SUSTAINABLE CHEMICAL PROCESSES

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A xylose-fermenting yeast hybridized by intergeneric fusion between *Saccharomyces cerevisiae* and *Candida intermedia* mutants for ethanol production

Prihardi Kahar[1] and Shuzo Tanaka[2*]

Abstract

Background: Bioethanol production from lignocellulosic biomass, in particular xylose, is currently of great concern, given the abundance of this sugar in the world, because *Saccharomyces cerevisiae*, which is widely used for bioethanol production, is unable to naturally ferment xylose. The aim of this study was to obtain a novel yeast capable of stably producing ethanol from biomass containing xylose by protoplast fusion between *S. cerevisiae* and xylose-utilizing yeast.

Results: We describe a novel xylose-fermenting yeast strain, FSC1, developed for ethanol production by intergeneric hybridization between *S. cerevisiae* and *Candida intermedia* mutants by using a protoplast fusion technique. The characteristics of the FSC1 strain are reported with respect to xylose fermentation, morphology, gene, and protein expression. Mutation of the parental strains prior to protoplast fusion endowed the FSC1 strain with the ability to convert xylose to ethanol. Microscopic analysis confirmed that the parental and FSC1 strains produced spores in the potassium acetate medium. The FSC1 strain is uninucleate diploid, has a stable metabolism, and expresses proteins at a higher level than the parental strains. We found that FSC1 strain could stably achieve an ethanol yield of 0.38 g/g-substrate in fermentation of a mixture of glucose and xylose. In addition, the fermentation ability of FSC1was improved by successive chemical mutation, resulting in a higher ethanol yield of 0.42 g/g-substrate, corresponding to 82% theoretical yield.

Conclusions: The mutation-fusion technique we have described here is very useful for the development of intergeneric hybrids capable of xylose fermentation, and the FSC strains generated using this technique have the potential for industrial use in ethanol production from lignocellulosic biomass.

Keywords: Xylose fermentation, Intergeneric hybridization, Mutation and fusion, *Saccharomyces cerevisiae*, *Candida intermedia*

Background

With the increasing appreciation of the problem of global warming, bioethanol has recently gained increasing attention as a renewable and carbon-neutral energy source. Bioethanol production through the fermentation of lignocellulosic biomass, in particular xylose, is currently of great concern, given the abundance of this sugar in wood and herbs. Given that *Saccharomyces cerevisiae*, which is widely used for bioethanol production, is unable to naturally ferment xylose, there is increasing investigation of its metabolic alteration to endow it with the ability to ferment xylose.

In general, fungal xylose fermentation initially requires two sequential reactions, namely, the conversion of xylose to xylitol, catalyzed by xylose reductase (XR), and the conversion of xylitol to xylulose, catalyzed by xylitol dehydrogenase (XDH). In a reaction catalyzed by xylulokinase (XK), xylulose is then phosphorylated

* Correspondence: tanakash@es.meisei-u.ac.jp
[2]Program in Environment and Ecology, Department of Interdisciplinary Science and Engineering, Faculty of Science and Engineering, Meisei University, 2-1-1 Hodokubo, Hino, Tokyo 191-8506, Japan
Full list of author information is available at the end of the article

to xylulose-5-phosphate before entering the pentose phosphate pathway (PPP). While *S. cerevisiae* is unable to ferment xylose because of the lack of XDH activity in the presence of glucose, it possesses the *XYL2* gene, encoding XDH [1], as well as the homologous gene *XKS1*, encoding XK [2-4], and *GRE3*, encoding aldose reductase, which is closely related to XR [5]. In addition, the ability of *S. cerevisiae* to take up xylose through hexose transporter, and its aldose reductase activity, can be enhanced by chemical mutation and intensive screening on the basis of 2-deoxyglucose (DOG) tolerance [6].

The metabolic alteration of the yeast for ethanol production has been attempted through mutation, fusion, and recombination. Improvements in yeast metabolism have been reported by mutation and fusion between *S. cerevisiae* and *Zygosaccharomyces fermentati* [7], *S. cerevisiae* and *Pichia stipitis* [8], *Kluyveromyces marxianus* TS8-1 and TS87-8 [9], and *S. cerevisiae* and *Candida shehatae* [10]. During culture passages, however, some fusants were dissociated into segregants resembling the parental strains [8], and fusant offspring are almost completely sterile mainly because of the inability of the chromosomes of the partner genomes to pair or to recombine [11]. More recently, several attempts have been made to transfer specific genes for xylose utilization to *S. cerevisiae* by construction of recombinant strains (reviewed by Matsushika et al., ref [12]). The recombinants are expected to be practically applied for ethanol production from lignocellulose by overcoming such problems as redox imbalance in the initial step of xylose fermentation and reverse flux in glycolysis [13].

The xylose transporter of yeast *Candida intermedia* PYCC 4715, which grows equally well in xylose and glucose and has a high xylose transport capacity [14], has been functionally expressed in recombinant *S. cerevisiae* to increase its xylose uptake [15]. From these studies, we think that cell fusion between *Saccharomyces* and *Candida* strains may yield a strain which can take up and utilize xylose, as well as glucose, for ethanol production. Since cell fusion allows the transfer of complete segments of genomic DNA from parental yeasts, a fusant rich in genetic information could be obtained by protoplast fusion and stabilized by routine mutation and screening techniques. *C. intermedia* is nonpathogenic and safe for use. Consequently, *C. intermedia* has potential as a cell fusion partner with *S. cerevisiae* for the transfer of genes for xylose fermentation.

The aim of this study was to obtain a novel yeast capable of stably producing ethanol from biomass containing xylose by harnessing recent progress in intergeneric hybridization techniques with proteomic analysis. We developed a novel xylose-fermenting strain by intergeneric protoplast fusion between *S. cerevisiae* and *C. intermedia* strains altered, in advance, by mutation. The fusant obtained was subsequently characterized with respect to xylose fermentation, ethanol yield, morphology, and gene and protein expression.

