Optimization of CDT-1 and XYL1 Expression for Balanced Co-Production of Ethanol and Xylitol from Cellobiose and Xylose by Engineered Saccharomyces cerevisiae

Jian Zha1, Bing-Zhi Li1, Ming-Hua Shen1, Meng-Long Hu1, Hao Song1,2, Ying-Jin Yuan1*

1 Key Laboratory of Systems Bioengineering (Tianjin University), Ministry of Education, Department of Pharmaceutical Engineering, School of Chemical Engineering & Technology, Tianjin University, Tianjin, P. R. China, 2 School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, Singapore

Abstract

Production of ethanol and xylitol from lignocellulosic hydrolysates is an alternative to the traditional production of ethanol in utilizing biomass. However, the conversion efficiency of xylose to xylitol is restricted by glucose repression, causing a low xylitol titer. To this end, we cloned genes CDT-1 (encoding a cellodextrin transporter) and gh1-1 (encoding an intracellular β-glucosidase) from Neurospora crassa and XYL1 (encoding a xylose reductase that converts xylose into xylitol) from Scheffersomyces stipitis into Saccharomyces cerevisiae, enabling simultaneous production of ethanol and xylitol from a mixture of cellobiose and xylose (main components of lignocellulosic hydrolysates). We further optimized the expression levels of CDT-1 and XYL1 by manipulating their promoters and copy-numbers, and constructed an engineered S. cerevisiae strain (carrying one copy of PGK1p-CDT1 and two copies of TDH3p-XYL1), which showed an 85.7% increase in xylitol production from the mixture of cellobiose and xylose than that from the mixture of glucose and xylose. Thus, we achieved a balanced co-fermentation of cellobiose (0.165 g/L/h) and xylitol (0.162 g/L/h) at similar rates to co-produce ethanol (0.36 g/g) and xylitol (1.00 g/g).

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* E-mail: yyuan@tju.edu.cn

Introduction

Lignocellulosic materials are renewable, abundant and inexpensive feedstock, which can be used to produce fuels and platform chemicals [1,2]. Hemicellulose, as a major component of lignocellulosic biomass, can be hydrolyzed to produce xylose, the second most abundant monosaccharide [3]. Xylose together with hexoses (mainly glucose) can be used for ethanol production [4]. Ethanol acts as a substitution of fossil fuels and has many environmental benefits such as minimization of carbon (CO) and nitrogen oxide (NOx) emission. As an alternative, xylose can be used to produce xylitol by microbial fermentation. Xylitol is widely used in medical and food industries, for example as a sugar substitute for diabetic and trauma patients [5,6,7]. Moreover, xylitol is a potential and promising approach to improving the economy of biomass conversion [9,10,11].

Xylose can be produced from xylose through NADPH-dependent xylose reductase (XR) encoded by XYL1 in yeasts such as Candida shehatae [12,13]. However, the xylitol yield by these yeasts is usually lower than 0.85 g/g xylose due to the fact that xylitol can be further metabolized [6]. Therefore, expression of XYL1 in Saccharomyces cerevisiae can improve the xylitol yield to its theoretically maximum value (1.00 g/g xylose) since xylitol cannot be further used as a carbon source [12,14].

Xylose utilization in S. cerevisiae suffers from glucose repression when glucose and xylose (i.e., main components of lignocellulosic hydrolysates) are both used as feedstock, which is caused by the preferential utilization of glucose [15,16,17,18,19]. There are no specific transporters for xylose uptake in S. cerevisiae. Transport of xylose into cytoplasm is mediated by hexose transporters which show much higher affinities to glucose than to xylose [20]. The discrimination on affinities causes differential uptake and utilization when multiple sugars are present, with glucose having the highest priority, thus leading to “glucose repression”. Such sequential utilization of xylose after glucose exhaustion presents several challenges to xylitol production. Firstly, xylose uptake is tremendously inhibited by glucose. Secondly, insufficient generation of cofactor such as NADPH (after glucose exhaustion) significantly reduces xylitol production since NAD(P)H is required for the conversion. Thus, xylitol production is dramatically impaired by delayed xylose utilization in recombinant S. cerevisiae. So far, no effective methods have been proposed to overcome the problems resulting from glucose repression in S. cerevisiae.

