Characterization of a Cellulose and Xylose Utilization *Bacillus cereus Yc-A1*

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Abstract Bioconversion of cellulose and xylose is critical for the economically utilization of lignocellulose resources. Cellulose and xylose utilized strain *Bacillus cereus Yc-A1* with highly ethanol endurance ability was present. The strain *Yc-A1* was identified according to the physiological and phylogenetic characteristics. The maximum carboxymethyl cellulase (CMCase) activity and filterpaperase (FPase) activity reached 0.261 and 0.194 U/ml at 48 and 96 h, respectively. The strain *Yc-A1* can use xylose as sole carbon source and its ethanol endurance ability was significantly superior to *Pichia stipitis 1960* and *Saccharomyces cerevisiae AADY* under 10% and 20% ethanol concentration. Ethanol and lactic acid was 0.72 g/L and 8.91 g/L for 72 cultivation analyzed by GC-MS and HPLC-MS, respectively. *Bacillus cereus Yc-A1* was inferred to have potential application in cellulose degradation process such as feed industry and paper making industry.

Introduction

Lignocellulose is the most abundant organic polymer on earth and its bioconversion is globally significant for the application of the renewable resources. During to the condensing structure and chemical composition, the efficient utilization of lignocellulose by microbes is challenging [1].Still, utilization of xylose, constitutes 10-40% of the total carbohydrate, is essential for the bioconversion of lignocellulose to fuels and chemicals [2, 3]. As the largest sources of hexose and pentose sugars from the hydrolysate of cellulose, the conversion of hexose (mainly glucose) and pentose (mainly xylose) has been the research focus for the past decades[4]. Many cellulose degradation microbes, mainly fungi and bacteria, were isolated or constructed such as *Ruminococcus*, *Clostridium*, *Acetivibrio cellulolyticus*, *Acinetobacter*, *Pseudomonas*, *Xanthomonas*, *Bacillus,* et al. [5, 6, 7, 8, 9,10]. To our best of knowledge, few data was available in terms of *Bacillus cereus sp.* with both cellulose and xylose utilization ability, also high ethanol endurance ability. These characteristics could simplify or strengthen certain crafts for the continuous bioconversion of cellulose and xylose or their mixture. The identification and characterization of *Bacillus cereus Yc-A1* was reported here.

Materials and methods

Strain and medium

The strain *Yc-A1* was isolated by CMC agar media from the corrupt tree bark in Fuliangang park located at Panyu district of Guangzhou city of China and kept at Microbial Culture Collection Center of Guangdong Institute of Microbiology (GIMCC1.842). *Pichia stipitis 1960* and *Saccharomyces cerevisiae AADY* was obtained from China Center of Industrial Culture Collection (CICC), Beijing, China and Angel Yeast Co Ltd, Yichang, China respectively. The molecular chemicals was purchased from Sangon Biotech of Shanghai. All medium chemicals are purchased from Beijing Chemical Reagents Company. Agar, peptone, yeast extraction are biochemical grade

reagents. The rest chemicals are analytic-grade. The universal primer pairs 27f and 1492r was synthesized by Sangon Biotech of Shanghai. CMC liquid medium contain 0.5% CMC-Na, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.15%Na_2SO_4, 0.05% NaCl, 0.04% KNO_3, 0.05% peptone, 0.05% yeast extract [7]. CMC solid medium contain 2% agar in CMC-Na liquid medium. Filter paper hydrolyzing medium contain 1×6 cm filter paper strips, 0.02% $CaCL_2.2H_2O$, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.15%Na_2SO_4, 0.05% peptone, 0.05% yeast extract [11]. YPD medium contain 2% glucose, 2% peptone, 1% yeast extract, 0.25% agar. Xylose liquid medium contain 6% Xylose, 0.02% $(NH_4)_2SO_4$, 0.5% KH_2PO_4, 0.04% $MgSO_4.7H_2O$, pH5.0. Glucose liquid medium contain 6% glucose, 0.02% $(NH_4)_2SO_4$, 0.5% KH_2PO_4, 0.04% $MgSO_4.7H_2O$, pH5.0. Mixed sugar medium contain 1.5% glucose, 4.5% xylose, 0.5% peptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.02% $(NH_4)_2SO_4$, 0.04% $MgSO_4.7H_2O$, pH5.0. mineral salt was sterilized by filtration, other medium was sterilized at 121°C for 20min.