Results

Mutation of wild-type strains and fermentation by mutants

The mutant M2 strain improved in xylose uptake had been selected from diverse mutant colonies of *S. cerevisiae* grown on medium containing DOG as described in a previous study [6]. Since the M2 strain lacks the XDH activity, *C. intermedia* was used as a donor of *xdh* gene in cell fusion of this study. *C. intermedia* can originally take and metabolize xylose into ethanol, but its ability of ethanol production is not high. Therefore, it is important to use the *C. intermedia* mutant that has no ability for taking xylose upon the fermentation. As described in the Methods, *C. intermedia* was mutated using ethyl methane sulfonate (EMS) to obtain a strain in which xylose uptake was strongly suppressed, but which contained the *xdh* gene. DOG was used for screening DOG-sensitive mutants to surely repress the growth of parental m11 in regeneration of fused protoplast cells. We finally selected a mutant m11 strain considered to have the potential to endow the fusant with the ability to metabolize xylose when hybridized with the M2 strain by protoplast fusion, as described in the following hybridization.

Next, fermentation by m11 and its wild-type strains were investigated in MMGX medium. The results are shown in Figure 1, and include those of the M2 strains for reference. As initially intended, the m11 strain of *C. intermedia* consumed glucose but not xylose, while the wild-type strain utilized both glucose and xylose, with xylitol accumulating at high levels in the supernatant (Figure 1b). Ethanol production was low but glycerol was produced in both m11 and wild-type strains. On the other hand, the M2 strain of *S. cerevisiae* did take up xylose at a rate of 5.26 g-xylose/g-cell, while the original strain did at 1.58 g-xylose/g-cell, and produced more ethanol with less glycerol formation as shown in Figure 1a, also detailed in the previous study [6].

Hybridization between *S. cerevisiae* M2 and *C. intermedia* m11 by protoplast fusion

Before protoplast fusion, we investigated the sporulation of *C. intermedia* m11 using the potassium acetate (KAc) medium. Using the Wirtz-Conklin spore stain method, m11 cells after sporulation were stained greenish-blue by Brilliant Green, but were not stained pink by safranin, indicating no growth of the vegetative cell (Figure 2a and c). Spores appeared as spheres under scanning electron microscope (SEM) (Figure 2e), and were different in shape from the m11 cell, which was ellipsoidal, as described below. We confirmed that the m11 strain formed

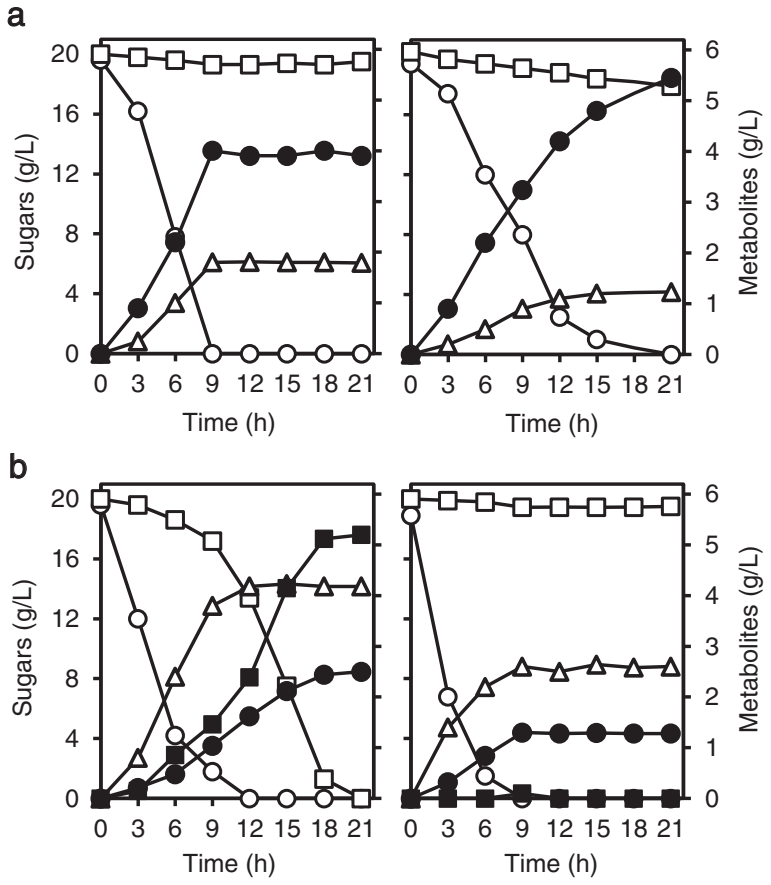

Figure 1 Fermentation of glucose and xylose by parental wild-type strains (left) and their mutant strains (right). (a) *S. cerevisiae* and the mutant M2 strains. **(b)** *C. intermedia* and the mutant m11 strains. (○) Glucose, (□) xylose, (●) ethanol, (■) xylitol, (Δ) glycerol.

spores, as reported for the US patent of the parental *C. intermedia* (originally designated as a *Kluyveromyces cellobiovorus*) [16,17]. Protoplasts of haploid cells obtained after sporulation of M2 and m11 strains were subjected to cell fusion and the regenerated cells were incubated in MMXDOG medium. Since the M2 strain is tolerant to DOG but is unable to use xylose, and the m11 strain is sensitive to DOG inhibition, only heterogenic fusants could form colonies in the medium. The suppression of xylose uptake in the m11 strain by mutation also allowed the selection of a target fusant without growth of the m11 strain in medium containing xylose as a carbon source.

A target strain that first appeared was selected from three colonies formed in MMXDOG medium and named FSC1, as a fusant between *S. cerevisiae* M2 and *C. intermedia* m11. Brilliant Green staining and SEM observation confirmed that the FSC1 strain produced spores in KAc medium (Figure 2b, d and f).

Colony formations of FSC1, M2 and m11 strains were investigated in YMG, YMFDOG and YMXDOG medium (Figure 3a, b and c, respectively). All strains grew in YMG, whereas FSC1 and M2 strains grew in YMFDOG, and only FSC1 strain grew in YMXDOG. These data indicated that the FSC1 cells were an intergeneric hybrid of the M2 and m11 cells. To investigate the possibility of normal mating of the partner strains instead of their haploid fusion for hybridization, we next attempted colony formation by mixing cultures of M2 and m11 strains cultivated in advance. As shown in Figure 3d, mixed cultures of M2 and m11 strains failed to form colonies in MMXDOG medium, indicating that the FSC1 strain was obtained by cell fusion, rather than normal mating between the M2 and m11 strains.

Next, the morphology of the M2, m11 and FSC1 strains was microscopically examined (Figure 4a, b and c, respectively). The M2 cells appeared ellipsoidal, while m11 cells were similar in shape but smaller in size. The FSC1 cells also appeared ellipsoidal and were much larger than the parental cells, with larger cells having a long axis of approximately 10 μm. 4′,6-diamidino-2-phenylindole (DAPI) stain confirmed that all FSC1 cells were uninucleate as shown by the arrow in Figure 4d.