To increase xylose utilization by recombinant S. cerevisiae expressing XYL1, repeated fed-batch fermentation was implemented to produce xylitol through continuously supplying glucose.

PLOS ONE | www.plosone.org 1

July 2013 | Volume 8 | Issue 7 | e68317
under aerobic or anaerobic conditions, but this increased operation costs and could not produce ethanol with high yields simultaneously [21,22]. A cellobiose metabolic pathway was incorporated into *S. cerevisiae* allowing for the simultaneous co-utilization of cellobiose (a dimer of glucose) and xylose [23,24]. This approach facilitates faster xylose consumption and ethanol production compared with the traditional method of producing ethanol from mixtures of glucose and xylose, providing a solution to bypassing glucose repression. Furthermore, Jin and coworkers recently cloned the cellobiose metabolic pathway into a recombinant *S. cerevisiae* expressing xylose reductase (*XYL1*) from *Sch. Stipitis* to produce xylitol at a high yield [25]. However, such a strategy of using cellobiose as a co-substrate for xylitol production under oxygen-limited or aerobic conditions may not be optimal because cellobiose can be used as a co-substrate for ethanol production. Xylose uptake decelerates with the decrease of aeration and the uptake rate of xylose is usually lower than that of cellobiose under anaerobic conditions [23,26], so xylitol production could then be reduced in the anaerobic co-fermentation of xylose and cellobiose. As a result, balanced consumption of cellobiose and xylose is required for efficient co-production of ethanol and xylitol under anaerobic conditions.

**Figure 1. Construction strategy of the recombinant *S. cerevisiae* strain to achieve the co-production of ethanol and xylitol from lignocellulosic biomass.** The uptake and hydrolysis of cellobiose (derived from cellulose) were accomplished by a cellodextrin transporter (encoded by *cdt-1*) and an intracellular β-glucosidase (encoded by *ghl-1*), respectively. The uptake and conversion of xylose (derived from hemicellulose) was accomplished by endogenous hexose transporters and a xylose reductase (encoded by *XYL1*), respectively. Thus, ethanol and xylitol were produced simultaneously by this engineered yeast. NAD(P)H for xylitol production was provided by the cellobiose metabolism. doi:10.1371/journal.pone.0068317.g001

**Table 1.** Strains and plasmids used in the study.

| Strains/plasmids | Relevant genotype | Source or reference |
|------------------|-------------------|---------------------|
| **strains** | | |
| L2612 | MAT alpha1,leu2,ura3, trp1 | [50] |
| SCX-1 | L2612, trp::pRS304-TDH3XYL1, ura3::YIp1ac211-cdt-1, pRS425-ghl-1 | This study |
| SCX-2 | L2612, trp::pRS304-PGK1XYL1, ura3::YIp1ac211 cdt-1, pRS425-ghl-1 | This study |
| SCX-3 | L2612, trp::pRS304-TDH3XYL1, pRS426-cdt-1, pRS425-ghl-1 | This study |
| SCX-4 | L2612, trp::pRS304-PGK1XYL1, pRS426-cdt-1, pRS425-ghl-1 | This study |
| SCX-5 | SCX-1, AUR1::pAUR101-TDH3XYL1 | This study |
| **Plasmids** | | |
| YIp1ac211 | URA3, an integrative plasmid | ATCC 87593 |
| pRS304 | TRP1, an integrative plasmid | [51] |
| pAUR101 | Aba1, an integrative plasmid | Takara |
| pRS425-ghl-1 | Expression of gh1-1 | [45] |
| pRS426-cdt-1 | Expression of cdt-1 under PGK1p | [45] |
| pRS304-PGK1 | pRS304, PGK1p | This study |
| pRS304-TDH3 | pRS304, TDH3p | This study |
| pRS304-PGK1XYL1 | Expression of YXL1 under PGK1p | This study |
| pRS304-TDH3XYL1 | Expression of YXL1 under TDH3p | This study |
| pAUR101-TDH3XYL1 | Expression of YXL1 under TDH3p | This study |

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Table 2. Detailed information on the promoter and copy number of the genes CDT-1 and XYL1 in the five recombinant strains.

