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Fed-batch saccharification and ethanol fermentation of Jerusalem artichoke stalks by an inulinase producing *Saccharomyces cerevisiae* MK01

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Jerusalem artichoke is a potential energy crop. While its tubers are being used to extract inulin, its stalks could be used for biofuel production. In this article, fed-batch saccharification and fermentation of Jerusalem artichoke stalks (JAS) was studied. Pretreatment with 2.0% (w/v) NaOH not only remained inulin, the unique component in JAS, but also increased cellulose content from 42.3% to 58.2% due to the removal of lignin. Batch-feeding of both the pretreated JAS and cellulases was an effective strategy for saccharification, through which 115.8 g/L total sugars including glucose, xylose, fructose and inulin were released from 20% (w/v) solids uploading with the supplementation of cellulases at 20 FPU (filter paper unit)/g dry biomass. An inulinase-producing yeast *Saccharomyces cerevisiae* MK01 was developed by the cell surface display of inulinase for ethanol fermentation from the pretreated JAS under the fed-batch conditions, and 38.3 g/L ethanol was produced at 96 h, with an ethanol yield of 0.361 g/g total sugars consumed, about 71% of the theoretical yield of 0.511, indicating that JAS would be a promising feedstock for ethanol production.

1. Introduction

Fossil fuels such as crude oil and coal are not renewable and environmentally friendly, which present a challenge for sustainable development. Biomass resources are one of the solutions. Currently, sugar and starch-based feedstocks are dominating biomass resources for this purpose [1], but they are not sustainable taking into account of global population and increased demand for food supply. Indeed, plant genetics provides a tool to engineer cell wall structure to address recalcitrant nature of lignocellulose, but it is not applicable for grain crops due to the risk of compromising grain yield. Therefore, dedicated energy crops that can grow well in marginal land without competing for arable land with grain production, and in the meantime be engineered for more amenable conversion are garnered attention [2].

Jerusalem artichoke (JA) is a perennial crop of the compositae family, which is tolerant to various environmental stresses such as drought, salt, pest invasion and infection of plant diseases [3,4]. Like switchgrass and *Miscanthus* selected as model herbaceous energy crops in the US and EU [2,5], JA might be developed as an energy crop due to its aforementioned agronomic traits. The major biomass of JA is from its tubers and stalks. Unlike grain crops that accumulate starch as carbohydrate, JA tubers contain inulin, a linear β (1, 2) linked oligo-fructan with a glucose terminal, which can be used to extract inulin for food use [6]. JA stalks (JAS) are another major biomass, but unfortunately related research is very limited. So far there are only two reports on ethanol production from JAS, in which pretreated JAS was mixed with grinded JA tubers [7,8]. Apparently, such a strategy compromised the advantage of JA tubers as the unique feedstock for inulin extraction. In addition, yeast strain used with these studies was from *Kluyveromyces marxianus*, a species not for ethanol fermentation in industry.

Like other lignocellulosic biomass, JAS exhibits a complex composed mainly of cellulose entangled with hemicelluloses and lignin [9], making the cellulose recalcitrant to enzymatic hydrolysis [10,11]. Therefore, pretreatment is required to deconstruct the complex, which impacts all subsequent steps such as cellulose hydrolysis and microbial fermentation [12]. On the other hand, JAS contains inulin and its content depends on harvesting season and climate conditions, which should be ultimately remained after pretreatment for ethaol production. Nowadays, acidic or alkaline pretreatment has been extensively studied for cellulosic ethanol production. While acid pretreatment presents the advantage of solubilizing hemicelluloses, alkaline pretreatment leads to

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significant removal of lignin [13], but their impact on inulin in JAS has never been reported.

