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Direct fermentation of xylan by *Clostridium* strain BOH3 for the production of butanol and hydrogen using optimized culture medium

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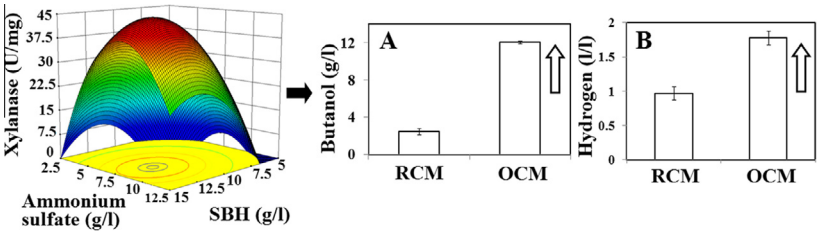
HIGHLIGHTS

- *Clostridium* strain BOH3 is able to use xylan for its growth and ABE fermentation.
- Optimized culture medium (OCM) enhances xylanase expression up to 1.6-fold.
- In OCM, strain BOH3 can effectively utilize high concentration (30–50 g/l) of xylan.
- BOH3 produces >14 g/l of butanol and >2.6 l/l of hydrogen from xylan in OCM.

GRAPHICAL ABSTRACT

An optimized culture medium (OCM) allows *Clostridium* strain BOH3 to ferment 30 g/l of xylan directly and produce (A) butanol and (B) hydrogen.



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ABSTRACT

Clostridium strain BOH3 is able to utilize 10 g/l of xylan in reinforced clostridial medium (RCM) and produce butanol and hydrogen. However, increasing xylan concentration to 30 g/l does not enhance the production of butanol and hydrogen due to insufficient expression of xylanase enzyme (27.5 U/mg). To enhance the xylanase activity, an optimized culture medium (OCM), which consists of sugarcane bagasse hydrolysate (11.75 g/l), ammonium sulfate (8.92 g/l) and iron (III) chloride (1.45 mM) is designed. In the optimized OCM, *Clostridium* strain BOH3 expresses more xylanase and shows higher xylanase activity (44.05 ± 0.25 U/mg) in the OCM. This activity is about 1.6-fold higher than that in the original RCM. Employing OCM as a medium, *Clostridium* strain BOH3 effectively ferments high concentration (30 and 50 g/l) of xylan and produces 12.05 ± 0.15 and 14.80 ± 0.15 g/l of butanol and 1.78 ± 0.08 and 2.65 ± 0.15 l/l of hydrogen, respectively.

1. Introduction

Xylan is the second most abundant polysaccharides on earth. It comprises β-1,4-linked xylose residues in its backbone. It can be hydrolyzed by a group of xylanases, including endoxylanase (EC 3.2.1.8), xylosidase (EC 3.2.1.37), arabinofuranosidase (EC 3.2.1.55), glucuronidase (EC 3.2.1.139) and acetylxylanesterases (EC 3.1.1.72) (Bastawde, 1992). Among them, endoxylanase plays a key role. It rapidly depolymerizes xylan into xylose, xylobiose and a small amount of xylooligosaccharides by hydrolyzing the β-1,4-D-xylosyl linkage. Due to this reason, endoxylanase is added as a major constituent in commercial xylanase preparation (Bajpai, 2009; Juturu and Wu, 2012). Xylanase has been used for different purposes in pulp and paper industry, wood industry, textile industry and food industry (Bajpai and Bajpai, 1992; Daneault et al., 1994; Kenealy and Jeffries, 2003). Additionally, xylanase is used for the production of biofuels such as ethanol, butanol and hydrogen from xylan-based feed stocks in recent years (Bhalla et al., 2013; Dodd and Cann, 2009; Qureshi et al., 2006).

