 was studied with the pH range of 3.0–10.0, at 70 °C for 48 h. The initial pH of the medium was adjusted with 1N HCl and 1N NaOH solutions prepared anaerobically. To investigate the effect of temperature, inoculated bottles were incubated at ranges of temperature from 45 °C to 85 °C with 5 °C intervals,

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