Saccharomyces cerevisiae has been used for ethanol fermentation from sugar- and starch-based feedstock for a long history, but this species is not efficient for ethanol production from inulin, presenting a necessity for engineering it with inulinase-producing ability for ethanol production from inulin in JAS. Heterologous expression of cellulases in *S. cerevisiae* by surface display has been developed for ethanol production from lignocellulosic biomass through consolidated bioprocessing strategy [14, 15], but unfortunately it has not been successful due to the intrinsic complexity of cellulases involving synergetic effect of different enzyme components as well as heterogeneous reaction with cellulose hydrolysis. In contrast, Inulinase is simple and enzymatic hydrolysis of inulin is homogeneous. Therefore, surface display of inulinase in *S. cerevisiae* maybe an effective strategy for ethanol production from inulin in JAS.

Although high-solids loading has potential in increasing ethanol titer to reduce energy consumption with product recovery and stillage treatment [16], there are many drawbacks such as poor mixing and mass transfer due to extremely high viscosity [17]. Fed-batch hydrolysis has been tested as a feasible strategy for addressing these problems [18, 19]. On the other hand, the batch-feeding of cellulases was also an effective strategy for cellulose hydrolysis[20].

In this study, we explored conditions for pretreatment and enzymatic hydrolysis of JAS to enhance sugar yield. Yeast strain expressing inulinase was developed by surface display for ethanol fermentation from the pretreated JAS.

2. Materials and Methods

2.1 Pretreatment

Raw JAS provided by Dalian Tianma Group Co. Ltd. (Dalian, China) was milled and sieved with 28 mesh screen. For acid pretreatment, 10.0% (w/v) feedstock was soaked in 0.5-5.0% (v/v) H_2SO_4 at 121 °C for 1 h. Then the biomass was filtered, washed to neutral pH, and dried at 50 °C for 24 h. For alkali pretreatment, 0.5-5.0% (w/v) NaOH was used and other conditions were same as those applied in the acid pretreatment. Solids recovery was calculated as a percentage of the total solids after pretreatment over the initial biomass.

2.2 Feeding strategies for the pretreated JAS and cellulases

Cellulases used in this study were generous gift from Youtell Biotechnology Co. Ltd. (Shandong, China) with 150 FPU/ml. Seven strategies illustrated in Table 1 were developed for feeding the pretreated JAS and cellulases. The total biomass loading was 20% and cellulases were supplemented at 20 FPU/g(dry biomass). The saccharification was performed in 100 ml reaction volume with 50 mmol/L citric acid buffer (pH 4.86) at 50 °C and 150 rpm for 48 h. The supernatant was then collected by centrifugation at 6000×g for 10 min, which was subsequently analyzed by HPLC.

Table 1 Feeding strategies for JAS and cellulases*.

Run	Feeding time (h)			
	0	12	24	36
Control	20/400	0/0	0/0	0/0
A-a	15/400	5/0	0/0	0/0
B-a	10/400	10/0	0/0	0/0
C-a	5/400	5/0	5/0	5/0
A-b	15/300	5/100	0/0	0/0
B-b	10/200	10/200	0/0	0/0
C-b	5/100	5/100	5/100	5/100

*Biomass loading (g)/cellulases (FPU) supplemented.

2.3 Strain development

The industrial strain *S. cerevisiae* from Angel Yeast (Hubei, China) for ethanol fermentation in China was used as the host to be engineered with INU1 from *K. marxianus* ATCC8554 by cell surface display. The primers used in the strain development were given in Table 2.

Table 2 Primers used in this study

Name	Sequence (5'-3')
G-418-F	CCCATATGGTTTAGCTTGCCTCGTC
G-418-R	GAAGGCCTGTTTTCGACACTGGATG
$SED1_A$-F	GAAGATCTAAATTATCAACTGTCCTATTATCTG
$SED1_A$-R	CCCAAGCTTTTATAAGAATAACATAGCAACACCA
INU1-F	GGACTAGTATGAAGTTCGCATACTCCCTCTTGC
INU1-R	GAAGATCTGATCAAACGTTAAATTGGGTAACGT
PCR-F	TCCCCGCGGGAGTGAGGAACTATCGCATACCTGC
PCR-R	TCCCCGCGGGCAAATTAAAGCCTTCGAGC