| Strain   | Promoter/Copy number (CDT-1) | Promoter/Copy number (XYL1) | Specific XR activity/ U/(mg protein) |
|----------|-------------------------------|-------------------------------|-------------------------------------|
| SCX-1    | PGK1/1                        | TDH3/1                        | 0.38±0.02                           |
| SCX-2    | PGK1/1                        | PGK1/1                        | 0.16±0.02                           |
| SCX-3    | PGK1/M                        | TDH3/1                        | 0.37±0.07                           |
| SCX-4    | PGK1/M                        | PGK1/1                        | 0.26±0.06                           |
| SCX-5    | PGK1/1                        | TDH3/2                        | 0.73±0.02                           |

M: multiple copies.

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In the present study, we proposed a method to simultaneously produce ethanol and xylitol from mixtures of cellobiose and xylose under anaerobic conditions by construction of a recombinant S. cerevisiae strain through expression of a cellobextrin transporter (CDT-1) and an intracellular β-glucosidase (gh1-1) from Neurospora crassa and the xylose reductase (XYL1) from Sch. stipitis (Figure 1). In order to balance the consumption of cellobiose and xylose, optimization of the expression of CDT-1 and XYL1 was performed by combination of various promoters and copy numbers. The optimization generated an efficient strain SCX-5 that can utilize cellobiose and xylose at similar rates.

Materials and Methods

Strains and Media

The strains used in this study are listed in Table 1. The host strain Saccharomyces cerevisiae L2612 (MATα, leu2-3, 112, ura3-52, trp1-298 can1 cyn1 gal1) was a gift from Professor Thomas Jeffries at University of Wisconsin–Madison. Escherichia coli DH5α was used for subcloning and was grown in LB medium supplemented with 100 mg/L ampicillin. Yeasts were grown in YP medium containing 6.7 g/L YNB (yeast nitrogen base), 20 g/L glucose, and 2 g/L amino acid powder mixture (lack of appropriate amino acids whenever necessary). 0.5 mg/L Aureothin (Takara Bio, Kyoto, Japan) was added for the selection of transformants. Anaerobic fermentation was carried out in YPXC medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose, and 20 g/L glucose) or YPXG medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose, and 20 g/L glucose). Inoculums were prepared in YPC medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L cellobiose).

Plasmid Construction

The plasmids and primers used in this study are listed in Table 1. The plasmid YIplac211 was transformed into the host strain SCX-2 and the strain with multiple copies of CDT-1 was yielded. Plasmid pRS425-gh1-1 was transformed, generating the strains capable of utilizing cellobiose (Table 1). The plasmids pRS304-PGK1XYL1, pRS304-TDH3XYL1 and pAUR101-TDH3XYL1 were linearized by appropriate restriction enzymes and transformed, forming strains SCX-1 to SCX-5 as described in Table 1. Yeast transformation was performed by the LiAc/SSDNA/PEG procedure [28].

Anaerobic Fermentation

Cells were cultivated in YPC medium to prepare inoculums for anaerobic fermentation. Cells at mid-exponential phase were harvested by centrifugation (4,000 rpm, 5 min) and inoculated into 50 mL YPXC or YPXG medium (initial OD600 = 1.0) in a 100-mL shaking flask (sealed with a rubber stopper with a syringe needle). Cells were cultivated in a rotary shaker at 30°C and 150 rpm (Honour, Tianjin, China). All fermentations were carried out in duplicates.

Analysis of Substrates and Fermentation Products

Cell growth was monitored by measuring OD600 of cell culture on a Model 2232 grating spectrometer (Shanghai No. 3 Analytical Equipment Factory, Shanghai, China). Samples were taken periodically from culture and centrifuged at 10,000 rpm for 5 min. Supernatant was collected for metabolite analysis. Concentrations of sugars, xylitol and ethanol were measured by an HPLC system consisting of an HPLC pump (Waters 1515, Milford, USA), a Bio-Rad HPX-87H column (Bio-Rad, Hercules, CA) and a refractive index detector (Waters 2414, Milford, USA). The column was eluted at 65°C with 5 mM sulfuric acid at 0.6 mL/min.

