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Characterization of inulin hydrolyzing enzyme(s) in commercial glucoamylases and its application in lactic acid production from Jerusalem artichoke tubers (Jat)

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HIGHLIGHTS

- Glucoamylase was identified as an inulin hydrolyzing enzyme.
- Commercial glucoamylase was used as inulinase for Jat hydrolysis.
- High lactic acid titer and yield were obtained in the SSF of Jat.

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ABSTRACT

A high inulinase activity was found in three commercially available glucoamylase enzymes. Its origin was investigated and two proteins in the commercial glucoamylases were identified as the potential enzymes showing inulinase activity. One of the commercial glucoamylases, GA-L New from Genencor, was used for Jerusalem artichoke tubers (Jat) hydrolysis and a high hydrolysis yield of fructose was obtained. The simultaneous saccharification and lactic acid fermentation (SSF) of Jat was carried out using GA-L New as the inulinase and *Pediococcus acidilactici* DQ2 as the fermenting strain. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 h. The enzyme cost using the commercial glucoamylase as inulinase was compared to that using the typical inulinase and a large profit margin was identified. The results provided a practical way of Jat application for lactic acid production using cheap commercial glucoamylase enzyme.

1. Introduction

Although current industrial production of lactic acid overwhelmingly uses starch feedstock, future need of lactic acid for biodegradable polylactide acid (PLA) certainly requires various feedstock for replacing petroleum based polymer materials, other than food-based starch only (Nampoothiri et al., 2010; Gao et al., 2011). Among many feedstock options, Jerusalem artichoke tubers (Jat) is a promising one because of its desirable growing traits such as cold and drought tolerance, wind and sand resistance, saline tolerance, strong fecundity, and high pest and disease resistant (Li and Chan-Halbrendt, 2009). Jat contains high carbohydrates (20%, w/w), in which 70–90% (w/w) are inulin, a polysaccharide composed of fructose unit chains and minor glucose unit (Szambelan et al., 2005). Jat had been widely planted in North America, Europe, and Asia countries besides its advantages (Chi et al., 2011; Li et al., 2013). In recent few years in China, Jerusalem artichoke started the large scale cultivation in the drought and coast regions as well as in the polluted regions by oil drilling or coal mining to replace the corn and wheat as feedstock of fermentation industry for production of ethanol, butanol, sugar alcohols and organic acids (Li et al., 2013). Although its total quantity is not comparable to lignocellulose biomass, Jat could be processed easily using the available technologies, or even easier than starch processing (Chi et al., 2011; Li et al., 2013), while lignocellulose processing is still a technology challenge and only used in the small scale demonstration stage (John et al., 2009; Zhao et al., 2013). Therefore, Jat has been considered as a readily available feedstock at present and a promising one in the future for industrial lactic acid production.

Jat can be hydrolyzed into fructose and glucose by inulinase enzyme or acid catalyst (Chi et al., 2011). Inulinase includes two enzymes, endo-inulinase (EC 3.2.1.7) cleaving middle sites of inulin oligos into even smaller oligos, and exo-inulinase (EC 3.2.1.80) cleaving fructose or glucose from the ends of inulin oligos. The recent studies showed that invertase (EC 3.2.1.26) from yeast and fungi also demonstrated strong inulin hydrolysis property (Wang

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and Li, 2013; Guo et al., 2013). However, these inulinase enzymes are expensive and not available as industrial enzyme for Jat hydrolysis at large scale (Sigma Product ID 16285, Novozym 960, $350 for 250 mL). Therefore, the cost reduction of inulinase is a key step for industrial application of Jat for production of biofuels and biochemicals.

