

Production of Cellulase by *Microbulbifer hydrolyticus* through Co-fermentation of Glucose and Xylose from Lignocellulose

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Cellulase is a compound enzyme that catalyzes cellulose into monosaccharides or oligosaccharides. Large amounts of cellulase are needed with the development of the lignocellulose processing industry, which necessitates faster methods to produce cellulase. In this work, the marine bacterium *Microbulbifer hydrolyticus* IRE-31-192 was selected to produce cellulase, due to its fast growth rate and short high space-time yield. Co-fermentation of glucose and xylose to produce cellulase was investigated on the basis of previous work. When the ratio of glucose/xylose was 2:1 (w/w), 294 U/L cellulase activity with highest space-time yield of 12.2 U/L h was obtained. The hydrolytic liquid of lignocellulose prepared from dried distiller's grains with solubles (DDGS) with the similar ratio of glucose/xylose was used as medium to produce cellulase. The efficiency of cellulase production from processed and unprocessed hydrolysates of DDGS was compared. Unprocessed hydrolysates were more beneficial for the production of cellulase, such that its activity was 261 U/L with a space-time yield of 14.5 U/L h. Thus, commonly used pure glucose and xylose could be replaced by hydrolysates of DDGS, and marine bacteria has potential application for cellulase production.

Keywords: Cellulase; Dried distiller's grains with solubles; Marine bacteria; Lignocellulose;

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INTRODUCTION

Global energy demand and fossil fuel depletion has intensified the need for alternative energy sources (Zhang *et al.* 2013). Lignocellulosic biomass is a significant renewable resource available worldwide for the production of sustainable biofuels and bio-products at competitive prices (Wen *et al.* 2013; Liu *et al.* 2015). A large market potential for cellulase production is projected due to its capability of converting lignocellulosic biomass to fermentable sugars for the production of high value chemicals, which has required faster methods to improve its productivity (Silva and Filho 2017).

Cellulases can be produced from both fungi and bacteria. Fungi show a higher enzyme performance but require a long growth cycle (Bansal *et al.* 2014; Payne *et al.* 2015). Bacterial production of cellulase has drawn much attention due to its fast growth rate and high space-time yield as well as the potential for scalable production (Dias *et al.* 2015; Paudel and Qin 2015). *Microbulbifer hydrolyticus* IRE-31 (ATCC 700072) is a marine bacterium isolated from marine pulp mill effluents that can grow in high salinity medium. It was identified in a previous study (Arens and Liu 2013). It has fast and strong

growth ability. Carbon sources are an energy source for the bacterial strain and essential inducers for cellulase production (González *et al.* 1997). In a previous study, the hydrolysis rate of carboxymethyl-cellulase (*i.e.* CMCase assay) was used to represent the activity of cellulase, and the effect of various carbon sources on cellulase production by *Microbulbifer hydrolyticus IRE-31* was explored (Liu *et al.* 2019). CMCase activities of 473 U/L and 266 U/L were obtained from glucose and xylose, respectively. However, commonly used glucose was mostly obtained from starch sources, which is expensive for industrial utilization, and it directly competes with human food. Hence, an accessible and cheaper carbon source needs to be explored.

Hydrolytic liquid from lignocellulose that contains both glucose and xylose is a substitute for common carbon resources to produce biochemicals (Guerriero *et al.* 2016), and thus was selected as an available carbon source to investigate its capability in cellulase production. The hydrolytic liquid prepared from dried distiller's grains with solubles (DDGS) as the lignocellulosic waste in wine production is used as a protein feedstuff in livestock, but the high fiber content in DDGS limits their utilization as livestock feeding because of possible health problems (Avelar *et al.* 2010). According to Liu *et al.* (2017b), DDGS contains glucose and xylose, but it has a high level of crude protein from yeast residue. The hydrolytic liquid of DDGS could be used to produce high value-added chemicals including ethanol, fumaric acid, *etc.* Therefore, it could be used to produce cellulase as well.

In this work, the co-fermentation process of glucose and xylose to produce cellulase was studied. The effect of hydrolytic liquid from DDGS on the production of cellulase was investigated.

EXPERIMENTAL

Preparation of the Hydrolytic Liquid

The DDGS was obtained from Shan Xi Xing Hua Cun wine factory and was used to prepare the hydrolytic liquid through acid hydrolysis, where the yields of monosaccharides reached 258 mg/g DDGS, including 15.88 g/L glucose, 7.53 g/L xylose, 2.35 g/L arabinose, and 0.116 g/L furfural. As *Microbulbifer hydrolyticus IRE-31-192* is a marine bacterium isolated from marine pulp mill effluents, it could tolerate inhibitors and high-concentrations of salt. Therefore, various DDGS hydrolytic liquids were prepared for cellulase production.

