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Development of sequential-co-culture system (*Pichia stipitis* and *Zymomonas mobilis*) for bioethanol production from Kans grass biomass

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ABSTRACT

Sequential-co-culture technique was investigated in this study for the production of bioethanol from relatively cheaper lignocellulosic biomass of Kans grass (*Saccharum spontaneum*). The consortium of *Pichia stipitis* and *Zymomonas mobilis* was used to develop a suitable sequential-co-culture system. The Kans grass biomass was hydrolyzed in such a manner that the two sugar fractions, xylose rich and glucose rich were generated (a separate study). The *P. stipitis* cells and respective fermentation media (xylose rich) were fed to the fermentation vessel, after the set fermentation time *Z. mobilis* cells and respective media were fed to the same vessel. Different strategies have been followed and experiments were conducted initially at flask level. The selected strategy was then applied at bioreactor level using both synthetic fermentation media and Kans grass hydrolysate media to compare the kinetic parameters. The sequential addition of cultures with their respective media and imposed process conditions, showed better utilization of total sugars added (>95%). Microaerobic condition for *P. stipitis* and strictly anaerobic condition for *Z. mobilis* fermentation were found significant. The average ethanol yield ($Y_{p/s}$) and overall volumetric productivity (r_{po}) were found as 0.453 g_p/g_s and 1.580 g/l/h respectively for Kans grass hydrolysate media and 0.474 g_p/g_s and 2.901 g/l/h respectively for synthetic fermentation media.

1. Introduction

The bioconversion of lignocellulosic biomass to fuel ethanol gaining the attention of researchers all over the globe, considering the fact that bioethanol is the cleanest liquid fuel alternative to fossil fuels. The lignocellulosic biomass is the largest resource of carbohydrates in nature. It is less expensive and more abundant than any other carbohydrate containing feedstocks (e.g. starch, sucrose). Moreover lignocellulosic feedstock does not compete the land use for food and feed production. The cellulose and hemicellulose fractions of lignocellulosic biomass typically comprise of two thirds of the dry mass by weight. These polysaccharides can be hydrolysed to soluble sugars and eventually be converted to valuable products mainly via fermentation. Kans grass (*Saccharum spontaneum*) biomass is believed to be a novel substrate with great prospective for the production fuel ethanol. It is a short-rotation perennial crop having the properties like high yields, low costs for establishing and managing, good suitability for low-quality land availability throughout the year. It is a C_4 plant which has the potential to achieve higher rates of photosynthesis and greater water use efficiency than their C_3 counterpart under the same environmental conditions [1]. The cell wall of Kans grass biomass (KGB) contained 68% total carbohydrate content on dry biomass basis wherein 43.78% cellulose and 24.22% hemicellulose fractions projecting its worth for fuel ethanol production [2]. Glucose from cellulose and xylose from hemicellulose fraction of lignocellulosic biomass are the two major soluble sugars present in the liquid hydrolysate. The prime requirement to make bioethanol commercially viable product from this route is the bioconversion of both the sugars to ethanol. No native microorganisms known to ferment both the sugars into ethanol at high yield [3,4]. The unavailability of industrially robust microbes for simultaneous fermentation of glucose and xylose in a single bioreactor system has been identified as the major technical barrier.

Saccharomyces cerevisiae and *Zymomonas mobilis* have been reported for glucose fermentation and are the most commonly used GRAS (generally recognized as safe) microbes for ethanol production. Among these two microorganisms *Z. mobilis* gives higher ethanol yield (5–10%) and is 2.5 times faster in terms of ethanol productivity than *S. cerevisiae* [5]. Also *Z. mobilis* follows homoethanol fermentation pathway, capable of producing almost a theoretical

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amount of ethanol from glucose via Entner–Doudoroff pathway under anaerobic conditions and tolerates up to 120 g/l ethanol [6]. Despite the robustness of these two microorganisms for ethanol production at industrial level, they are not well suited for fermenting sugars obtained from lignocellulosic biomass to ethanol. They are unable to ferment xylose, which in general contributes up to 30–40% of the total available fermentable sugar in the liquid hydrolysate of lignocellulosic biomass.

Mainly two approaches have been identified from literature for bioconversion of both type of sugars (pentose and hexose) to ethanol. First approach relied on the use of recombinant microorganism developed with the aim to ferment both xylose and glucose to ethanol. These include genetically modified strains of *S. cerevisiae*, *Z. mobilis*, *Escherichia coli* and *Klebsiella oxytoca* [7–12]. Though the high yield could be achieved from these genetically modified organisms (GMO's), their developmental cost, narrow and neutral pH range, long term stability and the utilization of residual cell mass as animal feed [4] like other GRAS organisms are the main disadvantages which restrict the use of GMO's at industrial level. The other approach lies in the use of two microorganisms simultaneously in a single fermentation system, defined as "co-culture". The ideal co-culture system involves such combination of microorganisms that do not affect each other's metabolic activities like their growth and, competitiveness towards substrate or nutrients should be minimal to maximize the production efficiency. Therefore, an efficient xylose converter along with glucose fermenting microorganism should be chosen carefully. *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* [13] are the three microbes reported for significant ethanol production from xylose. Among these three *P. stipitis* is the most efficient and highly productive [14]. Further Olsson and Hahn-Hagerdal [15] reported that several *P. stipitis* strains exhibit a killer character towards *S. cerevisiae* (due to presence of mitochondrial inhibitors like erythromycin and diuron) [16]. Thus this combination was ruled out and combination of *P. stipitis* with *Z. mobilis* [3] was explored in this study for co-culture system development to ferment KGB hydrolysate.