In this study, a high inulinase activity was found from a typical commercial glucoamylase GA-L New of Genencor produced by *Aspergillus niger* fermentation. The inulin hydrolyzing enzyme(s) in glucoamylase GA-L New were characterized and the results showed that the activity was from glucoamylase itself, other than other protein components in this mixed commercial enzyme, although the catalytic properties of the two enzymes were different (glucoamylase cleaves 1,4-α-D-glucosidic linkage, while inulinase cleaves 1,2-β-D-fructosidic linkage). Then the glucoamylase was used for Jat hydrolysis and a high yield of fructose from Jat was obtained. The simultaneous saccharification and lactic acid fermentation (SSF) of Jat was carried out using glucoamylase GA-L New as the inulinase and *Pediococcus acidilactici* DQ2 as the lactic acid fermenting strain. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 h. The results provided a practical way of Jat application for lactic acid production using cheap commercial glucoamylase enzyme.

2. Methods

2.1. Enzyme and strain

The commercial glucoamylase enzymes include glucoamylase GA-L New from DuPont Genencor Science, Wuxi, China, (http://biosciences.dupont.com/duponttm-genencorr-science/), amyloglucosidase A107823 from Aladdin Industrial Co., Ltd., Shanghai, China (http://www.aladdin-e.com), and a local product, glucoamylase YY0515 from Shanghai Yuanye Biological Technology Co., Ltd., Shanghai, China (www.shyuanye.com). All these glucoamylases were produced using *A. niger* as the production strain.

The lactic acid fermentation strain, *P. acidilactici* DQ2 (CGMCC 7471), was isolated in our previous study (Zhao et al., 2013) and stored in China General Microbial Collection Center (CGMCC), Beijing, China. The simplified MRS medium contained 10 g/L of peptone, 5 g/L of yeast extract, 1 g/L of $(NH_4)_2SO_4$, 2 g/L of KH_2PO_4, 0.58 g/L of $MgSO_4{\cdot}7H_2O$, 0.25 g/L of $MnSO_4{\cdot}4H_2O$, 5 g/L of sodium acetate, and 2 g/L of diammonium hydrogen citrate. The medium was autoclaved at 115 °C for 20 min. One vial of *P. acidilactici* DQ2 was inoculated into simplified MRS broth and cultured at 42 °C, 150 rpm for 12 h as inoculum seeds with the inoculum was 10%. pH was maintained by adding 60 g of $CaCO_3$ per 100 g of glucose. All experiments were repeated twice.

2.2. Raw materials chemicals

Jerusalem artichoke tubers (Jat) were purchased from Xinnong Technology Co., Ltd. (Qinghai, China). The Jat was washed, sliced, and dried at 60 °C until constant weight, then ground to fine powder. The composition of the dried Jat was determined using the same method by Kaldy et al. (1980). One gram of the dried Jat composed of 0.75 g inulin (glucose and fructose), 0.03 g cellulose, 0.02 g hemicellulose, 0.02 g protein, 0.01 g fat, and 0.05 g water.

The inulin powder was purchased from Langrui Fine Chemical Co., Ltd. (Shanghai, China). One gram of inulin powder was composed of 0.78 g of fructose and 0.23 g of glucose obtained from 1 g of inulin (dry base) according to the method by Gao et al. (2010).

All chemicals including peptone, yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}4H_2O$, sodium acetate, diammonium hydrogen citrate, were purchased from Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Albumin, Fraction V (CAS Number: PB10056, 69 kDa) was purchased from Beijing Probe bioscience Co., Ltd. (Shanghai, China). Coomassie Brilliant Blue G-250 (CAS Number: 0006192525), Coomassie Brilliant Blue R-250 (CAS Number: 0006104592) was obtained from Sigma–Aldrich.

2.3. Inulinase assay

The inulinase activity in the glucoamylase enzymes was measured using the modified method of Jing et al. (2003). 100 μL of the crude enzyme solution was mixing with 900 μL of 2% inulin powder in a 0.1 M sodium acetate buffer (pH 4.5). The solution was incubated at 60 °C for 10 min, then mixed with 3 mL of 3,5-dinitrosalicylic acid (DNS) solution. The mixture was placed in an ice bath, heated for 5 min at 100 °C to deactivate the enzyme, and the reducing sugars were assayed by observing the change of absorbance at 540 nm. One unit (U) of inulinase activity was defined as the amount of enzymes that produced 1 μmol of reducing sugars per minute from inulin under the assay conditions.