The hydrolysate from DDGS was first neutralized with Ca(OH)₂ to pH 7 and then adsorbed with active carbon; this product was defined as processed-hydrolytic liquid after being filtrated (Schuster and Chinn 2013; Liu *et al.* 2017b). The product neutralized with KOH to pH 7 without any other pretreated process was defined as unprocessed-hydrolytic liquid (Liu *et al.* 2018).

Microorganism and Culture Medium

Microbulbifer hydrolyticus IRE-31-192 from the authors' laboratory was used in this study. The strain was maintained on 2216E agar slant culture medium kept away from light by periodic transfers following incubation at 37 °C for 12 h to 18 h (Yiheng Instrument Co. Ltd, Shanghai, China) and storing at 4 °C. The medium 2216E was purchased from Qingdao Nissui Biotechnology Co. Ltd. (Qingdao, China). It is a special culture medium for marine bacteria, containing (per liter): peptone (5 g), yeast extract (1

g), ferric citrate (0.1 g), sodium chloride (19.45 g), magnesium chloride (5.9 g), magnesium sulfate (3.24 g), calcium chloride (1.8 g), potassium chloride (0.55 g), sodium bicarbonate (0.16 g), potassium bromide (0.08 g), strontium chloride (0.034 g), boric acid (0.022 g), sodium silicate (0.004 g), sodium fluoride (0.0024 g), ammonium nitrate (0.0016 g), and disodium hydrogen phosphate (0.008 g). The composition of agar medium was (per liter): 37.4 g 2216E, and 20 g agar, which was also used as seed medium without agar. The composition of the common fermentation medium was (per liter): 37.4 g 2216E, glucose, and xylose. When the hydrolytic liquid of DDGS was used as fermentation medium, the other nutrient was avoided. All media were sterilized by autoclaving at 116 °C for 25 min.

Fermentation Process

A single colony of strain *Microbulbifer hydrolyticus* IRE-31-192 grown on agar slant culture medium was selected and cultured using seed culture liquid. It was then kept away from light for 12 h at 37 °C and 180 rpm. A 5% (v/v) seed culture liquid was added into a 250 mL flask with 50 mL fermentation medium, which was then incubated for 3 h in a rotary shaker (Taicang instrument Co. Ltd, Jiangsu, China) at 37 °C and 180 rpm.

The use of the strain *M. hydrolyticus* IRE-31-192 in co-fermentation of glucose and xylose was subsequently investigated. The total sugar content was 30 g/L and the ratio of glucose was gradually decreased with the addition of xylose. The proportions of glucose and xylose (glucose/xylose (w/w): 5:1, 2:1, and 1:1) were investigated with the aim of optimization and highest production of CMCCase. As the optimum proportion of glucose/xylose was studied in the co-fermentation process, the capability of *M. hydrolyticus* IRE-31-192 to produce CMCCase from processed and unprocessed hydrolysates of DDGS was finally investigated. The fermentation broth was sampled at different times from the shake flask and centrifuged at 4 °C and 9,000 × g for 15 min (Yancheng instrument Co. Ltd, Jiangsu, China). The supernatant was used to measure the CMCCase activity.

Analysis Methods

The biomass was analyzed using UV spectrophotometry (Meipuda instrument Co. Ltd, Shanghai, China) at 600 nm with results ranging from 0.1 A to 0.8 A. CMCCase activity was defined as the amount of cellulase enzyme that liberates 1.0 μmol of reducing sugar (glucose) per minute under the assay conditions of pH 7.0 at 50 °C (Singh *et al.* 2015). The CMCCase activity in fermentation broth was thus expressed with enzyme activity per liter of fermentation broth (U/L).

The reducing sugar content was determined by the 3,5-dinitrosalicylic acid solution method (DNS reagent) for color development and the absorbance was measured at 540 nm (Hu *et al.* 2008). The consumption of glucose and xylose in fermentation process were quantified by high-performance liquid chromatography (HPLC) with a Bio-Rad Aminex HPX-87 H ion exclusion column (Beijing, China) with a refractive index detector and UV detector at 210 nm.

The column was eluted with 0.005 M H₂SO₄ at a column temperature of 50 °C and a flow rate of 0.6 mL/min (Liu *et al.* 2017a). The p-values of the data shown in this work were p < 0.05 after significance testing.