Morphological and physiological identification

The bacterial *Yc-A1* were identified by means of morphological examination and some biochemical characterization. The parameters investigated included colony characteristics, shape, size, Gram's reaction, Voges-Proskauer (V-P) reaction, Indole production, carbohydrate metabolism (acid-gas production) were carried out following the standard methods described in Bergey's Manual of Determinative Bacteria [12].

Molecular identification

Bacterial 16S rDNAs were amplified by PCR using the primers 27f/1492r. The PCR reaction was performed with a thermal program comprised of 30 cycles at 94°C for 30s, 55°C for 30s, and 72°C for 1 min. The amplified products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and was sequenced by Shenzhen Huada Gene Research Company. Phylogenetic trees were calculated by the Kimura two-parameter model and the neighbor-joining algorithm using the PAUP software (version 4.0 b8). One thousand bootstraps were performed to assign confidence levels to the nodes in the trees.

CMC degradation

Fermentation broth of strain *Yc-A1* was inoculated to CMC agar by sterilized toothpick after culturing at 30°C, 160 rpm for 26 h in 15 cm tube. The plates were incubated at 30°C for 72h. To visualize the hydrolysis zone, the plates were flooded with 0.1% Congo red solution and washed with 1 M NaCl. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter (hydrolysis capacity) was measured in order to evaluate the cellulose degradation ability [5].

Filter paper disintegration

Strain *Yc-A1* was inoculated into filter paper hydrolyzing medium and cultured at 30°C, 160 rpm for 72 h. The degradation degree was observed comparing with the control without inoculation.

Fermentation experiments

Strain Yc-A1 was inoculated into 3 ml YPD medium at 30°C, 160 rpm for 40 h. Then, one milliliter fermentation broth was inoculated into 250 ml Erlenmeyer flasks containing 50 mL of YPD medium. Two milliliter broth was sampled every 24 h till 120 h and was applied to CMCase activity, FPase activity and biomass (OD600) . One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol product per minute. Each experiment was performed in triplicate.

CMCase activity

Cellulase is a complex of enzymes containing mainly exo and endo β-glucanases plus cellobiase. CMCase activity and FPase activity was adopted to evaluate the cellulose activity. CMCase was estimated by using 1% CMC-Na as the sole carbon source. The reaction mixture was composed of 0.5ml enzyme sample and 1.5 ml CMC-Na (1%) dissolved in 0.1 mol/l sodium acetate buffer (pH 4.8). The reaction was kept at 50°C for 30 minutes. The amount of reducing sugar was determined by the DNS (3,5-dinitrosalicylic) method using glucose as a standard at 550 nm [13]. As a control, 0.5 ml enzyme sample and 1.5 ml CMC-Na (1%) was incubated in boiling water for 5min.

FPase activity

FPase activity were measured according to the method of Eveleigh with minor modification [11].

Briefly, 0.5 ml enzyme solution and 1.5 ml sodium citrate buffer (0.05 mol/L, pH4.8) was filled in 15 cm tube, then sterilized Whatman No. 1 filter paper cutted into 1×6 cm strips was immersed. The reaction was incubated 30 min at 50°C and was stopped by adding 3 ml DNS reagent. Place tubes in boiling water for 5 min and cool down to room temperature, distilled water was added in the tube up to the volume of 20 ml as test solution. A blank tube (without filter paper) was set to correct for any reducing sugar present in enzyme preparation. The enzyme solution sample was triplicate and read at OD540 with spectrometer. One unit of FPase activity was expressed as 1 μ mol of glucose liberated per ml enzyme per minute. The values obtained are compared with glucose standard curve.

Ethanol endurance

Growth curve of strain *Yc-A1* was observed on xylose liquid medium and glucose liquid medium at 30°C 170 rpm during 96 h. For the ethanol endurance test, ethanol with final concentration of 10% and 20% was added to the mixed sugar medium respectively. OD_{600} was recorded every 24 h till 96 h. *Pichia stipitis 1960* and *Sacharomyces cerevisiae AADY* were set as a control.