The protein concentration of the three commercially available glucoamylases was measured using Bradford method.

2.4. Purification of glucoamylase proteins

The crude glucoamylase GA-L New solution was filtered through a 0.22 μm membrane and purified using a chromatography column packed with Sephadex G-100 (Life Sciences, Uppsala, Sweden). The column was pre-equilibrated and eluted with citrate buffer (0.1 M, pH 5.0) at a flow rate of 1.0 mL/min and the eluent protein monitored at 280 nm. The SDS–PAGE electrophoresis of the fractions were performed with 12% polyacrylamide denaturing SDS gel and stained with Coomassie Brilliant Blue R-250. The inulinase activity of the fractions was measured using the method above in Section 2.5.

2.5. SSF bioreactor and its operation

SSF operation was carried out in 5-L bioreactor with pH and temperature control. A specially designed helical ribbon impeller was installed for well mixing at high Jat solids loading as described by Zhang et al. (2010) and Zhao et al. (2013). The SSF operation continued to 72 h and samples were withdrawn at regular intervals.

2.6. Analytical methods

Glucose, fructose and lactic acid was analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-Rad Aminex HPX-87H column at 65 °C. The mobile phase setup 5 mM H_2SO_4 with the rate of 0.6 mL/min. All samples were diluted and filtered before analysis.

3. Results and discussion

3.1. Characterization of inulin hydrolyzing enzyme(s) in commercially available glucoamylases

To find a cheap inulin hydrolyzing enzyme for replacement of expensive inulinase, various available industrial hydrolase enzymes were screened, and a high inulinase activity was found from a typical glucoamylase GA-L New, a product of Genencor widely used in starch processing industry (www.genenco.cn). The inulinase activity data of glucoamylase GA-L New were using inulin powder as substrate in Table 1, indicates that the inulinase activity of

Table 1
Effect of pH and temperature on inulinase activity and stability of glucoamylase GA-L New.

	Relative activity (%)	Relative stability (%)
pH		
3.6	88.7 ± 0.18	74.2 ± 0.32
4.0	100.0 ± 0.00	100.0 ± 0.00
4.6	91.6 ± 3.28	95.1 ± 7.49
5.0	87.2 ± 4.10	84.5 ± 4.56
5.6	76.1 ± 2.64	27.9 ± 3.75
Temperature (°C)		
30	38.13 ± 2.22	95.48 ± 4.74
40	49.34 ± 0.72	98.20 ± 6.9
45	62.69 ± 2.49	100.91 ± 5.87
50	84.85 ± 3.67	94.79 ± 2.11
55	89.07 ± 4.00	91.83 ± 1.59
60	100.00 ± 0.00	96.39 ± 1.86
65	95.59 ± 3.07	52.76 ± 5.44
70	91.47 ± 1.62	17.18 ± 2.66
80	40.90 ± 3.01	5.36 ± 0.0

pH stability was tested by pre-incubating 120 min at 4 °C and reaction mixture containing 0.9 mL of 1% inulin and 0.1 mL enzyme solution in sodium acetate buffer at 60 °C for 10 min. The thermo stability was tested by pre-incubating the enzyme at different temperatures (30–80 °C) for 2 h. The inulinase activity of the final concentrated elute with setting time and pH or temperature was regarded as 100%.

Table 2
Inulinase activity of three commercially available glucoamylase enzymes.

Glucoamylase type	Inulinase activity per mL (U/mL)	Protein concentration (mg/mL)	Specific inulinase activity (U/mg protein)
GA-L New (Genencor)	18.50	40.75	0.45
A107823 (Aladdin)	19.58	46.12	0.42
YY10515 (Shanghai Yuanye)	5.86	40.03	0.15

100 μL of the crude enzyme solution was mixing with 900 μL of 2% inulin powder in a 0.1 M sodium acetate buffer (pH 4.5). The solution was incubated at 60 °C for 10 min.

glucoamylase GA-L New maintained high at pH from 3.6 to 5.0, but decreased gradually with increasing pH; the stability was also relatively stable at pH from 3.6 to 5.0, then decreased when pH increased to 5.6. Inulinase activity of glucoamylase GA-L New increased with increasing temperature from 30 to 60 °C, then decreased at 70 °C and sharply decreased at 80 °C; the inulinase stability showed an optimal range from 30 to 60 °C, then decreased quickly above 60 °C was shown in Table 1.