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Butanol and hydrogen have several advantages compared to other biofuels (Jin et al., 2011; Show et al., 2012). They can be simultaneously produced from anaerobic fermentation of glucose or xylose. For example, some solventogenic *Clostridium* strains can produce acetone, butanol and ethanol (ABE), volatile fatty acids (VFAs), hydrogen and CO_2 during ABE fermentation (Gheshlaghi et al., 2009; Jang et al., 2012). However, most solventogenic *Clostridium* strains including *Clostridium acetobutylicum* and *Clostridium beijerinckii* can only utilize simple sugars such as glucose and xylose for their growth. They cannot use xylan as a carbon source due to the lack of xylanolytic enzymes (Jang et al., 2012; Jin et al., 2011; Qureshi et al., 2006). For instance, *C. acetobutylicum* P260 failed to grow in a xylan-containing medium unless the medium was amended with 5 g/l of xylose (or glucose) and xylanase (Qureshi et al., 2006). Meanwhile, *C. acteobutylicum* ATCC39236 cultured in a xylan-based medium showed a longer lag phase up to 240 h due to inadequate xylanase expression (Lemmel et al., 1986). In fact, in most cases xylan was already autoclaved (121 °C for 15–30 min) before adding to the medium, but most solventogenic *Clostridium* strains still could not effectively utilize the autoclaved xylan as a carbon source. To ferment xylan, pretreatment of xylan by using heat, high pressure or acid is necessary. In the literature, *Clostridium* strains such as *C. beijerinckii* BA101 and *C. acteobutylicum* ATCC824 can produce 8.9 g/l of organic solvents (acetone, butanol and ethanol) from the pretreated xylan (Qureshi et al., 2008; Sun and Liu, 2012). Alternatively, xylanase can be added to the medium for hydrolyzing xylan in a so-called simultaneous saccharification and fermentation (SSF) process. (Qureshi et al., 2006; Shah and Lee, 1994). However, in these cases xylan was pretreated with aqueous monoethanolamine (20%) solution at 186 °C for 3 h followed by moist heat (121 °C for 30 min) before the SSF process. On the other hand, a few solventogenic *Clostridium* strains including *Clostridium algidixylanolyticum*, *C. beijerinckii* LU-1, *C. acetobutylicum* LU-1, *Clostridium butyricum* and *Clostridium bifermentans* LU-1 are able to produce xylanase on their own. This means that it is possible for some *Clostridium* strains to utilize xylan directly without any pretreatment. However, most *Clostridium* strains do not express xylanase actively, and their potential to use xylan directly for the production of butanol and hydrogen is largely unknown (Broda et al., 2000; Marichamy and Mattiasson, 2005).

A common problem in the direct fermentation of xylan by using *Clostridium* strains is insufficient expression of xylanase by solventogenic *Clostridial* strains. Therefore, it is desirable to identify a new solventogenic *Clostridium* strain which is capable of expressing xylanase and utilizing unpretreated xylan as a substrate for fermentation. Recently, a solventogenic *Clostridium* strain BOH3 was reported to express xylanase in the culture medium with high activity (21.89 ± 0.1 U/mg) such that it can utilize xylan effectively at low concentration (10 g/l) to produce both butanol and hydrogen (Bramono et al., 2011; Rajagopalan et al., 2013b) However, when the xylan concentration was increased to 30 g/l, the final butanol and hydrogen concentration did not increase accordingly. One possible explanation is that the xylanase excreted by BOH3 is insufficient to hydrolyze 30 g/l of xylan completely. Therefore, we hypothesize that by increasing the expression level of xylanase, more xylan can be hydrolyzed and that will lead to higher butanol and hydrogen concentrations. Since the xylanase expression from strain BOH3 is supported by sugarcane bagasse hydrolysate (SBH), a production medium consisting of SBH as carbon source is designed based on response surface methodology to enhance the production of xylanase. Furthermore, the potential of this optimized culture medium (OCM) for supporting butanol and hydrogen production from xylan (native and autoclaved) is investigated. This is the first report dealing with production of xylanase enzyme by using a solventogenic *Clostridium* strain and a consolidated bioprocess for effective utilization of xylan for the production of butanol and hydrogen.

2. Methods

2.1. Materials

All chemicals including ammonium chloride, ammonium sulfate, sodium nitrate, potassium nitrate, magnesium chloride, calcium chloride, cobalt chloride, copper (II) sulfate, iron (II) sulfate, iron (III) chloride, manganese (II) chloride, nickel (II) chloride and zinc chloride were purchased from Sigma–Aldrich, USA. Reinforced clostridial medium was procured from Difco, USA. Beechwood xylan and xylose were obtained from Sigma–Aldrich, USA.