RESULTS AND DISCUSSION

CMCase Production from Co-fermentation of Glucose and Xylose

Liu *et al.* (2019) showed that high CMCase activities were obtained from a mixture of 30 g/L glucose and 10 g/L xylose. Based on this result, the CMCase production from co-fermentation process of glucose and xylose was investigated using 30 g/L mixture as carbon source. As shown in Fig. 1, when the content of glucose was decreased, the CMCase activity was reduced.

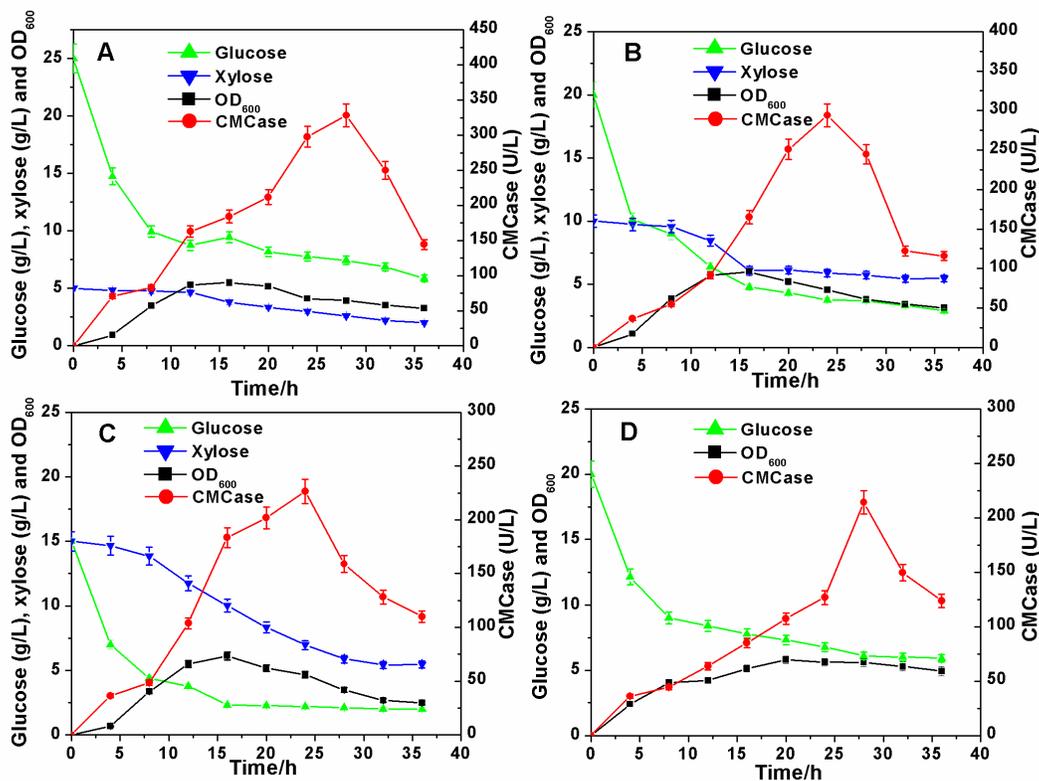


Fig. 1. The effect of different glucose/xylose ratio on the cellulase production process. (A: glucose/xylose 5:1 (w/w); B: glucose/xylose 2:1 (w/w); C: glucose/xylose 1:1 (w/w); and D: 20 g/L glucose)

When 25 g/L glucose and 5 g/L xylose were co-fermented (Fig. 1A), the highest CMCase activity reached up to 328 U/L, while 294 and 226 U/L CMCase activities were achieved after the ratio of glucose/xylose were decreased to 2:1 (Fig. 1B) and 1:1 (Fig. 1C), respectively. These findings indicated that glucose was beneficial for the production of CMCase. However, even though the CMCase activity decreased with the addition of xylose, the increasing OD₆₀₀ value demonstrated that xylose was helpful for cell growth. An appropriate cell growth could enhance the cellulase accumulation rate. Therefore, in comparison of the volumetric productivity, the highest value of 12.25 U/L h was obtained in co-fermentation of 20 g/L glucose and 10 g/L xylose, which was similar to the results obtained in previous work using glucose (16.85 U/L h) or xylose (16.6 U/L h) as solo carbon source, respectively (Liu *et al.* 2019). As shown in Fig. 1D, when 20 g/L glucose was used as solo carbon source, the OD₆₀₀ increased more slowly than it did in a co-fermentation process, yielding to 214 U/L CMCase activities. This showed that the addition

of xylose could improve the fermentation process, owing to that an optimum content of xylose benefited to reach a suitable cell growth, leading to an enhancement of CMCase production. Therefore, 20 g/L glucose and 10 g/L xylose was the optimum addition level in co-fermentation process.