The inulinase activity of three commercially available glucoamylase enzymes was tested to further confirm the inulinase activity in glucoamylase. The three commercially available glucoamylase enzymes include glucoamylase GA-L New from DuPont Genencor Science, Wuxi, China, (http://biosciences.dupont.com/duponttm-genencorr-science/); amyloglucosidase A107823 from Aladdin Industrial Co., Shanghai, China (http://www.aladdin-e.com); and a local product, glucoamylase YY0515 from Shanghai Yuanye Biological Technology Co., Shanghai, China (www.shyuanye.com). The inulinase activity per milliliter enzyme solution and the specific activity per milligram of total protein were measured and the results were shown in a newly added Table 2.

Table 2 indicates that all the three commercially available glucoamylase enzymes demonstrated high inulinase activity, with the close activity of the first two enzymes GA-L from Genencor and A107823 from Aladdin, and the relatively low activity of YY10515 from the local Shanghai Yuanye. The finding suggests that this phenomenon of inulinase activity in commercial glucoamylase enzymes might be a universal property of general glucoamylase enzymes, instead of a unique property of a specific glucoamylase such as GA-L New of Genencor product.

The glucoamylase GA-L New is the crude enzyme product extracted from *A. niger* fermentation broth without further purification. Multiple enzyme components were secreted into the crude enzyme product, besides the major glucoamylase component. These enzyme components may include inulinase enzyme(s) such as endoinulinase (EC 3.2.1.7), exoinulinase (EC 3.2.1.80), and invertase (EC 3.2.1.26) based on the information in the Brenda Enzyme Database (http://www.brenda-enzymes.org/). One evidence is that the inulinase properties were similar to that of the typical inulinase from *A. niger*, with the pH and temperature optima at pH 4.0–7.0 and at 35–60 °C, respectively (Kango, 2008; Derycke and Vandamme, 1984; Gaye et al., 1994). Still, glucoamylase itself might behave a substrate promiscuity of inulin hydrolysis and then degrade the Jat into fructose and glucose.

Identifying source of inulinase activity in Fig. 1, glucoamylase GA-L New, its crude enzyme solution was purified chromatographically by Sephadex G-100 gel and fractions were sent to test its proteins and the inulinase activity, respectively. Fig. S1, there were two protein peaks at 50 and 150 min of the chromatography purification, respectively; in addition to two inulinase activity peaks at 50 and 80 min, both were included in the first protein peak, while almost no inulinase activity was found in the second protein peak. Fig. 1(a), the SDS–PAGE of the fraction at 50 min of the chromatography purification, and two protein bands appeared and both were close to the molecular weight of 66.2 kDa. Fig. 1(b) shows the SDS–PAGE of the crude glucoamylase GA-L New and the result indicates that the major protein component of the crude enzyme also had the molecular weight close to 66.2 kDa, which should be the major enzyme of glucoamylase (EC 3.2.1.7).

To identify the protein close to 66.2 kDa in Fig. 1, the molecular weight data of the related proteins from *A. niger*, including glucoamylase (EC 3.2.1.3), endoinulinase (EC 3.2.1.7), exoinulinase (EC 3.2.1.80), and invertase (EC 3.2.1.26), were mined from the Brenda Enzyme Database (http://www.brenda-enzymes.org/) and the data were placed in order in Fig. 2. Fig. 2 shows that among the three typical inulinase enzymes and glucoamylase from *A. niger*, the molecular weights of both exoinulinase (EC 3.2.1.80) and invertase (EC 3.2.1.26) were far from the sample value of 66.2 kDa, thus could be deleted from the possible inulin hydrolyzing enzymes. Only endoinulinase (EC 3.2.1.7) and glucoamylase (EC 3.2.1.3) had the molecular weight close to 66.2 kDa. Therefore, the high inulinase activity in GA-L New should come from either the major glucoamylase fraction, or the minor endoinulinase fraction, or the both. Glucoamylase generally cleaves 1,4-α-D-glucosidic linkage of starch, and the mechanism of cleaving 1,2-β-D-fructosidic linkage is under investigation.