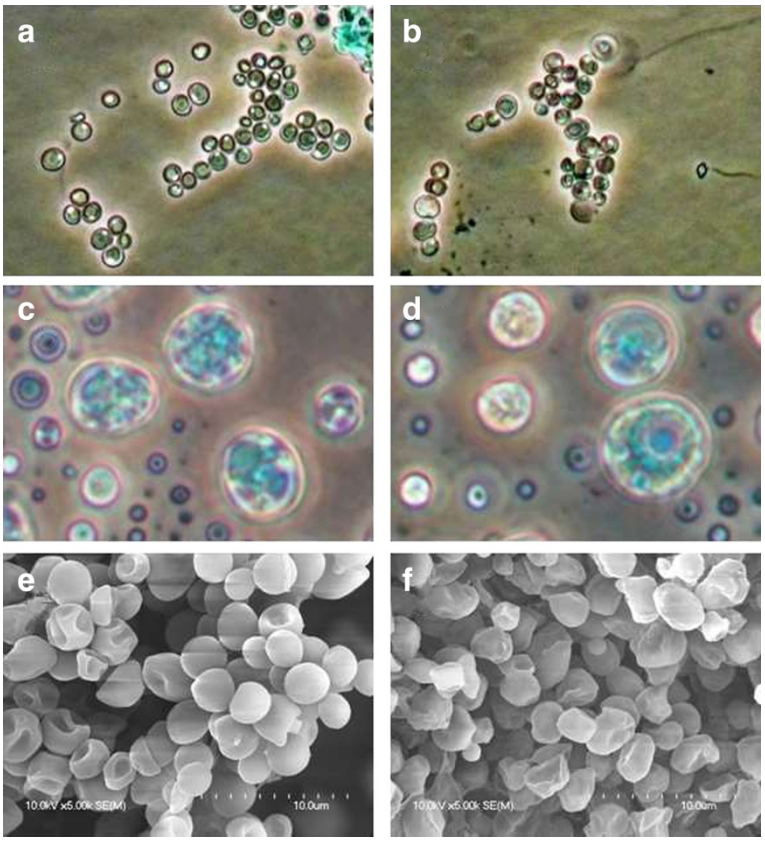

Figure 2 Spore formations of *C. intermedia* m11 (left) and FSC1 (right) strains in KAc medium. Microscopic observation with brilliant green staining before **(a, b)** and after **(c, d)** swelling the cell by soaking in saline solution, and SEM of the spores **(e, f)**.

Metabolism by xylose-fermenting fusant FSC1

We investigated fermentation by the FSC1 strain in MMGX medium, as conducted for the parental strains. Both glucose and xylose were completely fermented and ethanol production was approximately 2.7-fold higher than in the M2 strain (Figures 5a and 1a). The FSC1 strain produced some xylitol, but much less glycerol than the m11 strain. The ethanol yield for the FSC1 strain (0.38 g/g-substrate) was high compared to the wild-type and mutant strains (0.10 and 0.14 g/g-substrate in *S. cerevisiae* and 0.07 and 0.03 g/g-substrate in *C. intermedia*, respectively) (Figure 5b). Fermentation characteristics of the FSC1 strain were quantitatively summarized in Table 1.

Stability of metabolism is also concern for strains altered by mutation and fusion. As shown in Figure 5c, the FSC1 strain showed a high stability in both ethanol production and xylose consumption over 14 generations.

Next, we investigated the amino acid requirements of the FSC1, M2 and m11 strains, using seven amino acids in MMG medium (Table 2). The M2 strain grew in MMG while the m11 strain did not, although both strains grew in the enriched medium. The FSC1 strain grew in MMG, indicating the strain was prototrophic, as was observed for the M2 strain. On the other hand, the m11 strain was auxotrophic absolutely for uracil and relatively for histidine.

Mutation is often used to generate improved yeast strains [18]. We tried to improve the fermentation ability of the FSC1 strain by mutating twice using EMS in the manner described for the parental strains in Methods. The FSC1 mutant obtained showed 0.42 g/g-substrate in ethanol yield, 10% higher than by the FSC1 strain, as shown in Table 1. A xylose consumption rate was also 6 times higher, improved from 0.18 to 1.07 g/g-cell.h.

mRNA and total protein expression

We used the reverse transcription polymerase chain reaction (RT-PCR) to analyze the mRNA expression levels of genes related to xylose fermentation (*xr*, *xdh*, *xk*, and *adh1*). As shown in Figure 6a, analysis of the FSC1 strain indicated that *xr* and *xdh* were transferred from *C. intermedia* (Cd*xr* and Cd*xdh*), with reduced expression of *gre3* from *S. cerevisiae* (Sc*gre3* corresponding to *xr*), while *xk* and *adh1* were transferred from *S. cerevisiae* (Sc*xks1* corresponding to *xk*, and Sc*adh1*). Expression levels of Cd*xr*, Cd*xdh*, Sc*xks1* and Sc*adh1* in the FSC1 strain were higher than those in the parental strains.