Enzyme Assays

To measure XR activity, cells at the mid-exponential phase in the SD medium (with 20 g/L glucose) were harvested and washed twice with ice-cold water. Then, cells were resuspended in the
detriethadmine buffer (with 1% PMSF) and disrupted by sonication for 20 min [29]. The protein in the cell lysate was separated by centrifugation for 15 min (4°C, 10,000 rpm). Protein concentration was determined by a Coomassie protein assay kit.

Figure 2. Time profiles of the concentrations of cellobiose (A), ethanol (B), xylose (C) and xylitol (D) by the five recombinant yeast strains (SCX-1 to -5, see Table 2). The fermentation was conducted under anaerobic conditions in 50 mL YPXC medium containing 20 g/L xylose and 20 g/L cellobiose. The initial cell density was OD<sub>600</sub> = 1.0. The data was the mean ± standard deviation of duplicate experiments. doi:10.1371/journal.pone.0068317.g002
Table 3. Co-fermentation of xylose and cellobiose by the five recombinant strains under anaerobic conditions.

| Strains    | xylose (g/L)* | Ethanol (g/L) | Xylitol (g/L) | Yield (g/g consumed sugars) |
|------------|--------------|---------------|---------------|----------------------------|
| SCX-1      | 0.159±0.002  | 7.15±0.04     | 18.87±0.12†   | 0.35±0.00                  |
| SCX-2      | 0.131±0.002  | 7.55±0.04     | 15.99±0.12†   | 0.37±0.00                  |
| SCX-3      | 0.150±0.003  | 7.65±0.08     | 18.41±0.11†   | 0.37±0.01                  |
| SCX-4      | 0.115±0.002  | 7.40±0.23     | 13.14±0.81    | 0.36±0.01                  |
| SCX-5      | 0.162±0.000  | 7.19±0.09     | 19.24±0.24    | 0.36±0.01                  |

The growth was conducted in 50 mL YPXC medium containing 20 g/L xylose and 20 g/L cellobiose. The initial cell density was OD600 = 1.0.

*Value was significantly different (p<0.05) from SCX-2 and SCX-3.
†The values were significantly different (p<0.05) from SCX-4.

The xylose reductase (XR) activities of the five recombinant strains were measured (Table 2). As expected, XR activities in the strains with YXLI under the TDH3p promoter were higher than those under PKI1p promoter. For example, XR activity in SCX-1 was 2.4-fold of that in SCX-2; XR activity in SCX-3 was 1.4-fold of that in SCX-4. Also, a higher copy number of YXLI enabled a higher XR activity. Compared with strain SCX-1 which has only one copy of YXLI, the specific XR activity of strain SCX-5 with two copies of YXLI almost doubled.

Optimization of the Co-fermentation of Cellobiose and Xylose

To examine the effect of different expression levels of YXLI and CDT-1 on co-fermentation of cellobiose and xylose, we compared the cellobiose and xylose consumption in the five recombinant strains (Figure 2). It was found that the modulation on expression levels of YXLI and CDT-1 had little effect on cellobiose consumption during the whole fermentation process (Figure 2A). Only the initial cellobiose uptake rate during the first 24 h varied a little among the five strains (Figure 2A). We further examined the time profiles of ethanol production by the five recombinant strains, which showed insignificant difference (Figure 2B). Thus, the productivity and yield of ethanol by the five recombinant strains were similar (Table 3).

In contrast, the expression levels of CDT-1 and YXLI had an evident influence on xylose conversion and xylitol formation. Interestingly, we found that a lower CDT-1 expression level led to a higher productivity of xylitol (Table 3). For example, SCX-1 and -2 carrying one copy of CDT-1 had higher xylose utilization rates than those of SCX-3 and -4, respectively. Moreover, a higher expression level of YXLI led to a higher xylose utilization and xylitol productivity. SCX-1 and -3 with YXLI under the control of TDH3p had 21.4% and 30.4% higher xylose consumption rates than SCX-2 and -4 with YXLI under PKI1p, respectively (Figure 2C, Table 3). Strain SCX-5 derived from SCX-1 bore the highest XR activity. The xylose uptake rate of strain SCX-5 was the highest among the strains (Figure 2C). After 72 h, it consumed 95.1% and 93.8% of the total cellobiose and xylose, respectively. In contrast, the residual xylose of other strains was much higher than that of SCX-5 (Figure 2C). The xylitol production showed consistent profiling with xylose utilization in the five strains (Figure 2D). Therefore, we obtained an efficient strain SCX-5 through fine-tuning the expression of CDT-1 and YXLI.