2.2. Sugarcane bagasse hydrolysate preparation

Sugarcane bagasse (SB) was collected from a local fruit stall in Singapore. It was thoroughly washed using double distilled water and dried at 60 °C overnight. Later, they were chopped into fine powder using a mixer grinder (Braun, India) and screened by using a mechanical sieve set with a pore size of 1 mm. Only SB particles smaller than 1 mm were used in this study. To prepare sugarcane bagasse hydrolysate (SBH), SB particles were treated with 0.005 M of sulfuric acid at 121 °C for 15 min. Then, they were filtered by using 0.45-μm membrane (Sartorius, USA). The pH of the filtrate was adjusted to pH 6.8 by addition of 2 M of NaOH. Total reducing sugars present in SBH were estimated by using 3,5-dinitro salicylic acid (DNS) method (Miller, 1959).

2.3. Microorganism and xylanase expression

The microorganism used in this study was *Clostridium* strain BOH3 (Bramono et al., 2011; Rajagopalan et al., 2013a). A commercial reinforced clostridial medium (RCM) was used for culturing strain BOH3. RCM consists (g/L) of peptone (10), beef extract (10), yeast extract (3), D-glucose (5), starch (1), NaCl (5), sodium acetate (3), cysteine HCl (0.5) and agar (0.5). Firstly, 20 ml (1.5 × concentrated) of RCM was dispensed into a 120 ml serum bottle while the bottle was purging with N_2 to remove O_2. Later, the bottle was sealed with a rubber septum and an aluminum cap to provide an anaerobic condition. Subsequently, the bottle containing RCM was autoclaved at 121 °C for 20 min. SBH was used as a carbon source. For the selection of nitrogen source, 5 g/l of inorganic nitrogen salts including ammonium chloride, ammonium sulfate, sodium nitrate, potassium nitrate and urea were individually added to RCM containing SBH (5 g/l). Similarly, 1 mM of mineral salts including magnesium chloride, calcium chloride, cobalt chloride, copper (II) sulfate, iron (II) sulfate, iron (III) chloride, manganese (II) chloride, nickel (II) chloride and zinc chloride were separately added to RCM containing 5 g/l of SBH and 5 g/l of ammonium sulfate. SBH was sterilized through 0.22 μm syringe filters (Sartorius, USA). Meanwhile, stock solutions of nitrogen and mineral salts were prepared and autoclaved separately. SBH, nitrogen salt and mineral salt were added to RCM just before the inoculation and the final volume was adjusted to 30 ml. The final pH of the culture medium was examined and adjusted to pH 6.8 ± 0.1 by using 2 M NaOH/HCl. After inoculation (10%v/v), the bottle were incubated at 35 °C under constant shaking (150 rpm). After 48 h of fermentation, the samples were centrifuged at 10,000 rpm for 15 min and the clear supernatant was used for xylanase assay.

2.4. Xylanase assay

One milliliter of reaction mixture containing 0.5% (w/v) of beechwood xylan and 100 μl of the enzyme sample dissolved in

sodium acetate buffer (50 mM, pH 5) was incubated at 35 °C for 15 min, and the amount of reducing sugars released was measured by using DNS method. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar as xylose equivalents in 1 min under the assay conditions. Protein concentration was determined by using Bradford assay according to manufacturer's (Bio-Rad) instructions.

2.5. Response surface methodology (RSM)

The experimental design was a 2^3 full factorial central composite rotatable experimental plan with three medium components, i.e. SBH, ammonium sulfate and iron (III) chloride. The experimental plan consisted of 20 trials including six center points, and the value of the dependent response was the mean of three independent experiments. The response variable obtained using RSM was fitted to the second order polynomial equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y_i is the predicted response, x_i, x_j are input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient. The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' (Version 8.0, Stat-Ease Inc., Minneapolis, USA) statistical software package. Statistical analysis of the model was performed by the analysis of variance (ANOVA).

2.6. Butanol and hydrogen production

Beechwood xylan in RCM was fermented by strain BOH3 for butanol and hydrogen production. To investigate the effect of xylanase on production of butanol and hydrogen, a certain amount (0.5–2.5 U/ml) of commercially available xylanase from *Thermomyces lanuginosus* (Sigma, USA) was added to fermentation at the beginning. On the other hand, beechwood xylan (30 and 50 g/l) was added into optimized culture medium (OCM) for the production of butanol and hydrogen. In some experiments, xylan was not autoclaved to investigate the potential of xylanase on hydrolyzing native xylan. Similar to xylanase production, butanol fermentation was conducted anaerobically at 35 °C for 5–7 days under the agitation speed of 150 rpm. Samples were drawn every 12 h to estimate the liquid and gaseous products.