Analytical method

Strain *YC-A1* was cultured in 250 ml flask with 50 ml mixed sugar medium at 30°C 170 rpm for 72 h, then the supernatant was collected by centrifugation at 4000 g, 4 °C for 10 min. Filtrated supernatant samples were analyzed on Shimadzu GC/MS-QP2010 equipped with DB-WAX (60 m×0.5 μm×0.25 mm) column for ethanol concentration with headspace sampling. Lactic acid was analyzed on HPLC-MS (Agilent 6130 Quadrupole, USA) equipped with Agilent SB C18 (5 μm 4.6 mm×250 mm). The procedure of lactic acid analysis described by Zhao was employed in this study[14]. Ethanol analysis was according to the method by Xiao with minor revision [15]. The gradient temperature program was set at 40 °Cfor 2 min, ramped to 100 °C at the rate of 10 °C/min, then again ramped to 220 °C at the rate of 40 °C/min, and maintained for 3 min.

Statistical analysis

Enzyme activity and ethanol endurance data were processed for correlations coefficients using bivariate analysis (SPSS 13.0, Inc., Chicago, IL).

Results and discussion

Biochemical test of strain *Yc-A1*

Strain *Yc-A1* is a gram-positive, rod-shaped, motile bacterium and the typical biochemical tests was followed: glucose (+),sucrose (+),arabinose (+), xylose(+), maltose(+), lactose(-), nitrate(+), methyl red(+), diastase(+), catalase(+), VP(+).

Degradation of CMC and filter paper

The colony diameter of strain *Yc-A1* on CMC solid medium was 0.5±0.1, 5.0±0.2, 8.4±0.3 mm at 24, 48, 72 h respectively. The plate with *Yc-A1* colony was stained with congo red after 72 h cultivation. The hydrolysis capacity was 1.9±0.1, which showed strain *Yc-A1* have certain degradation ability of CMC. Filter paper disintegrating degree increased from 24-96 h according to Table 1. In this work, the degradation observed was not significant comparing to previous report [16]. It can be inferred the filter paper degradation of strain *Yc-A1* was not strong in test conditions, which was affected by incubation time, medium, et al.

Table 1 Degradation of filter paper by strain *Yc-A1* in tubes during 24-96 h

Incubate time(h)	24	48	72	96
filter paper disintegrating degree	+	++	+++	+++

"+"means brim of the filter paper expand, "++" means little fragments was observed in the bottom of the tube, "+++" means a little fragments was observed in the bottom of the tube, "++++" means the filter paper appeared pasty.

Growth curve and enzyme activity

The relationship among CMCase activity, FPase activity and biomass are presented in Fig. 1. The CMCase activity reached the maximum 0.29 U/ml at 48 h, while FPase activity reached the

maximum 0.08 U/ml at 96 h. No positive associations were noted for CMCase activity and biomass (p=0.158) or FPase activity and biomass (p=0.982), while the CMCase activity variation trend is similar with the biomass according to Fig3. Pourramezan et al. recently isolated two *Bacillaceae* strain *B2A* and *B5B* with cellulose degrading ability from the gut of xylophagous termite microcerotermes diversus [14]. The reported maximum CMCase activity was 1.58 U/ml/h (equals to 0.03 U/ml/min), which was nearly one tenth of that of strain *Yc-A1*. Ariffin reported *Bacillus pumilus EB3* of the maximum enzyme activity of 0.011 and 0.079 U/mL for FPase and CMCase respectively after 24-hour of fermentation, which accounts for 5.6% and 30.3% respectively of the FPase and CMCase activity of strain *Yc-A1*. Comparing to the *Trichoderma harzianum FJ1*(fungus), the CMCase activity reached 41.2 U/ml in 7 days. The maximal CMCase activity of *Trichoderma harzianum FJ1* was 158 times more than that of strain *Yc-A1*. Screened bacteria and fungus with cellulose degradation ability may cooperate to degrade lignocellulose efficiently. It was confirmed by BMC-9, a composite microbial system, which has a strong ability to rapidly degrade the lignocelluloses of rice straw [14]. Interestingly, *Bacillus sp.* was present in the microbial community of BMC-9