3.2. Saccharification of Jat by glucoamylase GA-L New and subsequent lactic acid fermentation

The findings suggest that glucoamylase GA-L New, a cheap, commercially available, and widely used industrial enzyme, could be used for practical Jat hydrolysis. In Fig. 3 shows the simultaneous saccharification and lactic acid fermentation (SSF) of Jat using glucoamylase GA-L New at the dosage from 1.0 to 2.5 U/g dry Jat (DM). Fructose accumulated slightly in the first 8 h of SSF, and then consumed completely in the next 16 h at all the enzyme dosage, while lactic acid constantly and rapidly increased in the first 16 h. In the next 56 h, fructose maintained at a low level with slight decrease, while lactic acid steadily increased to its maximum. The results indicate that fructose and glucose were released from Jat continuously by glucoamylase GA-L New, then the released fructose and glucose were converted into lactic acid by *P. acidilactici* DQ2 simultaneously. The results suggest that the

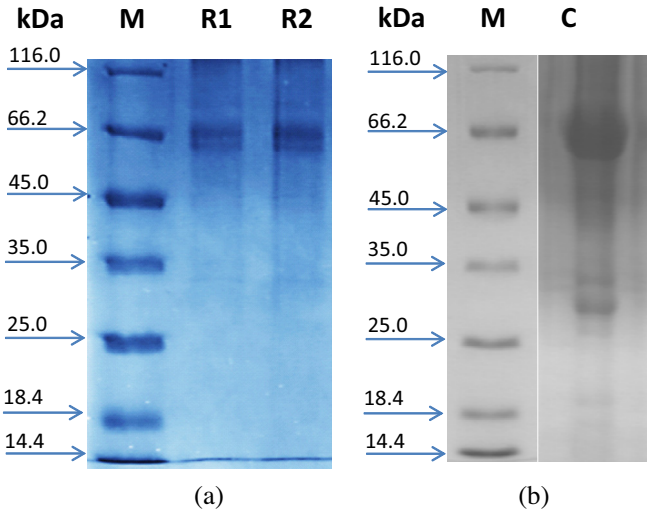


Fig. 1. Purification of glucoamylase GA-L New. (a) SDS–PAGE of the chromatograph Sephadex G-100 fractions at 50 min; Lane M: protein ladders; Lanes R1 and R2: samples; (b) SDS–PAGE of crude solution of glucoamylase GA-L New; Lane M: protein ladders; Lanes C: original crude enzyme samples.