2.7. Product analysis

Liquid samples were analyzed by using gas chromatography (GC; model 7890A; Agilent technologies, USA). The GC is equipped with a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm, model 123–7334; J&W, USA) and a flame ionization detector (FID). The oven temperature was set at 60 °C for 2 min, then it was increased with a ramp of 10 °C/min to 230 °C and held for 2 min. Helium was used as carrier gas with a flow rate of 2.5 ml/min. Acetone, butanol, ethanol, acetic and butyric acids (Sigma, USA) were added into liquid samples as internal standards for qualitative analysis. Based on increase in peak areas, the specific component was identified in the liquid samples. For quantitative analysis of these products, the standard curves were obtained by running known concentrations (0.5–25 g/l) of standard solutions by using the same method. On the other side, gaseous samples were analyzed for hydrogen production with another GC (model GC-17A; Shimadzu, Japan) equipped with a thermal conductivity detector (TCD) and a Supelco 80/100, Porapak-N column, (2 m × 1/8 inch stainless steel column). The oven temperature was kept constant at 110 °C for 2.3 min and argon (15 ml/min) was used as the carrier gas. One hundred microliter of gas was drawn from the head space of fermentation bottle by using a gas-tight syringe, and immediately injected into GC–TCD to begin the analysis. For every fermentation bottle, this procedure was repeated for three times and the average was computed for hydrogen production. Standard gaseous mixtures consisting of hydrogen (5%), nitrogen (60%), carbon dioxide (15%), carbon monoxide (15%) and methane (5%) at known proportions were used to obtain a calibration curve.

3. Results and discussion

3.1. Fermentation of xylan and xylose by BOH3

Clostridium strain BOH3 was cultured in RCM supplemented with xylan (30 g/l) for the production of butanol and hydrogen. For comparison, RCM supplemented with 30 g/l of xylose was also tested. Since the strain BOH3 is capable of excreting xylanase enzyme to the culture medium, it can utilize xylan effectively and attain maximum cell density (OD_{600} = 3.8) after 12 h of fermentation (Fig 1A). While the fermentation took place, pH of the medium was dropping down because of the release of acetic and butyric acids. At 48 h, the medium pH was 4.7 ± 0.2. After this, the pH slowly increased during butanol production phase and eventually reached 4.9 ± 0.1 (Fig 1B). A similar phenomenon was reported for other butanol producing *Clostridium* strains (Lemmel et al., 1986; Qureshi et al., 2006). In the culture supernatant, xylanase activity was observed after 6 h and it reached the maximum level of 27.5 U/mg after 48 h (Fig 1C). In addition to xylanase, BOH3 also produced a significant amount of hydrogen (0.87 l/l) from xylan. During the exponential growth phase, hydrogen production rate was approximately 250–270 ml/l h, and then diminished to 1–1.5 ml/l h after 48 h when BOH3 entered a stationary phase (Fig 1E). Meanwhile, butanol production started after 24 h. Its concentration reached 2.5 g/l after 5 days of fermentation. Eventually, fermentation of xylan (30 g/l) by strain BOH3 produced 2.5 g/l of butanol and 0.98 l/l of hydrogen. For comparison, strain BOH3 cultured in RCM with 30 g/l of xylose produced 10.5 g/l of butanol and 1.7 l/l of hydrogen (Fig 1D and E). The final cell density was also higher (OD_{600} = 5.5) when xylose was used. Apparently, xylose is a better substrate than xylan (Fig 1A). Theoretically, if xylan can be hydrolyzed to xylose completely, then the final butanol and hydrogen obtained from xylan should be comparable to that from xylose (Gheshlaghi et al., 2009; Lemm et al., 1986). Unfortunately, most solventogenic *Clostridium* strains either do not produce or only produce a little xylanase. In the case of strain BOH3, it has a xylanase gene (*xynb*) encoded in the genome (data not shown). Consequently, strain BOH3 expresses xylanase enzyme consistently and supports the butanol and hydrogen production when it is cultured in 10 g/l of xylan (Bramono et al., 2011; Rajagopalan et al., 2013b). However, in the presence of 30 g/l of xylan, the xylanase activity (27.5 or 2.43 U/ml) is insufficient for complete hydrolysis of xylan. That is why the cell density is low (OD_{600} = 3.8).