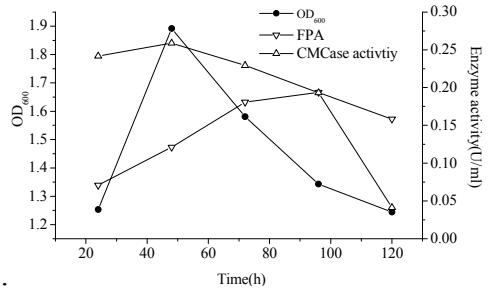

Fig. 1 Growth curve and enzyme activity of strain *Yc-A1* on CMC liquid medium

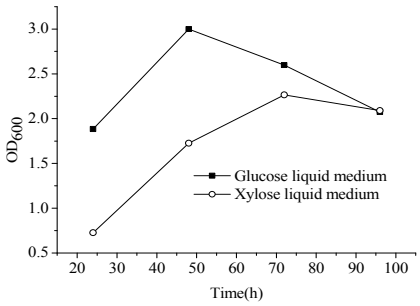

Fig. 2 Growth curves of strain *Yc-A1* on glucose and xylose liquid medium

Culture of strain *Yc-A1* on xylose and glucose medium

Strain *Yc-A1* can utilize both xylose and glucose as sole carbon source, while glucose is superior to xylose for cell growth (Fig. 2) The maximal biomass of strain *Yc-A1* was observed at 48 h (OD_{600}=3.0) on glucose liquid medium, while the maximal biomass was observed at 72 h (OD_{600}=2.3) on xylose liquid medium, which indicated strain *Yc-A1* grow faster on glucose liquid medium comparing to xylose liquid medium. Comparing the growth curve of strain *Yc-A1* on different medium (Fig.1,2), glucose and xylose carbon source is superior to CMC-Na for strain *Yc-A1* growth. Thus, the CMCase activity and FPase activity of strain *Yc-A1* could be improved by the optimization of the medium.

Ethanol endurance of strain *Yc-A1*

Comparing with the biomass showed in Fig.1 and Fig.2, high concentration ethanol (10% and 20%) will inhibit the growth of strain *Yc-A1* (Fig. 3). Meanwhile, the biomass of strain on mixed sugar medium containing 10% and 20% ethanol were significantly higher than that of *Saccharomyces cerevisiae AADY* and *Pichia stipitis 1960*, respectively by independent sample T test. It is generally considered *Pichia stipitis* can utilize xylose but with poor ethanol fermentation ability, while *Saccharomyces cerevisiae* can utilize glucose well with certain ethanol endurance ability [18, 19]. Strain *Yc-A1* can endure 10% and 20% ethanol, which was ascribed to the unique cells structure of *Bacillus sp*. Notably, the biomass of strain *Yc-A1* on 20% ethanol was higher than that of 10% ethanol during 48 to 96 h, it can be inferred ethanol can be used as carbon source for strain *Yc-A1* growth. The high ethanol endurance ability make strain *Yc-A1* possible to be applied in simultaneous saccharification and cofermentation (SSCF) craft.

Molecular identification of strain *Yc-A1*

Genome DNA of strain *Yc-A1* was extracted and 16S rRNA gene with12 Kb was amplified successfully with genbank accession number KR611620. Based on physiological analysis and 16S rRNA sequence homology (Fig. 4), Strain *Yc-A1* was identified as a member of *Bacillus cereus*.

CMCase was isolated from a marine bacterium, *Bacillus subtilis subsp. subtilis A-53* by Kim, which indicated certain *Bacillus cereus sp.* take part in the biodegradation process of cellulose in the natural world[20]. Interestingly, a large number of *Bacillus* strains have important economic relevance are used as probiotics, besides members of the genus was attributed to pathogens for agricultural application [21, 22]. Thus, whether toxins could be secrete by strain *Ac-A1* have to be determined for possible application in feed industry.

Determination of ethanol and lactic acid

Ethanol was identified according to the parent molecule equal to 45.0 *m*/*z* of $[M-H]^-$ with 7.6 min retention time by GC-MS and the ethanol concentration was 0.72 g/L. Meanwhile, lactic acid was identified according to the parent molecule equal to 89.1 *m*/*z* of $[M-H]^-$ with 3.2 min retention time by HPLC-MS analysis with the concentration 8.91 g/L. Ethanol and lactic acid production could be enhanced by culture condition optimization. So far, Most report about *Bacillus cereus* was well known as the cause of food poisoning. However, *Bacillus cereus Ac-A1*, which involved the degradation of cellulose and xylose, was present here. In view of the widespread of *Bacillus cereus sp.* in nature, *Bacillus cereus sp.* may play important role in the bioconversion of lignocellulose in certain habitat, which was not clear.