The inhibition of xylose uptake metabolism of *P. stipitis* by catabolite repression in the presence of high glucose concentration and ethanol inhibition due to rapid ethanol production by *Z. mobilis* are major problems identified in the existing co-culture systems where these microorganisms were co-cultured simultaneously. The countermeasures against these problems were taken by using sequential addition of cultures and their respective sugar media. The sequential-co-culture system of *P. stipitis* and *Z. mobilis* was developed at two levels based on the total working volume of fermentation media, first in flask (300 ml) then in bioreactor (4000 ml) using synthetic fermentation media. Finally the selected strategy was implemented for KGB hydrolysate fermentation at bioreactor level. Very limited data is available for lignocellulosic biomass to ethanol production using sequential-co-culture system particularly at bioreactor level [17]. Therefore, in the present investigation efforts were made to develop a novel sequential-co-culture system of high ethanol yield and volumetric productivity by utilizing sugars from KGB hydrolysate.

2. Materials and methods

2.1. Microorganisms and maintenance

P. stipitis NCIM 3498 used in the study was procured from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The culture was grown at 30 ± 0.2 °C and stored at 4 °C on agar slants of MGYP medium contained (g/l): malt extract 3; glucose 10; yeast extract 3; peptone 5 and agar 20, pH 6.0 ± 0.2. *Z. mobilis* MTCC 91 was procured from MTCC, IMT Chandigarh, India. The growth media of *Z. mobilis* consisted (g/l): glucose 20; yeast extract 5; $MgCl_2$ 0.1; $(NH_4)_2SO_4$ 0.1; KH_2PO_4 0.1. pH was adjusted to 5.0 ± 0.2. The media were sterilized by autoclaving at 121 °C for 15 min wherein xylose and glucose were sterilized separately and mixed with other respective media components under aseptic condition.

2.2. Inoculum preparation for sequential culture system development

For seed culture preparation, the composition of media was same as mentioned above for *Z. mobilis* whereas, glucose was replaced by same amount of xylose in the growth media of *P. stipitis*. The inocula of *P. stipitis* and *Z. mobilis* were grown in *Erlenmeyer* flasks separately at 30 ± 0.2 °C in an incubator (Lab Therm LT-X Kuhner, Switzerland). The *P. stipitis* flasks were kept under agitation (150 rpm) to grow cells aerobically, whereas *Z. mobilis* culture was grown under static condition anaerobically (grown in air tight flasks). For xylose fermentation 5% (v/v) inoculum (18 h, 1×10^7 cells/ml) of *P. stipitis* and for glucose fermentation 5% (v/v) inoculum (10 h, 1×10^8 cells/ml) of *Z. mobilis* was used.

2.3. Preparation of fermentation media

2.3.1. Synthetic media

P. stipitis fermentation media contained 60 g/l xylose, supplemented with (g/l): yeast extract 1.0; $(NH_4)_2HPO_4$ 2.0; $(NH_4)_2SO_4$ 1.0; $MgSO_4{\cdot}7H_2O$ 0.25; and 1 ml/l of trace element solution. The trace element solution contained (g/l): $CuSO_4{\cdot}H_2O$ 2.5; $FeCl_3{\cdot}6H_2O$ 2.7; $MnSO_4{\cdot}H_2O$ 1.7; $Na_2Mo_2O_4{\cdot}2H_2O$ 2.42; $ZnSO_4{\cdot}7H_2O$ 2.87; $CaCl_2{\cdot}6H_2O$ 2.4, pH adjusted to 6.0 ± 0.2 with H_2SO_4 solution (0.5 ml concentrated (98%) H_2SO_4 to 1 l). *Z. mobilis* fermentation media contained 100 g/l glucose, supplemented with (g/l): $(NH_4)_2SO_4$ 1.0; KH_2PO_4 0.02; $MgSO_4$ 0.5; yeast extract 6.5, pH was adjusted to 5.0 ± 0.2. The media were sterilized by autoclaving at 121 °C for 15 min wherein sugar solutions (xylose and glucose) were sterilized separately and added directly into the culture flasks.

2.3.2. KGB hydrolysate media

The KGB was hydrolyzed in such a manner that the hemicellulosic and cellulosic fractions were hydrolyzed sequentially by the addition of variable concentration of an inorganic acid (1–35%, v/v; total nine steps) along with direct steam insertion at atmospheric pressure to the reaction vessel for 30 min of reaction time. After each reaction time the liquid fraction was withdrawn from the reaction system. Total nine liquid hydrolysate fractions were collected in accordance to the different acid concentrations applied. The single vessel multi-step technique was successfully used to extract 95.3% of the total reducing sugars (TRS) available in the KGB in the form of carbohydrate polymer. After analysis of the desired compounds, the fractions were mixed in certain way to generate two main streams of sugars, first xylose rich fraction (XRF) and second glucose rich fraction (GRF). The compositions of the fractions were as follows: XRF (g/l): total reducing sugar (TRS) 60 (54 g/l xylose and 6 g/l other reducing sugars); furfural 0.362; hydroxymethyl furfural 0.118; phenolics 0.271 and GRF (g/l): TRS 100 (glucose only); hydroxymethyl furfural 0.972; phenolics 0.613. The fermentation media with XRF (xylose rich fermentation medium, XRFM) and GRF (glucose rich fermentation medium, GRFM) were prepared in similar manner as described in synthetic media preparation with the exception that xylose was replaced with XRF and glucose was replaced with GRF in corresponding media.