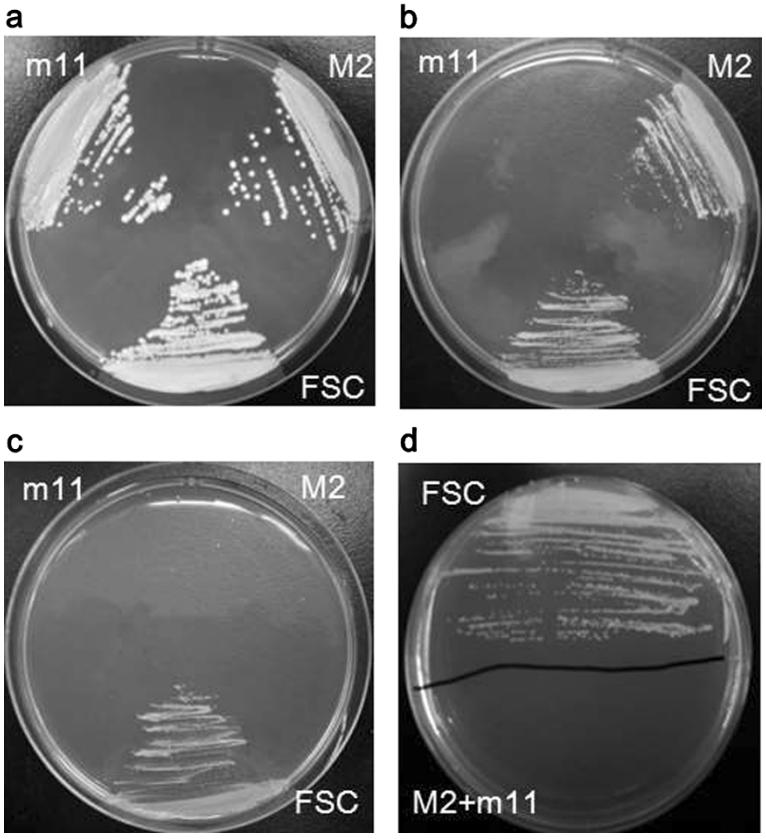

Figure 3 Colony formations of the M2, m11, and FSC1 strains on various YM agar media. (a) Colony formations of each strain on YMG, **(b)** YMFDOG, **(c)** YMXDOG, and **(d)** the mixed culture of M2 and m11 strains cultivated in advance and the FSC strain as a control on MMXDOG at 30°C for 3 days.

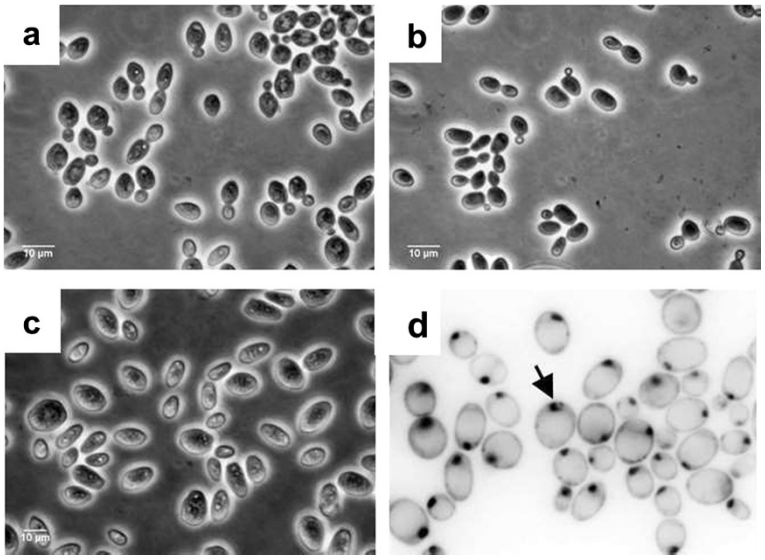

Figure 4 Microscopic observations of parental and fusant strains. (a) *S. cerevisiae* M2, **(b)** *C. intermedia* m11, and **(c)** FSC1 strains. Scale bars indicate 10 µm. **(d)** DAPI-stained image of the FSC1 strain. An arrow shows the representative position of nucleus inside the cell.

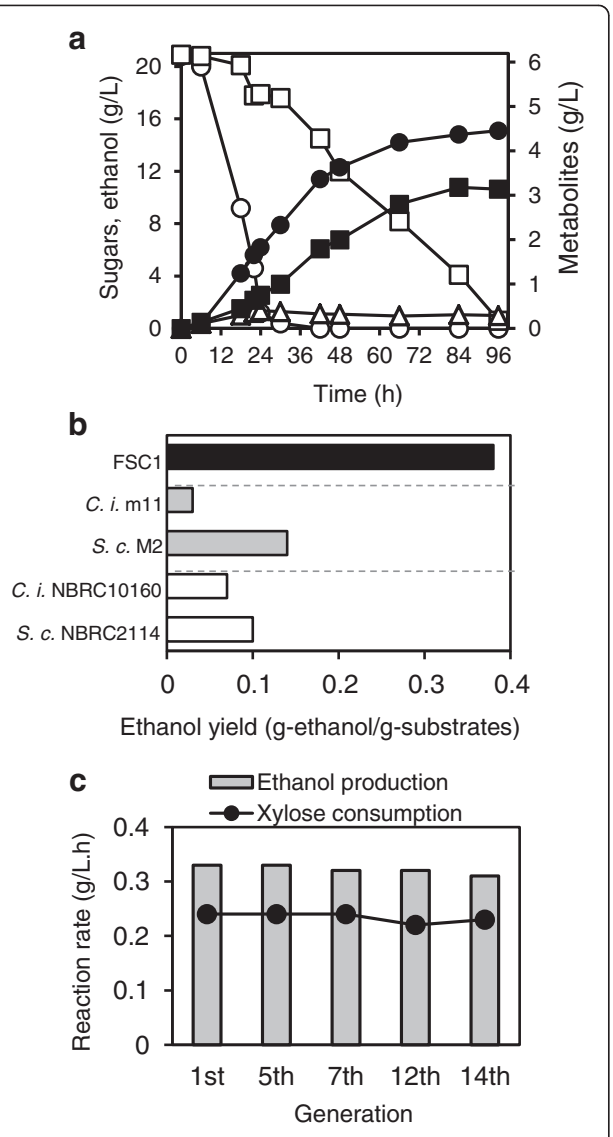

Figure 5 Fermentation by the fusant FSC1 strain using glucose and xylose as carbon sources. (a) Ethanol and other metabolites production by the FSC1 strain, (○) glucose, (□) xylose, (●) ethanol, (■) xylitol, (Δ) glycerol. **(b)** Ethanol yields of the FSC1 and parental strains. **(c)** Fermentation stability of the FSC1 strain.

With regard to total protein expression analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), most of the protein spots detected in the M2 and m11 strains were present at higher levels in the FSC1 strain (Figure 6b). Based on a search using Mascot, two of the proteins were considered to match to XR (score = 72, required score > 43, $p < 0.05$) and glyceraldehyde-3-phosphate dehydrogenase (TDH) (score = 130, required score > 59, $p < 0.05$).

Discussion

Here, intergeneric hybridization between the *S. cerevisiae* M2 and *C. intermedia* m11 strains was conducted by protoplast fusion. The resulting xylose-fermenting FSC1 strain was characterized in terms of xylose metabolism, protein expression and ethanol yield.

Fusants are generally less stable in metabolism because of the loss of non-homologous genes and chromosomes in chromosome segregations. To overcome this problem, we used haploid cells formed under suppression of mating by exogenous α factor in the M2 strain [19], anticipating a similar effect in the m11 strain, and performed electrofusion to attain more stable uninucleate polyploids [20] after chemical fusion using polyethylene glycol (PEG). Exogenous α factor inhibits mating when present in excess [19], though it is reported that prior activation of cells by α factor induces nuclear fusion [21]. In addition, we employed *C. intermedia* as a donator of the genes for xylose fermentation on the basis of a study reporting that genes and proteins necessary for xylose fermentation from *C. intermedia* can be functionally expressed in recombinant *S. cerevisiae* [15].