To test whether insufficient xylanase expression is the main cause of the low cell density, additional xylanase (0.5–2.5 U/ml) was added to the medium at the beginning of fermentation. Supplementation of xylanase significantly increased the cell density and final concentrations of butanol and hydrogen (Fig 2 A–C). When the xylanase concentration was ~2 U/ml, the cell density was 5.35 (OD_{600}). Consequently, the final butanol and hydrogen concentrations increased to 10.1 g/l and 1.65 l/l, respectively, which are almost the same as the previous results when xylose was used as the substrate. Therefore, optimizing culture medium to enhance the xylanase expression would presumably improve the utilization of xylan substrate for butanol and hydrogen production.

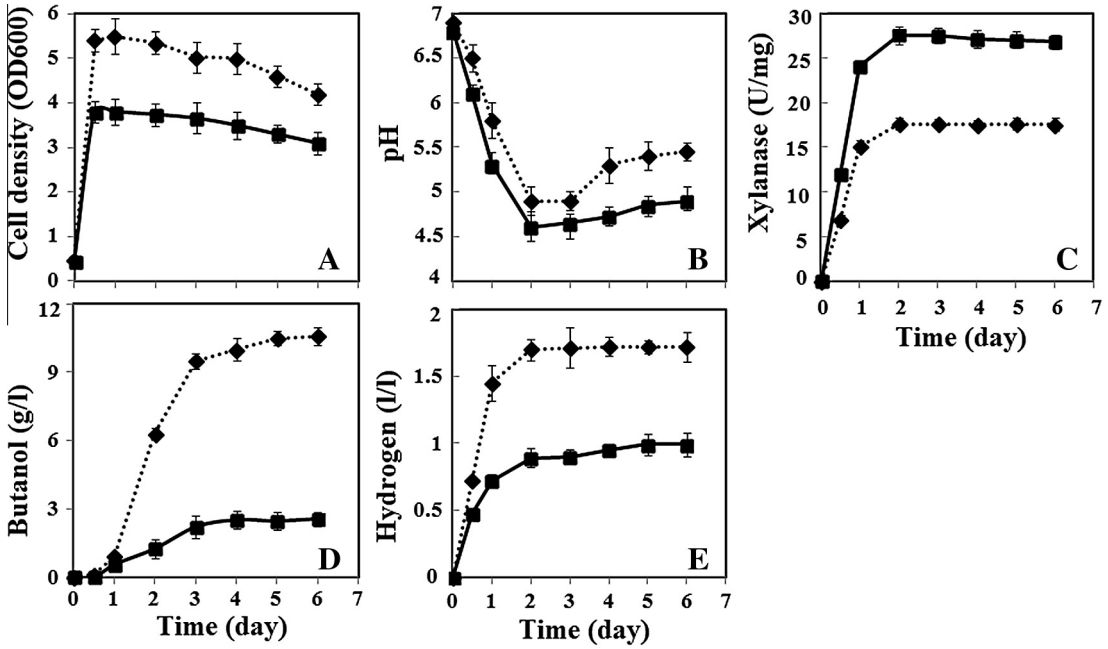

Fig. 1. Fermentation profile for *Clostridium* strain BOH3 cultured in RCM supplemented with 30 g/l of xylan (solid line) or xylose (dotted line). (A) Cell density, (B) pH profile (C) xylanase activity, (D) butanol concentration and (E) hydrogen concentration.