The G418 resistant gene *KanMX4* was used for screening transformants. The *Nde*I-*Stu*I DNA fragment containing *KanMX4* was amplified from the HO-poly-KanMX4-HO vector by PCR using the G418-F and G418-R primers [21]. The fragment was then subcloned into the *Nde*I and *Stu*I sites of pRS316 to obtain pRS316-KanMX4, which was used as the starting plasmid for the cell-surface expression of *INU1*. The inulinase gene (Genebank accession number X57202.1) fragment without the termination codon was amplified from *K. marxianus* genome by PCR with the primers INU1-F (SpeI) and INU1-R (BglII). The *SED1* (Genebank accession number NM_001180385) fragment lacking of only the start codon was used as anchoring region, which was amplified from *S. cerevisiae* S288c genomic DNA by PCR using the $SED1_A$-F

(*Bgl*II) and $SED1_A$-R (*Hind*III) primers, and designed to situate to the 3'-terminal of the *INU1* fragment. The two fragments were inserted into the plasmid pRS425-β-glucosidase [22, 23]. The *INU1-SED1* fusion protein was inserted between the *PGK1* promoter and the *CYC1* terminator of pRS425-β-glucosidase, and the resulting plasmid was named pRS425-IS. The *Sac*II-*Sac*II DNA fragment encoding PGK_P-INU1-$SED1_A$-$CYC1_T$ was cut from pRS425-IS, which was then subcloned into the *Sac*II site of pRS316-KanMX4. The final plasmid was named pRS316-IS (Fig. 1), and the correctness of the *INU1* fragment and the *SED1* fragment was confirmed by sequencing (Sangon Biotech, China).

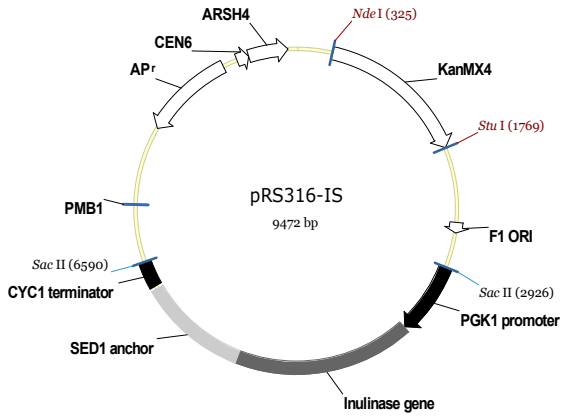

Fig. 1 Development of the inulinase expressing plasmid.

The plasmid pRS316-IS was transformed into *S. cerevisiae* Angel using the electroporation method following the instruction of the Bio-Rad Gene Pulser System (Bio-Rad, USA). The host stain transformed with the empty plasmid pRS316-KanMX4 containing KanMX4 resistant gene was used as the control. Positive transformants carrying *INU1* were screened in the YPD agar medium containing 1% yeast extract, 2% peptone and 2% glucose supplemented with 2% agar and 300 μg/mL G-418. To confirm the existence of the target gene, *INU1* fragment was amplified by PCR with the primers shown in Table 2, which was confirmed by gel electrophoresis. The constructed strain was designated as *S. cerevisiae* MK01.

In addition to the INU1-bearing recombinant, *S. cerevisiae* XL engineered with xylose metabolic pathway by inserting a tandem PsmXR-*PsXDH-ScXK* expression cassette into the chromosome of *S. cerevisiae* for co-fermentation of glucose and xylose was also examined [24].

2.4. Simulated medium

Simulated medium was developed based on the composition analysis of JAS hydrolysate containing 6.5% glucose, 1.6% inulin and 1.1% xylose with 0.4% peptone and 0.3% yeast extract supplemented to evaluate ethanol fermentation performance of yeast strains. Yeast culture with OD_{620} ~0.5 was inoculated into 100 ml simulated medium in 250 ml Erlenmeyer flask, and fermentation was performed at 37 °C and 150 rpm for 48 h. All fermentations were carried out in duplicate.