The FSC1 strain possesses high and stable rates of xylose fermentation and ethanol production from a substrate containing glucose and xylose. We consider that the mutation of the parental strains enabled their fusion to transfer genes for xylose fermentation of *C. intermedia* and for ethanol production of *S. cerevisiae*. Since the FSC1 strain was apparently uninucleate (Figure 4d), we confirmed that our mutation-fusion technique resulted in nuclear fusion of protoplasts, which is desirable since homocaryons are metabolically more stable than heterocaryons. Since the FSC1 strain produces spores in KAc medium for sporulation (Figure 2b, d and f), we believe that the FSC1 strain is diploid, resulting from fusion between M2 and m11

Table 1 Fermentation characteristics of FSC strains determined by batch fermentation in YMGX medium at 30°C under microaerobic condition

Strain	Cells†	Time*	R_{xylose}	υ_{EtOH}	Y_P (g g-substrate^{-1})		
	(g L^{-1})	(h)	(g g-cells^{-1} h^{-1})	(g L^{-1} h^{-1})	Ethanol	Xylitol	Cells
FSC1	1.3	96	0.18	0.33	0.38	0.08	0.06
FSC1 mutant	1.4	30	1.07	0.65	0.42	0.03	0.06

†, initial concentration; *, Time to completely consume sugars; R_{xylose}, Specific xylose consumption rate; υ_{EtOH}, Ethanol production rate; Y_P, Yield of products based on glucose and xylose added.

Table 2 Requirements of *S. cerevisae* M2, *C. intermedia* m11 and FSC1 strains on amino acids in MMG medium at 30°C

Media		Growth (+, grow; –, not grow)		
		S. c. M2	*C. i.* m11	FSC1
Minimal (MMG)		+++	-	+++
Enriched with amino acids		+++	+++	+++
Deficient in	Adenine	+++	++	+++
	Uracil	+++	-	+++
	L-Tryptophan	+++	+++	+++
	L-Histidine	+++	+	+++
	L-Methionine	+++	+++	+++
	L-Lysine	+++	+++	+++
	L-Leucine	+++	+++	+++

haploids. This is not contradictory to previous studies reporting that several *Candida* species have *MTL* loci with two idiomorphs, namely *a* and *α*, which are mating type-like loci similar to *MATa* and *MATα* of *S. cerevisiae* [22], although *Candida* is a large and heterogeneous taxon.

All genes necessary for xylose metabolism *xr*, *xdh*, and *xk* (*xks1*) were expressed at a higher level in FSC1 than in the parental strains (Figure 6a). Moreover, the level of total protein expression in FSC1 was also higher than a simple summation of the parental strains when the same amount of crude cell extracts were loaded on gel (Figure 6b), most likely due to the activation in FSC1 of numerous metabolic pathways during fermentation. RT-PCR and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) analysis indicated that XR derived from *C. intermedia* m11, and TDH from *S. cerevisiae* M2, were both expressed in FSC1. Since TDH is required for glycolysis and, by extension, cell viability [23], the expression of TDH in the FSC1 strain is important to convert xylose to ethanol via glycolysis. These results indicate that the FSC1 strain is an intergeneric hybrid between *S. cerevisiae* M2 and *C. intermedia* m11, a fact supported by the observation that normal mating of the parental strains did not occur (Figure 3d).

The metabolic properties of the FSC1 strain indicate that it maintains the redox balance required for fermentation inside the cell. Redox imbalance, which is thought to be caused by coenzyme specificity differences between heterogenic XR (with NADPH) and XDH (with NAD^+) enzymes, is a major cause of xylitol accumulation inside and outside the cell, resulting in the reduction of ethanol yield [24-26]. Since cell fusion allows the transfer of complete segments of genomic DNA from parental cells, a fusant will be rich in genetic information. We suggest that the higher expression of proteins in the FSC1 strain is not caused simply by the activation of metabolic pathways (PPP) for xylose fermentation, but by the presence of overall metabolic (glycolysis and PPP) regulation to maintain the redox balance for fermentation inside the cell. This is a key point behind the improved xylose utilization, which was supposed from the protein level.

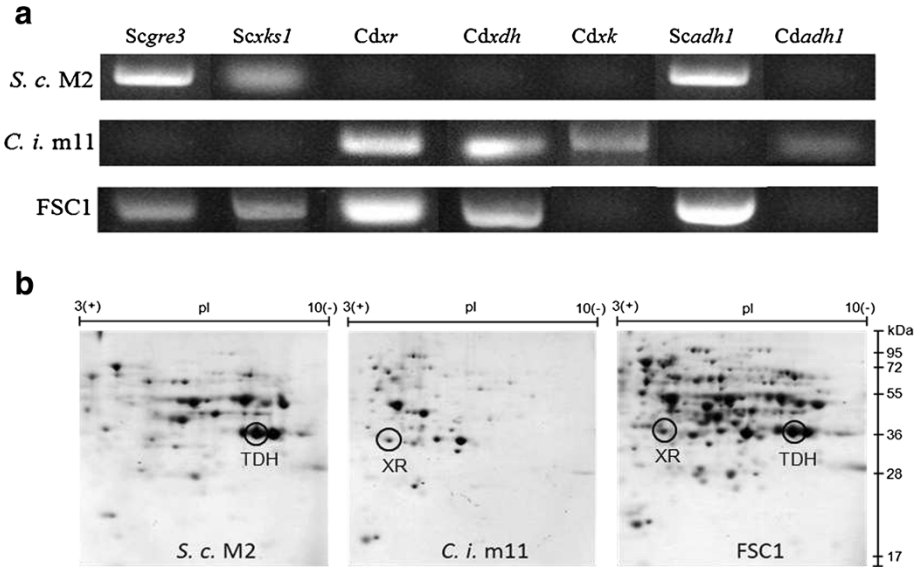

Figure 6 Expressions of genes for xylose fermentation analyzed by RT-PCR and proteins by 2D-PAGE. (a) Genes for xylose fermentation by *S. cerevisiae* M2, *C. intermedia* m11, and FSC1 strains microaerobically grown on glucose and xylose as carbon sources. **(b)** Protein spots of each strain (100 μg protein extracts on the gels), in which the horizontal axis is the isoelectric focusing dimension from pH3 to pH10 and the vertical axis is 15% (w/v) polyacrylamide gel dimension from 11 kDa to 250 kDa.