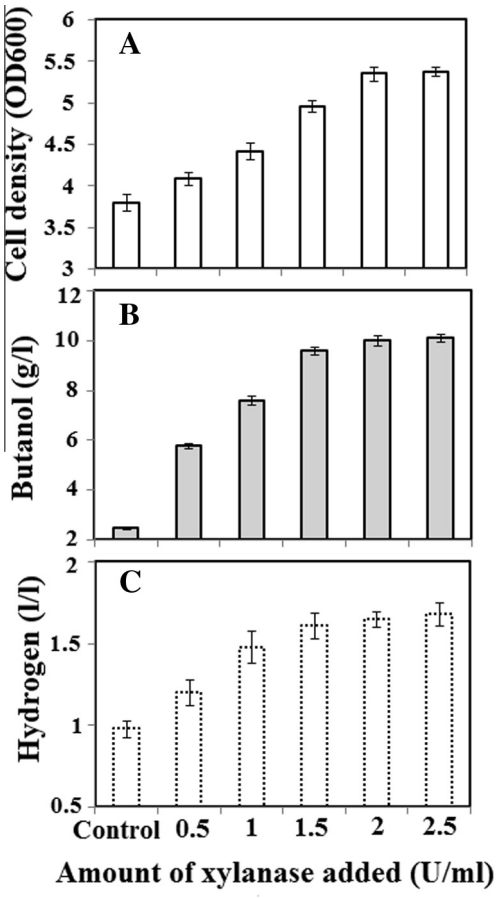

Fig. 2. Effect of adding xylanase on promoting (A) cell density, (B) butanol concentration and (C) hydrogen concentration during fermentation of 30 g/l of xylan.

3.2. Optimized culture medium for maximizing xylanase activity

Initially, the effect of medium components on xylanase activity in the culture medium was studied. In general, some medium components, such as nitrogen sources and metal ions, can induce expression of xylanase and increase the xylanase activity. Table 1 show that addition of inorganic nitrogen salts to the medium enhanced the production of xylanase by BOH3. Noticeably, ammonium sulfate enhances the xylanase activity to 7.34 ± 0.21 U/mg, which is better than other nitrogen salts selected for the study. It seems that ammonium sulfate is a preferred nitrogen source for the production of xylanase. Meanwhile, addition of metal ions except copper (II) and zinc (II) also enhanced the xylanase activity. Among various metal ions studied, adding iron (II) and (III) to the medium increases the xylanase activity to 8.93 ± 0.14 and 9.12 ± 0.15 U/mg, respectively. Iron is an important element for almost all microbes, because it plays a key role in numerous cellular functions and metabolic pathways (Andrews et al., 2003; Vasileva et al., 2012). There are several iron-containing microbial proteins which participate in energy metabolism processes. In the case of strict anaerobe such as *Clostridium*, iron is present in redox enzymes such as hydrogenases and nitrogenases, which help to maintain redox potential, and fight against oxidative stress. In a typical case, nearly 14 to 20 iron atoms are present in iron hydrogenases I and II of Clostridium strains (Adams and Mortenson, 1984; Lovenber and Sobel, 1965; Watanabe et al., 2009). Additionally, iron (II) and (III) transporter proteins such as feo (A and B) proteins and ATP binding cassette transporter (iron (III) ABC transporter) protein are identified in *Clostridium* (Metacyc, 2013). Therefore, addition of iron presumably favors certain metabolic pathways and accelerates the xylanase expression machinery in strain BOH3. However, it requires further molecular level investigation to support above statement and also for better understanding.

3.3. Response surface analysis

The effect of SBH, ammonium sulfate and iron (III) chloride on xylanase activity was studied by employing response surface methodology. According to experimental strategy, the concentra-

Table 1
Effect of medium component on xylanase activity.

Medium component	Xylanase activity (U/mg)[a]	Medium component	Xylanase activity(U/mg)[a]
No nitrogen source	3.6	No metal ions	7.3
Nitrogen source (5 g/l)		*Metal ions* (5 mM)	
Ammonium chloride	5.7	Mg^{2+}	7.8
Ammonium sulfate	7.3	Mn^{2+}	7.8
Sodium nitrate	5.2	Fe^{2+}	8.9
Potassium nitrate	5.5	Ca^{2+}	7.9
Urea	6.7	Cu^{2+}	5.0
	3.6	Co^{2+}	8.1
		Ni^{2+}	8.1
		Fe^{3+}	9.1
		Zn^{2+}	4.5

[a] Average values obtained from three independent experiments.

Table 2
Experimental plan and xylanase activity.