2.5 Analysis

A HPLC system (Waters 410, Waters, MA, USA) with the column (Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm, Hercules CA) and Waters 2414 refractive detector was used to analyze glucose, fructose, xylose and ethanol. The mobile phase was 5 mmol/L H_2SO_4 at a flow rate of 0.5 ml/min. The lignocellulosic components were determined by the standard methods developed by the National Renewable Energy Laboratory (NREL) [25], and the cellulose, hemicelluloses and lignin contents were calculated using equations below:

Cellulose (%) = (Glucose×0.9)/biomass×100% (1)

Hemicellulose (%) =(Xylose×0.88)/biomass×100% (2)

Lignin(%)=(Soluble & insoluble lignin)/biomass×100% (3)

The degree of polymerization (DP) of inulin varied depending on the origin and season of harvesting of Jerusalem artichoke [26]. Herein inulin was quantified by fructose content and the DP was chosen as 5 (fructose: glucose = 4:1) in safe [27].

Inulin (%) = (Fructose×1.15)/biomass×100% (4)

3. Results and discussion

3.1 Pretreatment of JAS with acid or alkali

The impact of H_2SO_4 and NaOH supplemented with different concentrations on the pretreatment of JAS was evaluated by measuring cellulose, hemicelluloses, lignin and inulin contents, which are summarized in Table 3.

With the increase of H_2SO_4 from 0.5% to 5.0%, hemicelluloses, inulin and total solids decreased from 12.0%, 13.3% and 85.0% to 2.1%, 4.2%, and 63.7%, respectively, indicating that the acid pretreatment significantly hydrolyzed hemicelluloses and inulin. However, when H_2SO_4 supplementation was in the range of 0.2% to 2.0%, cellulose content was increased from 42.2% to 55.4%, but more H_2SO_4 supplementation significantly decreased cellulose content due to hydrolysis. Besides, lignin content in the pretreated JAS increased concomitantly from 19.2% to 27.1% due to the decrease of hemicelluloses. By comparing solids recovery, cellulose, hemicelluloses and inulin contents, 2.0% H_2SO_4 treatment showed the best result among all acid dosages.

Investigations to explore the effect of NaOH pretreatment on JAS were also performed. With the increase of NaOH from 0.5% to 5.0%, significant decreases of lignin from 13.7% to 5.2%, inulin from 15.8% to 4.9% and solids recovery from 90.0% to 66.5% were observed, but the change in hemicelluloses content was comparatively small. These results showed that NaOH pretreatment of the JAS could effectively increase cellulose content from 42.3% to 60.8%. The optimal condition was 2.0% NaOH based on overall evaluation on solid recovery and cellulose and inulin contents.

Comparing to H_2SO_4, NaOH pretreatment removed lignin and enriched cellulose and hemicelluloses. Thus, 2.0% NaOH pretreatment was considered to be more effective for JAS.

Table 3 Evaluation of JAS pretreatment by H_2SO_4 or NaOH

	Solids recovery (%)	Cellulose (%)	Hemicelluloses (%)	Lignin (%)	Inulin (%)
Raw JAS	100.0	39.4±1.5	14.9±1.3	18.1±1.1	18.3±0.4
H_2SO_4(%)					
0.5	85.0±1.8	42.2±0.5	12.0±1.1	19.2±1.1	13.3±0.1
1.0	83.1±1.1	48.3±2.0	8.9± 0.2	20.2±1.6	11.2±0.1
2.0	78.3±1.8	55.4±1.1	5.3± 0.3	21.8±1.3	10.1±0.1
3.0	70.2±1.5	41.5±0.9	3.3± 0.5	23.5±0.8	8.4± 0.8
4.0	68.5±1.5	38.7±0.6	2.5±1.1	26.0± 0.1	7.5±0.1
5.0	63.7±1.7	35.8±1.9	2.1±1.3	27.1±1.7	4.2±0.2
NaOH(%)					
0.5	90.0±1.1	42.3±1.6	13.7±2.5	13.7±1.7	15.8±0.5
1.0	87.1±1.8	49.8±0.7	12.3±2.7	10.6±1.5	13.4±0.1
2.0	85.4±2.1	58.2±1.2	11.1±1.9	7.1± 0.9	13.0±0.6
3.0	75.5±1.5	58.9±1.4	10.8±1.9	6.1±1.3	10.7±0.0
4.0	70.7±1.1	60.7±1.4	10.0±1.4	5.9±0.1	8.2±0.1
5.0	66.5±1.9	60.8±1.4	9.9±0.2	5.2±0.1	4.9±0.1