Optimization of CDT-1 expression is a useful method to balance the consumption of cellobiose and xylose. Cellobiose transport has been considered to be a limiting factor in cellobiose utilization. In one previous study, overexpression of CDT-1 increased cellobiose utilization and ethanol production [23]. Another study of improving cellobiose utilization by introduction of protein engineered cellobiose transporters with increased transport kinetics (Nmax) also independently demonstrates the importance of enhancing cellobiose transport [32]. However, excessive overexpression of CDT-1 might not be beneficial for cellobiose consumption and xylose utilization. A previous study showed that a higher expression level of Gsf1 (xylose transporter gene) decreased xylose consumption rate compared with its lower expression [33]. Overexpression of CDT-1 can lead to imbalance.
between CDT-1 and gh1-1, which decreases cellobiose consumption. Du and coworkers observed that a more balanced CDT-1 and gh1-1 can increase cellobiose utilization [34]. Because cellobiose metabolism supplies NAD(P)H for xylitol formation, decreased cellobiose metabolism will result in insufficient NAD(P)H supply and subsequently will slow down xylose consumption. On the
other hand, overexpression of CDT-1 might cause the excessive occupation of cellodextrin transporters on cell membrane, restricting the normal location of hexose transporters and therefore leading to inefficient xylose uptake. Thus, optimal expression of CDT-1 is required for efficient co-metabolism of cellobiose and xylose.

Notably, it was observed that uptake rates of cellobiose and xylose decreased dramatically when sugar concentration was low (Figure 2A and 2C), which has been reported in literature [19,35]. Increasing the affinity of cellobiose transporters or hexose transporters to cellobiose or xylose at low sugar concentrations can be a solution to improving the fermentation. Expression of heterologous xylose transporters to cellobiose or xylose at low sugar concentrations can significantly improve transport kinetics in batch cultivation at 4 g/L xylose concentration [36].

Previous studies have demonstrated that higher expression of XYL1 enables faster xylose fermentation [12,37,38]. Integration of one extra copy of XYL1 increased the xylose consumption rate by 1.7 fold [37]. Matsushika and coworkers also observed that XYL1 under the control of the promoter PGK1 enabled 9% more xylose consumption than that under the promoter ADH1, which is considered to be a weaker promoter [38]. The increased xylose consumption rate was most probably due to increased xylitol productivity which accelerated the downstream reactions and thus enhanced the carbon flux. In the present study, xylose reductase encoded by XYL1 catalyzes the conversion of xylose to xylitol. Enhanced expression of XYL1 resulted in a higher XR activity that could accelerate xylose conversion. A similar result was previously observed in optimization of xylitol production by a recombinant S. cerevisiae in a fed-batch culture [39].

Recently, Jin's group used a similar strategy for enhanced xylitol production [25]. Different from their work, the aim of the present work is coproduction of ethanol and xylitol with high yields from mixtures of cellobiose and xylose rather than just production of xylitol. Additionally, the strategy applied here is combinatorial optimization of the expression levels of CDT-1 and XYL1, which has been proven to be effective in improving the efficiency of fermentation. However, Jin and coworkers did not optimize these genes but enhanced NADPH supply through overexpression of ALD6, IDP2, and S.2WF1.

Taken together, the co-production of ethanol and xylitol were affected by the combinatorial expression of XYL1 and CDT-1, a fine tuning of which enabled the construction of an efficient strain SCX-5 carrying one copy of CDT-1 and two copies of XYL1 under the control of TDH3 promoter. Such an engineered strain SCX-5 could co-utilize cellobiose and xylitol at similar rates (~0.16 g/L/h) and co-produce ethanol and xylitol with high yields (0.36 g/g and 1.00 g/g, respectively).