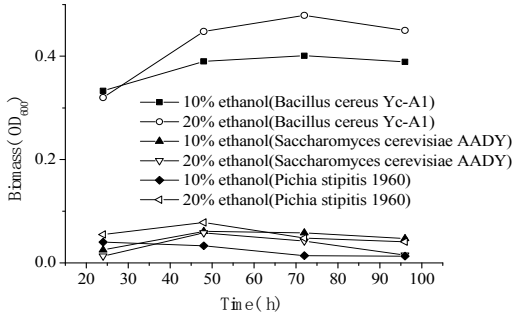

Fig. 3 Ethanol tolerance of strain *Yc-A1* on xylose liquid medium compared with *S.cerevisiae* and *P. stipitis*

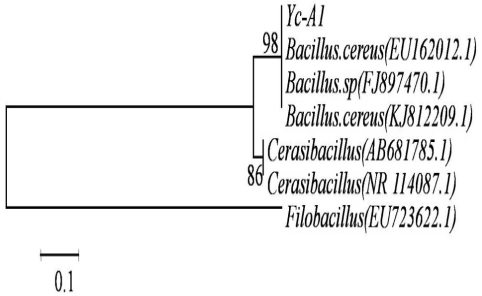

Fig. 4 Phylogenetic tree of strain *Yc-A1* based on 16S rDNA sequences

Conclusions

Strain *Yc-A1* was identified as *Bacillus cereus* by physiological and phylogenetic analysis. *Bacillus cereus Yc-A1* can utilize cellulose or xylose as sole carbon source and can endure 20% ethanol, which is superior to *Pichia stipitis 1960* and *Saccharomyces cerevisiae AADY* in the test condition. Due to the cellulose and xylose degradation ability and good lactic acid production ability, *Bacillus cereus Yc-A1* have the potential application in feed industry and paper making industry.

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References

[1] Menon V, Rao M. *Prog Energ Combust* Vol.38 (2012) , p. 522

[2] Jeffries TW, Jin YS. *Appl Microbiol Biotechnol* Vol.63 (2004) 495-509.

[3] Ladisch MR, Lin KW, Voloch M, et al. Enzyme and Microbial Technology Vol.5 (1983) , p. 82

[4] Harner NK, Wen X, Bajwa PK, et al. J Ind Microbiol Biotechnol Vol.42 (2015) , p. 1

[5] Ariffin HN, Abdullah MS, Umi Kalsom Y, et al. *Int J Eng Tech* Vol.3 (2006) , p. 47

[6] Dassa B, Borovok I, Lamed R, et al. *BMC genomics* Vol.13 (2012) , p. 1

[7] Latham MJ, Wolin MJ. *Appl Microbiol Biotechnol* Vol.34 (1977) , p. 297
[8] Maria de Vries, Anne Schöler, Julia Ertl, et al. *FEMS Microbio Ecol* Vol.91 (2015) , p. 1
[9] Patel GB, Khan AW, Agnew BJ, Colvin JR. *Int J Syst Bacteril* Vol.30 (1980) , p. 179
[10] Ray AK, Bairagi A, Ghosh KS, Sen SK. *Acta Ichthyologica ET Piscatoria* Vol.37 (2007, p. 47
[11] Eveleigh DE, Mandels M, Andreotti R, Roche C. *Biotechnol Biofuels* 2 (2009) , p. 1
[12] Buchanan RE, Gibbons NE. Bergey's manual of determinative bacteriology, 8th Ed. The Williams and Wilkins Co, Baltimore (1974).
[13] Miller GL. *Anal Chem* 31 (1959) , p. 426
[14] Zhao HY, Yu HR, Yuan XF, et al. *J. Microbiol. Biotechnol* Vol.24 (2014) , p. 585
[15] Xiao Z, Dai S, Niu Y, et al. *J Food Sci* Vol.76 (2011) , p. 1125
[16] Teather RM, Wood PJ. *Appl Environ Microb* Vol.43 (1982) , p. 777
[17] Pourramezan Z, Ghezelbash GR, Romani B, et al. *Mikrobiologiia* Vol.81 (2012) , p. 796
[18] Delgenes JP, Moletta R, Navarro J M. *Enzyme Microb Technol* Vol.19 (1996) , p. 220
[19] Nigam JN. *J Appl Microbiol* Vol.90 (2001) , p. 208
[20] Kim BK, Lee BH, Lee YJ, et al. *Enzyme Microb Technol* Vol.*44* (2009), p. 411
[21] Hong HA, Duc LH, Cutting SM. *FEMS Microbiol. Rev* Vol. 29 (2005), p. 813
[22] Kim MJ, Han JK, Park JS, et al. *J. Microbiol. Biotechnol* Vol. 25 (2015). p. 872