Although fermentation was conducted under micro-aerobic conditions, undesirable byproducts as glycerol and acetate were not produced. As summarized in Table 1, the ethanol yield and production rate of the FSC1 strain were 0.38 g/g-substrate, corresponding to 75% theoretical yield, and 0.33 g/L · h in fermentation of the mixture of glucose and xylose, respectively. In addition, the fermentation ability of FSC1was further improved by successive mutation, achieving a higher ethanol yield (0.42 g/g-substrate). The ethanol yields of the FSC strains are comparable to 0.34 g/g-substrate of engineered strains of the recombinant *S. cerevisiae* TMB3400, generated by introducing the gene for xylose transporter from *C. intermedia* [27], in a mixture of xylose and glucose, and 0.05-0.46 g/g-substrate in fermentation of xylose as a sole carbon source by various recombinant *S. cerevisiae* strains [12]. Although the xylose consumption rate (1.07 g/g-cell. h, i.e., 7.1 mmol/g-cell. h) of FSC1 mutant was lower than those with previous reports such as the recombinant *S. cerevisiae* TMB3400 [27], the substrate was completely consumed within 30 h, shorter than a normal reaction time 48 h in ethanol fermentation for practical use. We believe that the mutation-fusion technique developed in this study is applicable for metabolic alteration of ethanol producing yeasts as a diverse method from recombination.

Conclusions

We developed a novel xylose-fermenting yeast strain, FSC1, for ethanol production by intergeneric hybridization between *Saccharomyces cerevisiae* and *Candida intermedia* mutants by using a protoplast fusion technique. The fermentation ability of the FSC1 strain was further improved by chemical mutation. The mutation-fusion technique we have described is useful for the development of an intergeneric fusant capable of xylose fermentation. The FSC strains obtained by this technique hold the potential for ethanol production from globally abundant lignocellulosic biomass.

Methods

Strains and cultivation media

Wild-type yeast strains *S. cerevisiae* NBRC 2114 and *C. intermedia* NBRC 10601 were obtained from the NITE Biological Resource Center (NBRC) at the National Institute of Technology and Evaluation (NITE) in Tsukuba, Japan. *C. intermedia* NBRC 1060 was originally designated as a *K. cellobiovorus* strain that was reported to be capable of producing ethanol from xylose in 1984 [16,17] but was later classified as a neotype of *C. intermedia* (Ciferri & Ashford) Langeron et Guerra [28]. *S. cerevisiae* is taxonomically classified into *Saccharomycetaceae* in family and *C. intermedia* NRRL Y-981 belongs to the *Metschnikowia* clade [29]. Because *S. cerevisiae* and *C. intermedia* belong to different taxonomical families, experiments on cell fusion were conducted taking the containment measures confirmed by the competent minister under the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Act No. 97 of 2003, Japan).

The cultivation media were YM medium and minimal medium (MM). YM medium containing 5 g/L Bacto peptone, 3 g/L Bacto yeast extract, and 3 g/L Bacto malt extract was supplemented with the following carbon sources: 10 g/L glucose (YMG liquid); 10 g/L glucose with 20 g/L Bacto agar (YMG); 10 g/L xylose and 5 g/L DOG with 20 g/L Bacto agar (YMXDOG); and 10 g/L fructose and appropriate amounts of DOG with 20 g/L Bacto agar (YMFDOG). MM agar containing 1.7 g/L yeast nitrogen base (without amino acids) with 5 g/L ammonium sulfate and 20 g/L Bacto agar was also prepared with carbon sources of 10 g/L xylose and 5 g/L DOG (MMXDOG). DOG was used for screening DOG-sensitive or DOG-tolerant mutants. MMXDOG and YMXDOG were used for screening the target strain in protoplast fusion. In fermentation tests, YM and MM liquid were supplemented with carbon sources of glucose and xylose at 10 g/L each for seed preparation (YMGX) and at 20 g/L each for fermentation (MMGX). Minimal liquid medium supplemented with 10 g/L glucose (MMG) was also used for the evaluation of amino acid requirements for growth.

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where mentioned otherwise.

Mutation and screening

To obtain a variety of species of different phenotypes for screening target mutants, *S. cerevisiae* and *C. intermedia* strains were individually mutated using EMS (Wako Pure Chemicals, Ltd., Osaka, Japan) as described in our previous study [6]. The strain was grown at 30°C for 6 h in 50 mL of YM medium in 1 L of distilled water. Next, a 1-mL aliquot of the cell suspension was transferred to a 1.5-mL tube. After centrifugation, the cell pellets were washed twice with cold 0.1 M sodium phosphate buffer (pH 7.0), then suspended in 1 mL of the same buffer. EMS (30 μL, purity ≥ 98%) was added to the suspension, giving a final volume of EMS at nearly 3% (v/v). The tube was incubated at 30°C on a roller shaker for 60 min. The reaction was stopped by adding 10% (w/v) sterile sodium thiosulfate solution at a final concentration of 1% (v/v), and the suspension was centrifuged at 5,000 × *g* for 1 min. After removal of the supernatant, 1 mL of 10% (w/v) sterile sodium thiosulfate solution was added to the pellet, and the suspension was mixed and incubated at room temperature for 15 min to completely terminate the reaction.

After mutation, the mutant cells were washed with 0.1 M sodium phosphate buffer (pH 7.0) three times,

and 100-μL aliquots of the suspension were spread on YMFDOG containing 3 g/L DOG. Replica plates were also prepared on YMG, and the plates were incubated at 30°C for colony formation. To screen a DOG-tolerant mutant (named the M2 strain) from *S. cerevisiae* strain, colonies that appeared were replicated on YMFDOG containing 5 g/L DOG and then screened after incubation at 30°C for 3 days. To screen a DOG-sensitive mutant (named the m11 strain) from *C. intermedia*, colonies that were unable to grow on YMFDOG containing 3 g/L DOG, were selected from the replica plates. Growth of both mutant strains was evaluated on YMG.