Co-fermentation of the Strain SCX-5 in Glucose and Xylose

To verify that the strategy of co-fermentation by SCX-5 could avoid glucose repression, we investigated the co-fermentation of SCX-5 in glucose and xylose for the co-production of ethanol and xylitol. As shown in Figure 3A, glucose was used up in 8 h. Meanwhile, the ethanol production reached maximum in 8 h with a yield of 0.37 g/g glucose (Figure 3B). The ethanol concentration decreased a little during the following period, which might be due to evaporation or oxidation by SCX-5. However, in the co-fermentation of cellobiose and xylose, the consumption of cellobiose and the production of ethanol were much slower, during which ethanol gradually accumulated in 120 h with a final yield of 0.36 g/g glucose (Figure 3A and 3B).

As for xylose, 98.5% of xylose was consumed in the co-fermentation of cellobiose and xylose (in 120 h), whereas only 54.2% of xylose was used in the co-fermentation of glucose and xylose (Figure 3C). Xylitol production rate was lower in the co-fermentation of glucose and xylose, and the final concentration of xylitol reached only 10.36 g/L (Figure 3D). In contrast, 85.7% more xylitol was produced in the co-fermentation of cellobiose and xylose. The results demonstrated that this strategy of co-utilizing cellobiose and xylose could bypass glucose repression and achieve co-production of ethanol and xylitol at a higher titer.

Diauxic growth pattern is a major obstacle for efficient co-production of ethanol and xylitol from glucose and xylose in lignocellulosic hydrolysates [14,40]. To eliminate glucose repression during utilization of multiple sugars in E. coli, disruption of ptsG, deletion of cya or introduction of a CRP* mutant (cyclic adenosine monophosphate receptor protein) have been performed [41]. However, these strategies have no effect on relieving glucose repression in S. cerevisiae, which has been thought to be related to the competition between glucose and other sugars during uptake process [18]. The entrance of xylose into cells is facilitated by hexose transporters, which show higher preference for glucose than xylose [42,43,44]. As a result, xylose uptake is competitively inhibited when glucose is present. In the present study we introduced the cellobiose transporter and β-glucosidase, allowing the utilization of cellobiose so that hexose transporters were solely used for xylose uptake [23,45]. Therefore, the method applied here provides a way to bypass glucose repression. The efficient ethanol production and xylitol formation proceeded simultaneously from a mixture of cellobiose and xylose, which has not been reported. Although Jin's group reported a similar method to produce xylitol with high productivity, their design mainly focused on xylitol production rather than ethanol [25]. Our study provides an applicable method to obtain valuable chemicals such as ethanol and xylitol from lignocellulosic hydrolysates, increasing the economic competitiveness of biomass-based bio refinery. In addition, the co-fermentation of cellobiose and xylose in lignocellulosic hydrolysates by SCX-5 can lower the usage of β-glucosidase in cellulase cocktails and thereby reduce the cost associated with the cellobiose saccharification [25,46,47]. Furthermore, the final products (ethanol and xylitol) can be easily separated by distillation and sequential crystallization, which is a great merit of this strategy [48]. Simultaneous utilization of glucose and xylose was observed for a lipid-producing yeast strain Trichosporon cutaneum [49]. However, identification of the specific xylose transporters and successful expression of them in S. cerevisiae is difficult to achieve nowadays. Thus, the strategy applied here is still one of the best approaches to overcoming glucose repression.

Conclusions

In this study, we constructed S. cerevisiae strains able to co-produce ethanol and xylitol by expressing the genes encoding cellobiose pathway enzymes (CDT-1 and gh1-1) and the gene encoding XR for xylitol production (XYL1). Furthermore, combinatorial modulation of the expression levels of CDT-1 and XYL1 resulted in an optimized strain SCX-5 capable of consuming xylose and cellobiose at almost identical rate with the xylitol yield of 1.00 g/g xylose and the ethanol yield of 0.36 g/g cellobiose. SCX-5 produced 85.7% more xylitol in the mixture of cellobiose and xylene than in glucose and xylose.

Supporting Information

Table S1 Primers used in the study. (DOCX)
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Author Contributions

Conceived and designed the experiments: JZ BZL YJY. Performed the experiments: JZ MHS MLH. Analyzed the data: JZ HS. Contributed reagents/materials/analysis tools: JZ MHS MLH. Wrote the paper: JZ HS.

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