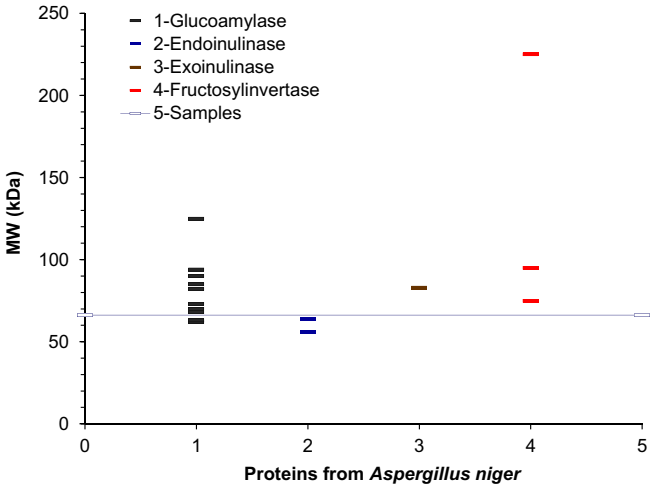
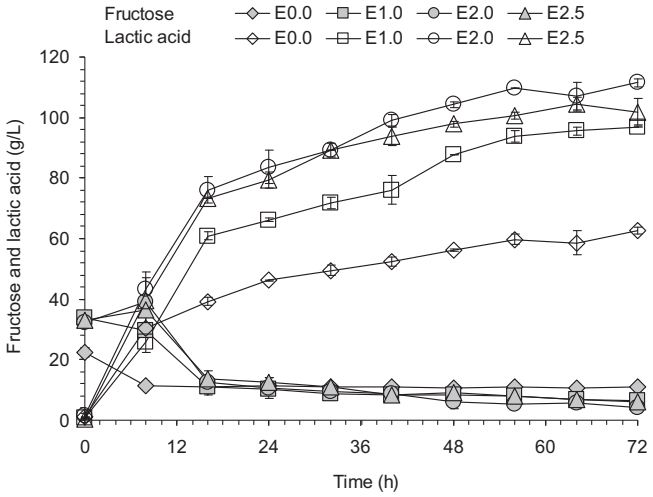


Fig. 2. Molecular weight analysis of proteins from *Aspergillus niger*.

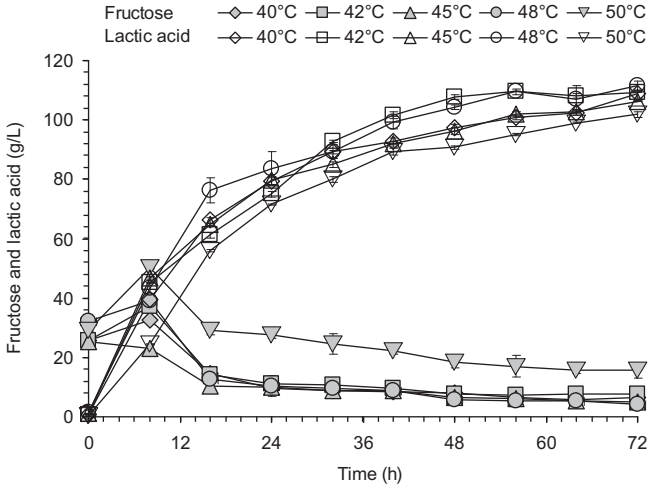
optimal glucoamylase dosage was 2.0 U/g DM, and the further increase of enzyme dosage led to the decrease of lactic acid fermentation performance. Interestingly, 62 g/L of lactic acid was produced from 30 g/L of fructose and glucose when no glucoamylase was added to the SSF system. The results may suggest that the lactic acid bacteria (LAB) used in this study, *P. acidilactici* DQ2, exhibited some natural inulinase activity.

Generally, lactic acid bacteria (LAB) strains including are mesophiles at elevated temperature (Rojan et al., 2009; Zhao et al., 2013). Glucoamylase GA-L New is also thermo-tolerant enzyme with high activity and constant stability up to 60 °C as shown in Table 1. Fig. 4 shows the SSF of Jat at different temperature profiles from 40 to 50 °C. The results indicate that the lactic acid production performance maintained high in the range of 40–50 °C, suggesting that both *P. acidilactici* DQ2 and glucoamylase GA-L New were strong thermo-tolerant. Similar results were obtained at the temperature range of the experiments, suggesting that the effect of temperature on the lactic acid fermentation was not significant. When SSF temperature increased to 50 °C, fructose began to accumulate and 15.6 g/L of fructose left at the end of the 72 h SSF. Among the temperature ranges tested, the SSF performance at 48



Glucoamylase GA-L New (U/g DM)	0.0	1.0	1.5	2.0	2.5
Lactic acid yield (g/g DM)	0.26	0.40	0.42	0.46	0.42
Lactic acid productivity (g/L/h)	0.87	1.34	1.41	1.55	1.40

Fig. 3. SSF of Jat at different glucoamylase GA-L New dosage. Conditions: 48 °C, pH 5.5, 25% (w/v) solids loading of Jat.