3.2 Saccharification of the pretreated JAS

Fig. 2(a) illustrates major sugars detected in the hydrolysate of JAS pretreated by 2% NaOH and saccharified with strategies illustrated in Table 1. As can be seen, the control with all biomass and enzyme loaded at the beginning produced the lowest total sugars of 57.3 g/L, since the mixing was poor due to the high solid uploading, and extremely viscous slurry was observed until 20 h. By applying the biomass feeding strategies (A-a, B-a and C-a), total sugars were increased by 25.1%, 53.4% and 40.4%, respectively, compared to that detected in the control. When the fed-batch of cellulases (A-b, B-b and C-b) was further applied, total sugars were increased by 17.2%, 48.6% and 10.3% compared to that detected with the fed-batch of the pretreated JAS only. The time-course of glucose releasing from the hydrolysis of the cellulose component during the process illustrated in Fig. 2(b) further supported the effectiveness of the saccharification strategy B-b.

For the batch feeding of cellulases, the decrease of their activities was alleviated. However, the fed-batch saccharification strategy C-b with four equally feeding of the feedstock and enzyme at 0, 12, 24 and 36 h performed worse than the strategy B-b with the twice loading of the feedstock and enzyme due to short time for the enzymatic hydrolysis and low enzyme dosage at the early saccharification stage. It is clearly indicated that total sugars produced was dependent on the synergistic feeding of the biomass and enzyme. Twice feeding of both biomass and enzyme with 10% initial biomass loading was validated as a more efficient strategy for the enzymatic hydrolysis, through which 83.7 g/L glucose, 11.2 g/L xylose and 4.3 g/L fructose were obtained from 200 g/L biomass. It is interesting that 16.4 g/L inulin were also detected after 48 h saccharification.

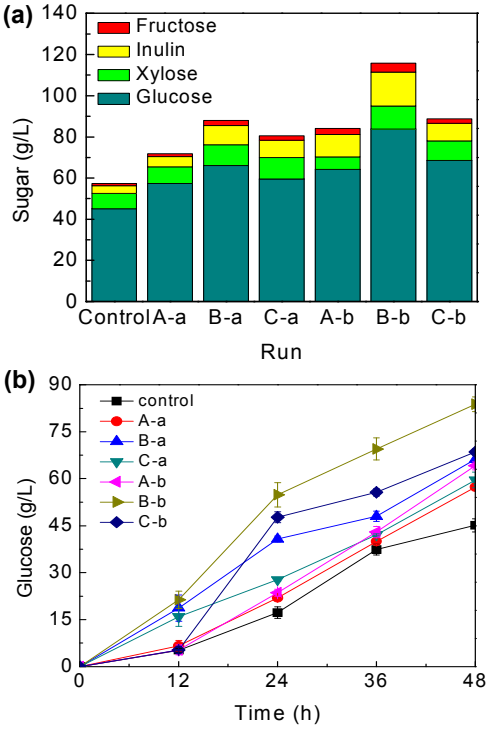

Fig. 2 Major sugars detected at 48 h for JAS pretreated by 2% NaOH and hydrolyzed with strategies illustrated in Table 1 (a) and time-course of glucose releasing from the hydrolysis of the cellulose component (b).