Protoplast fusion and regeneration

Before protoplast fusion, M2 and m11 strains were separately plated onto agar medium containing 1 g/L Bacto yeast extract, 0.5 g/L glucose, 10 g/L KAc, and 20 g/L Bacto agar, and incubated for 24 h for sporulation. A lump of asci containing haploid spores in each strain was cultivated at 30°C for 2–4 h in YMG liquid medium containing 2 mg/L α factor to inhibit mating in M2 strain, with expectation of a similar effect in the m11 strain. Nuclear fusion of *S. cerevisiae* requires prior activation by α factor, leading to arrest in the G1 portion of the cell cycle, in conjugation [21], but exogenous α factor inhibits mating when present in excess [19]. Haploid cells in early logarithmic growth phase (α cells) were collected by centrifugation at 5,000 × *g* for 10 min. Cell pellets were then treated with 20 mM Tris/HCl buffer (pH 7.5) containing 1% (v/v) β-mercaptoethanol and 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) for 10 min.

After centrifugation at 5,000 × *g* for 10 min, the cell pellets were suspended in 20 mM phosphate citrate buffer (pH 6.5) containing 0.6 M sorbitol, 0.6 M KCl, and 10 mM β-mercaptoethanol. Zymolyase 20-T (Takara Bio Inc., Shiga, Japan) was added into the mixture at a final density of 20 mU/mg-cell, and cell pellets were incubated at 30°C for 1 h to obtain protoplasts. Each protoplast was suspended for purification in equal amounts of 20 mM phosphate citrate buffer (pH 6.5) containing 30% (w/v) $MgSO_4$ and 20 mM Tris–HCl buffer (pH 7.5) with 1 M Sorbitol and 0.1 M EDTA, and collected by centrifugation at 3,000 × *g* for 10 min. Protoplast fusion was carried out by mixing purified protoplasts of both strains at a 1:1 ratio in 1 mL fusion buffer containing 1.2 M sorbitol, 30% (w/v) PEG4000, 0.1 M calcium propionate, 10 mM Tris/HCl (pH 7.2), 1 g/L bovine serum albumin (BSA), and 15% (v/v) dimethyl sulfoxide (DMSO). The suspension was incubated at 30°C for 1 h to enable protoplast fusion, transferred to an electrophoresis apparatus, then exposed to direct current at 50 V for two seconds in triplicate to ensure complete protoplast fusion.

To screen for strains tolerant to DOG, fused protoplast cells were suspended in regeneration medium containing 1.2 M sorbitol, 5× YM (YM enriched with 5-fold additions of Bacto peptone, Bacto yeast extract, and Bacto malt extract), 2% glycerol, 0.6 M potassium chloride, 1 mg/mL colchicine and 5 g/L DOG, and incubated at 28°C for 6–8 h. Colchicine was used as a mitosis inhibitor [30] for fused protoplast cells, although it has been reported that colchicine does not bind with *S. cerevisiae* tubulin [31]. Partially regenerated cells were collected by centrifugation at 3,000 × *g* for 10 min and suspended within 1.5% (w/v) soft agar containing 0.4 M calcium propionate, and then immediately layered onto MMXDOG containing 0.6 M potassium chloride, to allow the complete regeneration of fused protoplast cells.

Seed preparation and fermentation

Seed preparation for fermentation was performed in two steps. In the first step, a single colony of the yeast was inoculated into 100 mL of YMG liquid medium in a 500-mL flask after autoclaving at 122°C for 20 min, and then incubated at 30°C overnight on a shaker at 150 rpm. In the second step, 10 mL of the first seed were transferred in quadruplicate to 100 mL of YMGX liquid medium in a 500-mL flask, which was then incubated at 30°C for 24 h on a shaker at 150 rpm. After collecting and washing twice with sterile phosphate-buffered saline (PBS), cells were resuspended in PBS in a minimal volume (50 ml).

Fermentation was started by adding 50 mL of cell suspension containing 1.3–1.5 g-dried weight (DW) of cells to 950 mL of MMGX medium in a jar fermenter. 5 N NaOH was used to maintain pH 5 in the culture. To maintain microaerobic conditions, air was pumped through a sterilized membrane filter into the reactor to maintain 5% dissolved oxygen under the air-saturated condition. Levels of glucose, xylose, xylitol, glycerol, and ethanol in the culture medium were quantified by high performance liquid chromatography (HPLC) as described in the previous study [6]. Stability of fermentation by fusant cells was confirmed in both ethanol production and xylose consumption over 14 generations. Generation in this context refers to the culture of glycerol stock cells obtained from one single colony appearing on appropriate cultivation agar YMGXDOG.

Gene and total protein expression analyses

Cells were harvested from the jar fermenter directly after depletion of the carbon sources, washed with cold sterile water twice and then freeze-dried. Freeze-dried cells (100 mg) were suspended in 250 μL Yeast protein extraction reagent (Y-PER) supplemented with 5 μL protease inhibitor (Wako Pure Chemicals, Ltd., Osaka, Japan),

incubated for 60 min, then centrifuged at 14,000 × g for 10 min. To remove excess salts, the supernatant was passed through a desalting column Bio-Gel P-6 (BioRad Lab., Inc., Hercules, CA) buffered with 10 mM Tris/HCl (pH7.4), and the eluate was used as a protein mixture sample for total protein analyses.

To confirm the expression levels of genes related to xylose utilization (*xr*, *xdh*, and *xk*) and conversion of acetaldehyde to ethanol (*adh1*) in M2, m11, and fusant cells, RT-PCR analyses were performed. Cells were microaerobically cultivated for 18 h then collected by centrifugation at 5,000 × *g* for 10 min at 4°C. Collected cells were washed twice with cold sterile water and immediately freeze-dried. After mechanical disruption with a sample grinding kit (GE Healthcare, Inc., Uppsala, Sweden), total RNA was extracted from cells (30 mg-cell DW) using NucleoSpin RNA II (Macherey-Nagel GmbH & Co. KG, Düren, Germany). mRNA was purified from total RNA using Oligotex-dT30 < super > mRNA purification kit (Takara Bio, Inc.). PCR primers were designed on the basis of NCBI databases of genomic DNA from *S. cerevisiae* (Sc) and *Candida* species (Ci) as follows: (forward) 5′-CCATCCAGGCAGTAC CACTT-3′ and (reverse) 5′-TACCATCCAACCAGG TCCAT-3′, for Sc-*gre3* (Accession no. CR382125.1); (forward) 5′-GGCTCAATTAACAGGGTCCA-3′ and (reverse) 5′-ACAGGCATCTGCCTCCTCTA-3′, for Sc-*xks1* (Accession no. NC_006041.1); (forward) 5′-AGGCCAA CGAATTGTTGATCA-3′ and (reverse) 5′-GTGTCAA CAACGTATCTACCA-3′, for Sc-*adh1* (Accession no. NC_006042.1); (forward) 5′-CCTGCTGTTTTGCAAG TTGA-3′ and (reverse) 5′-CTCTTTGAGCGGACCATC TC-3′, for Ci-*xr* (Accession no. AF278715.1); (forward) 5′-AATGGTCTTGGGTCACGAATCC-3′ and (reverse) 5′-GCTCTGACCAAGTCGTAGGCTTC-3′, for Ci-*xdh* (Accession no. JN578088.1); (forward) 5′-GGATTCG ACTTATCCACCCAACAA-3′ and (reverse) 5′-CCA GTACACGGATCCATGTTG-3′, for Ci-*xk* (Accession no. FM992691.1); (forward) 5′-CACTCACGATGGTT CATTCG-3′ and (reverse) 5′-AAGATGGTGCGACA TTGG-3′, for Ci-*adh1* (Accession no. KC236900.1). RT-PCR amplification of purified mRNA was carried out using a One Step PrimeScript RT-PCR Kit (Takara Bio, Inc.). RT-PCR gene products were separated electrophoretically in a 1% (w/v) TAE agarose gel and viewed using a UV transilluminator.