Expt. no.	Variables and their coded values			Xylanase (U/mg protein)			
	SBH (g/l) [X_1]	Ammonium sulfate (g/l) [X_2]	Iron (III) chloride (mM) [X_3]	Predicted	Actual	Residual	% Value
1	7.5	5.0	1.0	14.1	11.3	−5.7	<18
2	12.5	5.0	2.0	26.6	26.3	−0.5	<1
3	7.5	10.0	1.0	16.6	17.4	1.5	>5
4	12.5	10.0	1.0	32.0	34.3	4.5	>7
5	7.5	5.0	2.0	14.4	13.4	−2.1	<7
6	12.5	5.0	2.0	29.2	29.3	0.1	>0.2
7	7.5	10.0	2.0	17.4	18.5	2.2	>6
8	12.5	10.0	2.0	35.2	39.4	8.4	>12
9	5.0	7.5	1.5	5.3	4.8	−1.0	<10
10	15.0	7.5	1.5	35.5	38.1	5.4	>8
11	10.0	2.5	1.5	8.1	6.8	−2.7	<17
12	10.0	12.5	1.5	16.6	19.4	5.7	>17
13	10.0	7.5	0.5	18.5	19.8	2.5	>7
14	10.0	7.5	2.5	22.0	22.6	1.1	>3
15	10.0	7.5	1.5	39.9	40.7	1.7	>2
16	10.0	7.5	1.5	39.9	40.8	1.8	>2
17	10.0	7.5	1.5	39.9	40.7	1.6	>2
18	10.0	7.5	1.5	39.9	40.7	1.6	>2
19	10.0	7.5	1.5	39.9	40.8	1.8	>2
20	10.0	7.5	1.5	39.9	40.9	2.0	>3

tions of SBH, ammonium sulfate and iron (III) chloride were varied. The actual values and the corresponding coded levels for each independent variable are given in Table 2. Based on preliminary results, values corresponding to center point on xylanase production were chosen (data not shown). All the trials according to experimental plan were performed, and the respective xylanase activity measured is given in Table 2. The regression equation describing the relationship between the xylanase activity and the test variables in coded units is:

$$Y = 4.66 + 8.46X_1 + 3.43X_2 + 1.07X_3 + 0.87X_1X_2 + 0.62X_1X_3 + 0.15X_2X_3 - 4.86X_3^2 - 6.95X_2^2 - 4.93X_3^2 \quad (2)$$

The coefficient of determination (R^2) was calculated as 0.9985 for xylanase production (Table 3), indicating that the statistical model can explain 99.85% of variability in the response (Box et al., 1978). Adequate precision measures signal to noise ratio. An adequate precision of 70.298 for xylanase production was recorded. The predicted R^2 of 0.9863 is in reasonable agreement with the adjusted R^2 of 0.9967. This indicates good agreement between the experimental and predicted values for xylanase activity. Notably, the model F-value of 642.34 and values of P (<0.0001) indicate that model terms are significant. The lack of fit F-value of 186.99 implies

Table 3
Result of ANOVA quadratic model.

Source	SS	DF	MS	F-value	Probability (P)
Model	3094.22	9	343.80	642.34	<0.0001
Residual (error)	5.35	10	0.54		
Lack of fit	5.32	5	1.06	186.99	<0.0001
Pure error	0.028	5	0.0057		
Total	3099.57	19			

R^2 = 0.9985; adjusted R^2 = 0.9967; predicted R^2 = 0.9863; adequate precision = 70.298; coefficient of variation (CV) = 2.68%; SS – sum of squares; DF – degree of freedom; MS – mean square.

Table 4
Significance of regression coefficients.

Model term	Parameter estimate	Standard error	Computed t-value	P-value
Intercept	40.66	0.29	140.2068	<0.0001
X_1	8.46	0.18	45.7567	<0.0001
X_2	3.43	0.18	18.5135	<0.0001
X_3	1.07	0.18	5.7567	0.0002
X_1X_2	0.87	0.26	3.3269	0.0073
X_1X_3	0.62	0.26	2.3653	0.0382
X_2X_3	0.15	0.26	0.5769	0.5787
X_1^2	−4.86	0.15	−33.5172	<0.0001
X_2^2	−6.95	0.15	−47.8965	<0.0001
X_3^2	−4.93	0.15	−34.0000	<0.0001

that the lack of fit is significant. Moreover, the coefficient of variation (CV = 2.68%) is low indicating a better precision and reliability of the experiments.

Student t-test and P-values of coefficients of linear, interactive and quadratic terms are listed in Table 4. The model terms express linear (X_1, X_2, and X_3), interactive ($X_{1,2}, X_{22}$, and X_{23}) and quadratic (X_1^2, X_2^2, and X_3^2) effects of medium components on xylanase expression. Based on t-value, the probability of the difference (P-value) in the data due to sampling error is calculated. If P-value measures lower than 0.05, the model term is significant and influences the response directly (Myers et al., 2008). Accordingly, linear and quadratic terms estimate much lower P-values (<0.0001) which indicate that xylanase activity correlates with SBH, ammonium sulfate and iron (III) chloride significantly. For interactive terms, X_1X_2 and X_1X_3 for SBH with ammonium sulfate (P = 0.0073) and SBH with iron (III) chloride (P = 0.0382) are found significant, whereas X_2X_3 for ammonium sulfate with iron (III) chloride is observed insignificant (P = 0.0.5782) on xylanase activity.