3.3 Evaluation of yeast strains

As can be seen in Fig. 3(a), inulinase activity was significantly enhanced in *S. cerevisiae* MK01, and the inulinase activity as high as 6000 U/g (DCW) was detected at 96 h with the engineered yeast strain, about 4 folds of that observed with the control, while the xylose-metabolizing strain *S. cerevisiae* XL exhibited even poorer inulinase activity than that with the control. Meanwhile, no significant difference was observed in growth between *S. cerevisiae* MK01 and the control (Fig. 3b).

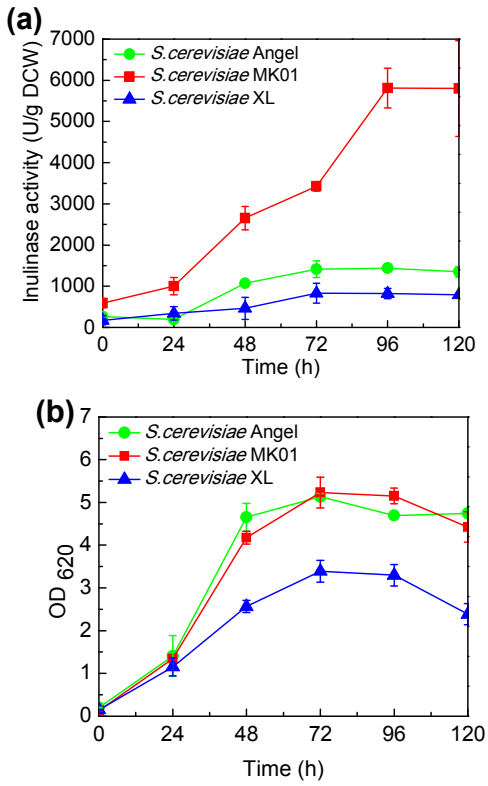

Fig. 3 Inulinase activities (a) and growth profiles (b) of *S. cerevisiae* MK01 and the control strain *S. cerevisiae* Angel.

Fig. 4 is the results of ethanol fermentation with simulated medium. *S. cerevisiae* MK01 grew faster than others, but no significant difference was observed in glucose consumption since all yeast strains almost completely consumed glucose within 24 h. However, *S. cerevisiae* MK01 consumed 13.9 g/L inulin, while less inulin (2.1–8.9 g/L) was consumed by other strains. In case of xylose conversion, *S. cerevisiae* XL showed better performance than other strains, with 6.3 g/L xylose consumed. It is clear that strains consumed more total sugars produced more ethanol. The highest ethanol of 34.2 g/L was produced by *S. cerevisiae* MK01. If concerning xylose conversion, *S. cerevisiae* XL was better, but for JAS hydrolysate containing more inulin than xylose, *S. cerevisiae* MK01 took advantages in converting more sugars that conferred by its improved inulinase activity for more efficient ethanol production. Therefore, *S. cerevisiae* MK01 was more suitable for ethanol production from JAS.

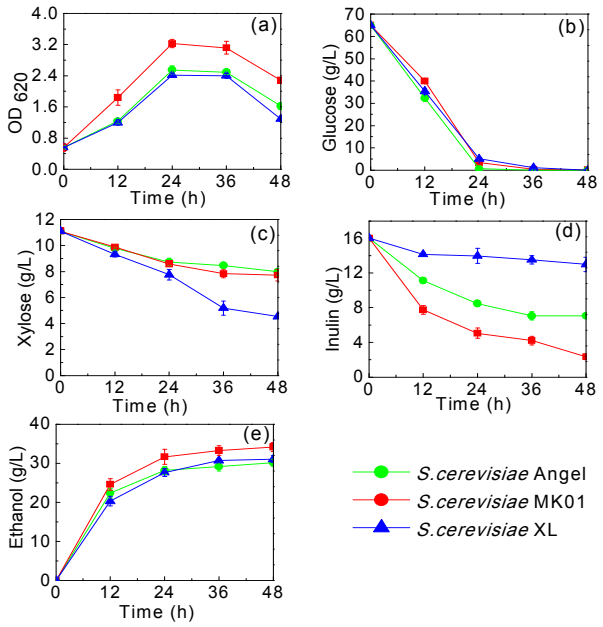

Fig. 4 Ethanol fermentation of the simulated medium by different yeast strains.