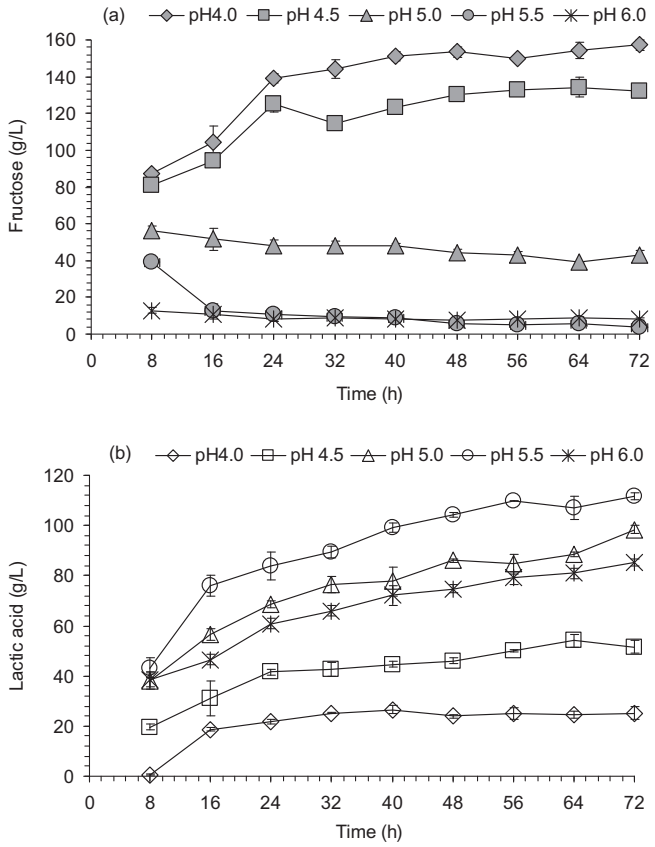


Temp (°C)	40	42	45	48	50
Lactic acid yield (g/g DM)	0.45	0.46	0.44	0.46	0.44
Lactic acid productivity (g/L/h)	1.51	1.52	1.48	1.55	1.46

Fig. 4. SSF of Jat at different temperature. Conditions: 25% (w/v) solid loading of Jat, glucoamylase GA-L new 2 U/g DM, pH 5.5.

and 42 °C was a slight advantage over others. The lower temperature of 42 °C preferred the cell growth and metabolism of *P. acidilactici* DQ2, while 48 °C preferred the Jat hydrolysis to give more fructose and glucose sugars. The higher temperature was selected because of the contamination control and energy saving.

Fig. 5 shows the SSF performance of Jat at different pH values and the results indicates that the effect of pH on the SSF performance of Jat was complicated. The fermenting strain *P. acidilactici* DQ2 showed poor performance at low pH (below 5.0) (Zhao et al., 2013), while the saccharification enzyme GA-L New showed a poor activity and stability at high pH (above 6.0) (Table 1). Clearly, there



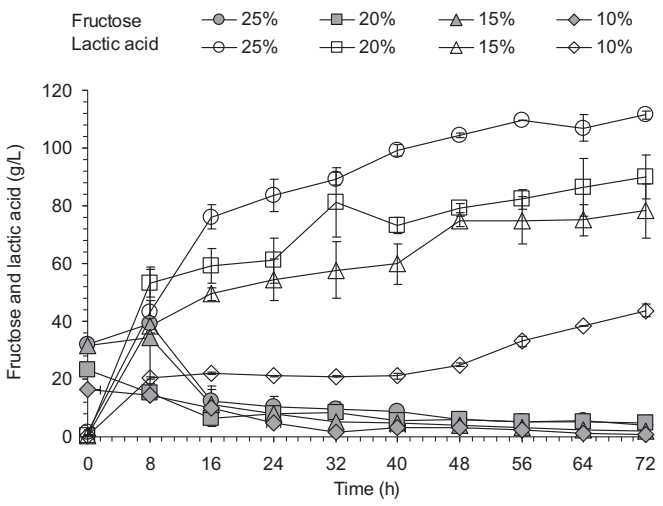
pH values	4.0	4.5	5.0	5.5	6.0
Lactic acid yield (g/g DM)	0.10	0.21	0.43	0.46	0.39
Lactic acid productivity (g/L/h)	0.33	0.72	1.37	1.55	1.18