The levels of variables for maximum xylanase activity estimated from the model by numerical method according to Meyers and Montgomery are 11.75 g/l for SBH, 8.92 g/l for ammonium sulfate and 1.45 mM for iron (III) chloride. These values fall within the range of experimental design (Box et al., 1978; Myers et al., 2008). In an optimized culture medium containing the components mentioned above, the xylanase activity is 44.05 U/mg which is close to a predicted value (43.85 U/mg). Compared to xylanase activity in RCM, there was about 1.6-fold higher enzyme xylanase activity from optimized medium.

3.4. Consolidated bioprocess for butanol and hydrogen production

The optimized culture medium (OCM) for xylanase expression was used for the production of butanol and hydrogen from 30 and 50 g/l of xylan. Table 5 shows the xylanase activity and concentration of butanol and hydrogen from BOH3 in OCM containing either autoclaved or native xylan. It shows that xylan in OCM supports the butanol and hydrogen is almost equivalent to the concentration obtained from xylose with OCM. Interestingly, strain BOH3

Table 5
Butanol and hydrogen production from xylan by *Clostridium* strain BOH3.

Products	Carbon source (30 g/l) in OCM[a]			Carbon source (50 g/l) in OCM[a]		
	Xylose	Xylan (autoclaved)	Xylan (native)	Xylose	Xylan (autoclaved)	Xylan (native)
Cell density (OD_{600})[b]	5.94 ± 0.30	5.50 ± 0.10	n.m	6.24 ± 0.20	5.70 ± 0.05	n.m
Xylanase (U/mg)[c]	49.50 ± 0.25	61.85 ± 0.25	57.37 ± 0.20	51.50 ± 0.15	70.65 ± 0.20	61.55 ± 0.10
Butanol (g/l)[d]	12.95 ± 0.15	12.05 ± 0.15	7.15 ± 0.10	16.05 ± 0.20	14.80 ± 0.15	8.85 ± 0.25
Total solvents (g/l)[d]	16.70 ± 0.10	15.85 ± 0.10	11.82 ± 0.29	21.70 ± 0.15	19.05 ± 0.25	13.05 ± 0.15
VFAs (g/l)[d]	1.22 ± 0.05	2.20 ± 0.05	4.05 ± 0.08	2.05 ± 0.25	3.55 ± 0.10	4.80 ± 0.12
Hydrogen (l/l)[d]	1.90 ± 0.05	1.78 ± 0.08	1.39 ± 0.10	3.10 ± 0.10	2.65 ± 0.15	1.95 ± 0.15

n.m – value not measured.
[a] Optimized culture medium for xylanase expression.
[b] Measured at 12 h of fermentation.
[c] Activity estimated at 48 h of fermentation.
[d] Measured after 5 days of fermentation, and in case of native xylan the values obtained after 7 days of fermentation.

grown in OCM produced 7.15 ± 0.10 and 8.85 ± 0.25 g/l of butanol and 1.39 ± 0.10 and 1.95 ± 0.15 l/l of hydrogen from 30 and 50 g/l of xylan, respectively. These concentrations are nearly equivalent to the cases in which xylose was used as a substrate. It shows that optimization of xylanase expression in clostridial fermentation significantly enhances the butanol and hydrogen production from xylan. Meanwhile, this process eliminates the addition of commercial xylanase into SSF process and facilitates one-pot fermentation for butanol and hydrogen production.

4. Conclusion

Clostridium strain BOH3 can utilize 10 g/l of xylan effectively for the production of butanol and hydrogen. To allow BOH3 utilize high concentration (30 and 50 g/l) of xylan effectively, an OCM is designed to obtain maximum xylanase activity in the medium. This OCM facilitates direct fermentation of xylan to produce butanol and hydrogen with a yield almost equivalent to that when xylose is used as a substrate. This study shows the potential of using BOH3 and OCM in a consolidated bioprocess to produce butanol and hydrogen.

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