3.4 Ethanol fermentation from JAS

Unit operations were integrated for ethanol fermentation from JAS: 2% NaOH pretreated JAS was saccharified with the loading strategy B-b for the biomass and enzyme. The fermentation was carried out by *S. cerevisiae* MK01 at 37°C for 96 h. The purpose of saccharification was to release sugars quickly at elevated temperature for the enzymatic hydrolysis, and in the meantime reduce viscosity with high biomass loading that caused diffusion and mixing limitations with the fermentation process[28]. The total sugars with the feedstock were analyzed to be 115.8 g/L. The experimental results are shown in Fig. 5.

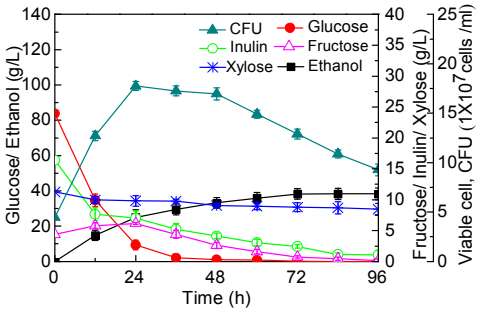

Fig. 5 Ethanol fermentation from JAS hydrolysate by *S. cerevisiae* MK01 (2% NaOH pretreated JAS was pre-saccharified under 50 °C for 48 h with the biomass and cellulase loading strategy B-b).

Yeast cells grew exponentially until 24 h, and then experienced a stationary phase for the next 24 h followed by the death phase. It was observed that glucose was quickly consumed. Meanwhile, inulin was gradually hydrolyzed by the inulinase displayed onto the surface of *S. cerevisiae* MK01, and fructose was released consistently, but was consumed as glucose was depleted. However, *S. cerevisiae* MK01 is not a good xylose utilizing strain and 8.5 g/L xylose was left at the end of the fermentation. Ethanol increased rapidly at the first 20 h as glucose was consumed. The maximum ethanol production of 38.3 g/L was achieved at 96 h, with 9.7 g/L total sugars unconverted, making the ethanol yield of 0.361 g/g total sugars consumed, about 71% of the theoretical yield of 0.511. Since xylose was the major residual sugar, it is expected that ethanol yield would be further improved with the development of yeast strains expressing both genes for inulinase production and xylose metabolism.

Ethanol production from JA tubers was explored in the 1980s to address the oil crisis [29, 30]. As a result, JA was grown in large quantities by Midwestern US farmers [31], but this project was interrupted shortly when cheap oil came back, making the farmers suffered big economic loss. Rocked oil prices at the beginning of the millennium, particularly in 2008 when oil prices hit the unprecedented record of USD147 per barrel, highlighted the importance of biofuels again [32, 33].

Although JA could be developed as an energy crop with advantages over other lignocellulosic biomass, how to process it without a repeat of the historic tragedy presents a challenge. We propose a roadmap for the biorefinery of JA in Fig. 6, and compare its advantages over other lignocellulosic biomass in Table 4.