Fig. 5. SSF of Jat at different pH values. Conditions: glucoamylase GA-L New 2.0 U/g DM, 25% (w/v) solid loading, 48 °C.

should be a trade-off between the efficiency of saccharification and lactic acid fermentation. In Fig. 5, a significant fructose accumulation occurred and led to a low lactic acid production when pH was less than or equal to 5.0, mainly due to the bad fermentability of *P. acidilactici* DQ2 at low pH levels. When pH increased to 6.0, no fructose accumulation was found but lactic acid formation was very limited, mainly due to the low activity and stability of glucoamylase GA-L New. Only when pH value maintained at 5.5, the satisfying performance of both Jat saccharification and lactic acid formation w obtained.

3.3. SSF at high solids loading of Jat for production of high titer lactic acid

A high lactic acid product concentration achievement, the solids loading of Jat feedstock should be increased to its maximum. In this study, a specially designed bioreactor with a helical ribbon stirrer for well mixing the high Jat solids loading was used and the possible high Jat content from 10% to 25% (w/v) in the bioreactor were investigated at the optimal enzyme dosage, pH, temperature conditions. In addition to reduce cost of lactic acid production only 5 g/L of yeast extract was used. Fig. 6 shows that both lactic acid titer and productivity increased with increasing Jat solids content from 10% to 25%, and up to 111.5 g/L and 1.55 g/L/h after 72 h SSF operation at 25% Jat solids content. However, almost 30% of lactic acid yield was found when the lactic acid yield decreased with



Solid conc. (%, w/v)	25	20	15	10
Lactic acid yield (g/g DM)	0.46	0.49	0.57	0.69
Lactic acid productivity (g/L/h)	1.55	1.25	1.09	0.78

Fig. 6. SSF of Jat at different solid loading. Conditions: glucoamylase GA-L New 2.0 U/g DM, pH 5.5, 48 °C.

increasing Jat solids loading from 0.69 g/g DM at 10% solids (w/v) to 0.46 g/g DM at 25% solids (w/v). Balancing the yield loss and the increase of titer and productivity, 25% (w/v) might be a proper solids content in the practical SSF of Jat.

Glucoamylase is one of the most widely used and the cheapest industrial enzymes in starch processing industry (Nghiem et al., 2011). The price of glucoamylase GA-L New was approximately $3.0 per kg (Inquiry from Genencor China, Wuxi, China, www.genencor.cn). Based on the inulinase activity data of GA-L New, which was 20 U/mL, the cost for one unit (U) of inulinase was $3.0/1000/20 = $0.00015, when glucoamylase GA-L New was used as the inulinase enzyme. On the other hand, the only available commercial inulinase enzyme was on the Sigma catalog (Product ID 16285, equivalent name: Novozym 960) with the inulinase activity of 285 U/mL (www.sigma.com). Its cheapest price with the largest package (250 mL) was approximately $350 per 250 mL, thus the cost for on unit (U) of inulinase was calculated as $350/250/285 = $0.005. The result indicates that the cost using glucoamylase GA-L New as inulinase enzyme was only 3% (=$0.00015/$0.005) than that using Novozyme 960, thus leaving a huge profit margin for glucoamylase use as inulinase enzyme in industrial application.

4. Conclusion

High inulinase activity was observed and characterized in three commercial glucoamylases. One of the glucoamylase GA-L New from Genencor was applied to lactic acid production from Jat. A high lactic acid titer, yield, and productivity of 111.5 g/L, 0.46 g/g DM, and 1.55 g/L/h, respectively, were obtained within 72 h. The cost using commercial glucoamylase was compared to that using inulinase and a large profit margin was identified.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.08.123.

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