For total protein expression, the protein extract of each strain was analyzed by 2D-PAGE as previously reported [32]. Isoelectric focusing (IEF) electrophoresis of desalted protein samples was performed using IPG ReadyStrip pH3-10 NL (BioRad Lab., Inc.) conditioned in Protean IEF system (BioRad Lab., Inc.). We prepared the crude cell extracts adjusted to 100 μg respectively from M2, m11 and FSC1 strains, under the same procedure. After treatment in an alkylation solution containing 100 mM iodoacetate, 6 M urea, 2% (w/v) sodium dodecyl sulfate (SDS) and 20% (v/v) glycerol in 0.375 M Tris/HCl (pH 8.8), the IPG strip was applied to a 15% non-gradient SDS-PAGE electrophoresis. Finally, the developed gel was stained using a sensitive colloidal Coomassie G-250 solution to observe significant changes of total protein expression.

Protein spots were identified by MALDI-TOF/TOF analyses. Spots were enzymatically digested in a manner similar to that previously described [33] using modified porcine trypsin (Promega Corp., Madison, WI). Gel pieces were washed with 50% (v/v) acetonitrile to remove SDS, salt, and stain. Washed and dehydrated spots were then vacuum-dried to remove solvent and rehydrated with trypsin (8–10 ng/μL) solution in 50 mM ammonium bicarbonate at pH 8.7 and incubated for 8–10 h at 37°C. The samples were analyzed using an Applied Biosystems 4700 proteomics analyzer with TOF/TOF ion optics (Genomine Inc. Pohang, Korea). Sequence tag searches were performed using Mascot search (http://www.matrixscience.com).

Microscopic observation

For microscopic observation of sporulation, each strain was sporulated using KAc agar containing 10 g/L KAc and 20 g/L Bacto agar for 7 days at 30°C in the manner described earlier. After incubation, spores were removed from the surface of the agar medium by washing with 0.05% Tween 80 in saline solution. The suspension was centrifuged at 3,000 × g for 10 min at 4°C. The supernatant was transferred into fresh tube and kept at 4°C until use. To confirm sporulation using the Wirtz-Conklin spore staining technique [34], the spores were strained with 5% brilliant (malachite) green (dye for staining spores) solution on a slide, heated with a Bunsen flame for 5 min and washed with MilliQ water, then counterstained with 0.5% safranin (dye for staining vegetative cells) solution for 1 min. Staining was conducted before and after swelling the cell by soaking in saline solution for a few days. After drying, the slide was observed under a light microscope. SEM observation was also performed to confirm the morphology of ascospores from each strain. The spores were fixed overnight at 4°C with 0.1% (vol./vol.-PBS buffer) glutaraldehyde, washed three times with PBS buffer, dehydrated in an ethanol series, then dried at room temperature. After coating with Pt-Pd using a sputter coater (Hitachi E102 Ion Sputter, Hitachi, Tokyo, Japan) for 2 min at DC20 mA, spore samples were observed with SEM (Hitachi S-4700 Type II, Hitachi, Tokyo, Japan) at 10 kV.

For microscopic observation of cell fusion, cells were harvested after 18 h cultivation under microaerobic conditions in YMG liquid medium. After washing with sterile 20 mM Tris/HCl (pH 8.0) twice, the cells were appropriately diluted in 20 mM Tris/HCl (pH 8.0) and placed on

acid-washed slide glasses. For DAPI staining, fusant cells on glass slides were covered with DAPI mounting solution containing 2 mg/mL DAPI (Lonza, Basel, Switzerland), 0.2 M 1,4-diazabicyclo-2,2,2-octane and 90% (v/v) glycerol in 20 mM Tris/HCl (pH 8.0).

Abbreviations

XR: Xylose reductase; XDH: Xylitol dehydrogenase; XK: Xylulokinase; PPP: Pentose phosphate pathway; DOG: 2-deoxyglucose; EMS: Ethyl methane sulfonate; KAc: Potassium acetate; SEM: Scanning electron microscope; DAPI: 4′,6-diamidino-2-phenylindole; RT-PCR: Reverse transcription polymerase chain reaction; 2D-PAGE: Two-dimensional polyacrylamide gel electrophoresis; TDH: Glyceraldehyde-3-phosphate dehydrogenase; PEG: Polyethylene glycol; MALDI-TOF/TOF: Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight; EDTA: Ethylenediaminetetraacetic acid; PBS: Phosphate-buffered saline; DW: Dried weight; HPLC: High performance liquid chromatography.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PK carried out the experiments and drafted the manuscript. ST conceived and designed the study as a research representative, and completed the manuscript. Both authors read and approved the final manuscript.

Authors' information

PK was a researcher of the Laboratory of Water Environment and Bioenergy at Meisei University, when the study was conducted from 2010–2012. ST is a professor of the laboratory and was a representative of the study.

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Author details

[1]Department of Chemical Science and Engineering, Faculty of Engineering, Kobe University, 1-1 Rokkodai, Nada-Ku, Kobe 657-8501, Japan. [2]Program in Environment and Ecology, Department of Interdisciplinary Science and Engineering, Faculty of Science and Engineering, Meisei University, 2-1-1 Hodokubo, Hino, Tokyo 191-8506, Japan.

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