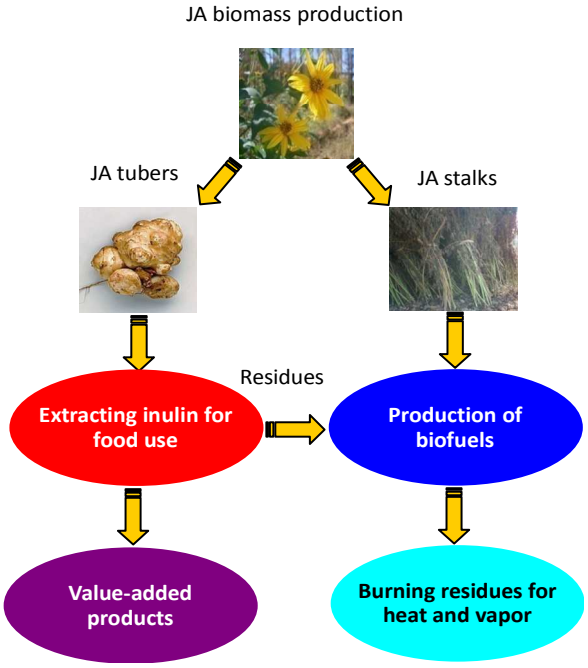

Fig. 6 Roadmap for the biorefinery of JA biomass.

Table 4 Biorefinery of JA over other lignocellulosic biomass

Biomass		Land usage	Advantages
Jerusalem artichoke	Potential energy crop	Marginal lands	Inulin extraction from tubers for food use, and modified to address the recalcitrance of stalks
Switchgrass and *Miscanthus*	Energy crops	Marginal lands	Modified to address their recalcitrance
Corn stover, rice/wheat straw and so on	Agricultural residues	No	No way to address their recalcitrance

JA presents potentials for producing value-added products to credit its biorefinery, but other lignocellulosic biomass is lacking of this advantage. Even inulin extracted directly from JA tubers without significant energy input compared to the pretreatment of lignocellulosic biomass and recovery of ethanol from fermentation broth can be sold in the food market at much higher prices than that for ethanol as biofuel, needless to say functional products and specialities that can be derived from inulin [34, 35]. Therefore, JA tubers should not be used for producing bulk commodities such as ethanol as biofuel. On the other hand, JAS with some inuin seems to be better than other lignocellulosic biomass for ethanol production through microbial fermentation in case significant progress is made in engineering strains with pentose utilization.

4. Conclusions

Experimental results suggest that the pretreatment with 2.0% (w/v) NaOH could remove lignin effectively from JAS and consequently increased cellulose content from 42.3% to 58.2%. Fed-batch for the feedstock and cellulase was proven to be a promising strategy for efficient saccharification, which produced 115.8 g/L total sugars. The inulinase-producing yeast *S. cerevisiae* MK01 was evaluated as a suitable strain at present to ferment the JAS hydrolysate, which produced 38.3 g/L ethanol at 96 h with 20% solids uploading, making the ethanol yield of 0.361 g/g total sugars consumed, about 71% of the theoretical yield of 0.511. With the development of yeast strains engineered with inulinase production and pentose metabolism as well, ethanol production from JAS is expected to be further improved, although many challenges are still ahead. Therefore, JAS is a promising feedstock for ethanol production, which would credit the biorefinery of JA as a potential energy crop.

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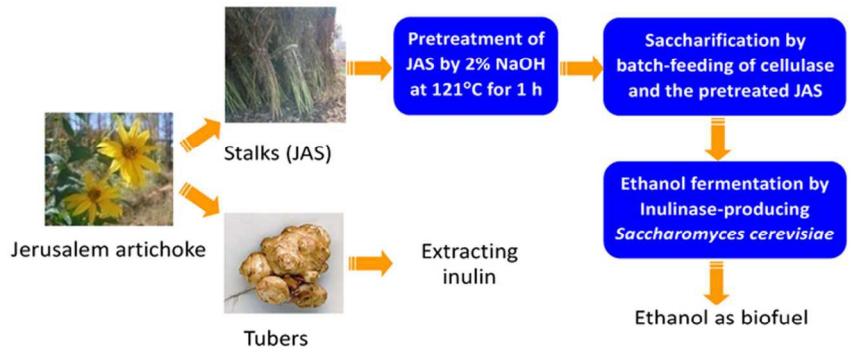

Biorefinery of Jerusalem artichoke to produce ethanol as biofuel and value-added product inulin

73x32mm (300 x 300 DPI)