CMCase Production from Hydrolytic Liquid of Lignocellulose

The original hydrolytic liquid prepared from DDGS contained 15.9 g/L glucose, 7.53 g/L xylose, and 7.16 g/L crude protein (Liu *et al.* 2017b), which was pretreated using the above mentioned method to obtain both processed and unprocessed hydrolytic liquids, respectively. The content of processed hydrolytic liquid changed to 12.6 g/L glucose, 6.25 g/L xylose, and 5.39 g/L crude protein. The ratio of glucose/xylose was almost 2:1 in both kind of hydrolytic liquids similar to that in the optimum co-fermentation process, and they were used as medium to investigate the effect on the production of CMCase, as shown in Fig. 2. When the unprocessed hydrolytic liquid was used as medium (Fig. 2A), the highest CMCase activity reached 261 U/L at 18 h with the volumetric productivity of 14.5 U/L per hour, which was higher than that got from processed hydrolytic liquid (11.31 U/L h). Meanwhile, OD₆₀₀ of the strain in both hydrolytic liquids also showed the same trend that it was higher in unprocessed hydrolytic liquid. It was presumed that the content of sugar in unprocessed hydrolytic liquid was higher than it was in the processed liquid, and that the unprocessed hydrolytic liquid with high-concentration of salt ions was more suitable for the strain to grow as well as to produce CMCase. In addition, the CMCase activities and its volumetric productivity obtained from unprocessed DDGS hydrolytic was similar with the value of the co-fermentation process, which meant that the hydrolytic liquid of lignocellulose could significantly replace the common carbon sources for producing cellulase.

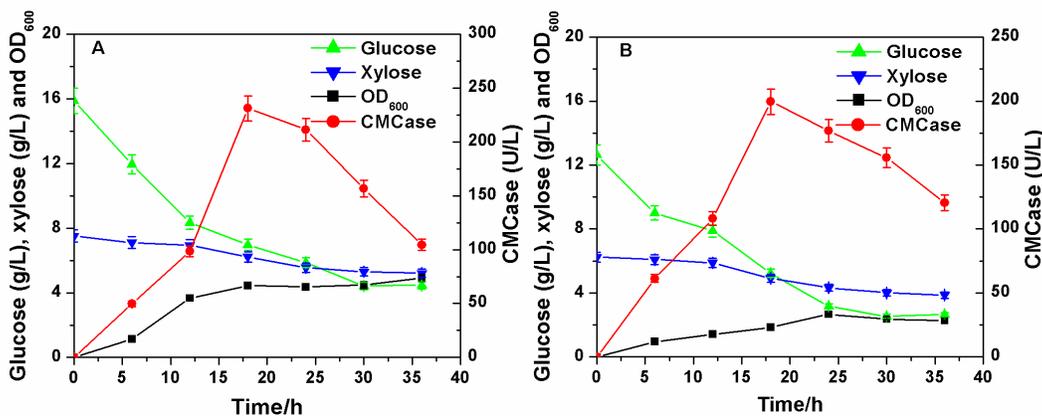


Fig. 2. The effect of unprocessed and processed hydrolytic liquid on the production of cellulase (A: unprocessed hydrolytic liquid; and B: processed hydrolytic liquid)

CONCLUSIONS

1. Marine bacteria *Microbulbifer hydrolyticus* IRE-31-192 was selected to investigate the production of cellulase using co-fermentation of a mixture of glucose and xylose. A cellulase activity of 293 U/L was obtained from the mixture with a glucose/xylose ratio

- of 2:1 (w/w).
2. The hydrolytic liquid of lignocellulose, prepared from dried distiller's grains with solubles (DDGS) with a similar glucose/xylose ratio, was used as medium to produce cellulase using *M. hydrolyticus IRE-31-192*. The highest CMCase activity reached 261.3 U/L at 18 h with the volumetric productivity of 14.52 U/L h, when the unprocessed hydrolytic liquid was used as medium.
 3. Unprocessed hydrolytic liquid with high content of monosaccharide and salt ions was more beneficial for the cellulase production process, which meant that the hydrolytic liquid of lignocellulose had a major potential utilization to replace common carbon sources in the cellulase production process.

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