Direct Fermentation of Raw Starch Using a *Kluyveromyces marxianus* Strain that Expresses Glucoamylase and Alpha-Amylase to Produce Ethanol

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Raw starch and raw cassava tuber powder were directly and efficiently fermented at elevated temperatures to produce ethanol using the thermotolerant yeast Kluyveromyces marxianus that expresses α-amylase from Aspergillus oryzae as well as α-amylase and glucoamylase from Debaryomyces occidentalis. Among the constructed K. marxianus strains, YRL 009 had the highest efficiency in direct starch fermentation. Raw starch from corn, potato, cassava, or wheat can be fermented at temperatures higher than 40°C. At the optimal fermentation temperature 42°C, YRL 009 produced 66.52 g/L ethanol from 200 g/L cassava starch, which was the highest production among the selected raw starches. This production increased to 79.75 g/L ethanol with a 78.3% theoretical yield (with all cassava starch were consumed) from raw cassava starch at higher initial cell densities. Fermentation was also carried out at 45 and 48°C. By using 200 g/L raw cassava starch, 137.11 and 87.71 g/L sugar were consumed with 55.36 and 32.16 g/L ethanol produced, respectively. Furthermore, this strain could directly ferment 200 g/L nonsterile raw cassava tuber powder (containing 178.52 g/L cassava starch) without additional nutritional supplements to produce 69.73 g/L ethanol by consuming 166.07 g/L sugar at 42°C. YRL 009, which has consolidated bioprocessing ability, is the best strain for fermenting starches at elevated temperatures that has been reported to date. © 2014 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:338–347, 2014
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Introduction

Bioethanol has attracted increasing attention because fossil fuel sources are being depleted. Starch is widespread and naturally occurring in organic compounds obtained from higher plants. Traditionally, alcoholic fermentation from raw starch can be separated into three individual processes. First, raw starch is gelatinized at 60 to 80°C or higher temperature[1]; then, it is liquefied by α-amylase and saccharified to glucose by glucoamylase (GAM1).[2] Finally, the glucose will be converted to ethanol by industrial yeasts, such as *Saccharomyces cerevisiae*.

To reduce the cost of the liquefaction of starch and exogenous hydrolytic enzymes, hydrolyzing starch, and subsequently using sugar in a single step (consolidated bioprocessing, CBP)[3] could render the process more cost-effective. Actually, more than 150 yeasts have been found to be capable of directly using starch; however, most of them are not suitable for ethanol production because of their low-tolerance for ethanol[4] and their slow fermentation rates. Heterologous amylolytic enzymes, which are expressed in *S. cerevisiae*, have been widely used in the ethanol fermentation industry to construct CBP strains. Alpha-amylase (AMY) and GAM1 from fungi and yeasts were successfully expressed in *S. cerevisiae*.[5–7] The AMY and GAM1 from

Additional Supporting Information may be found in the online version of this article.

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Table 1. Primers Used in This Study

Primer	Sequence (5′-3′)*
AoAMY-EcoRI-F	ACGTGAATTCATGATGGTCGCGTGGTGGTCTC
AoAMY-NotI-R	ATAAGAATGCGGCCGCTTACGAGCTACTACAGATCTTGCTACC
DoAMY-EcoRI-F	ACGTGAATTCATGAGATTTTCAACTGAAGGATTTAC
DoAMY-NotI-R	ATAAGAATGCGGCCGCTTACTATTGATTGCAGATGCCAGATCCCGAG
DoGAM1-EcoRI-F	ACGTGAATTCATGATTTTTCTGAAGCTGATTAAAAG
DoGAM1-NotI-R	ATAAGAATGCGGCCGCTTACCAAGTAATGGTGAAATCCTTAG
GAP-StuI-F	GAGAAGGCCTACCAGTTCTCACACGGAACACCAC
GAP-StuI-R	GAGAAGGCCTTCAATGAATCGAAAATGTC
Km26sRNA-F	CCAGTTATTTGTAAAGTGCTTTC
Km26sRNA-R	ACAAATCAGACAACGAAGGCTTAATC
RT-KmLAC4-F	TCTTGCCTTATTCCTGAGAAT
RT-KmLAC4-R	AAGACGTGCATCAAACAACGCAAAAG
RT-AoAMY-F	CGTGGTGGTCTCTATTTCTGTACGG
RT-AoAMY-R	CACCACAGTATTTCCGATCCGCAG
RT-DoAMY-F	CAACTGAAGGATTTACAAGTAAAG
RT-DoAMY-R	CAGATCTGGCAAACCTATCAGTAACG
RT-DoGAM1-F	AATTGGTTTGGGATTAGTTAGTG
RT-DoGAM1-R	GCAGAGTCATTAAAGATATTAGG

*Restriction enzyme sites are underlined.

D. occidentalis show remarkable debranching activity[8] and a high expression level in *S. cerevisiae*. Many engineered yeast strains that express AMY and GAM1 genes from *D. occidentalis* can produce ethanol from starch.[6,9,10]

Most of the previous studies of CBP reported that the optimal temperature of the exogenous amylolytic enzymes was ∼50°C, while the optimal growth and fermentation temperature of most starch fermentation microorganisms is between 28 and 40°C.[11] Consequently, thermotolerant yeasts that can produce ethanol at elevated temperatures have received attention. Previous studies showed that the thermotolerant yeast *Kluyveromyces marxianus* can grow at 52°C and shows a high growth rate at 40°C (0.86–0.99 h^{-1}).[12,13] Although *K. marxianus* cannot use starch directly, it can ferment glucose at elevated temperatures (45°C) with a high productivity (∼2 g/L/h).[14]

Cassava is the world's fourth important crop and the cheapest source of calories for both human nutrition and animal feeding,[15] which is grown in Africa, Asia, and Latin America. The starch content in fresh cassava is >30%, and 3.0 tons of dry cassava or 7.2 tons of fresh cassava has been estimated to produce 1 ton of fuel ethanol.[16] In the case of dry corn, it also needs 3 tons to produce the same amount of fuel ethanol.[17] In this study, nonsterile cassava tuber powder without additional nutritional supplements was used to evaluate the CBP ability of constructed yeast.

Alpha-amylase genes (*amy*) form *A. oryzae*[18,19] and *D. occidentalis*, together with the glucoamylase gene (*gam1*) from *D. occidentalis* were expressed in *K. marxianus* YHJ 010. The resultant strain could assimilate and ferment raw starch and cassava tuber powder at elevated temperatures better than previously reported thermo-tolerant yeasts.

Materials and Methods

Chemicals and strains

All chemicals were reagent grade or higher and purchased from Sangon Biotech (Shanghai, China) unless otherwise noted. Yeast extract, peptone, and tryptone were purchased from Oxoid (Cambridge, UK). All restriction enzymes and T4 DNA Ligase were obtained from Thermo Fisher Scientific (MA). PrimeStar HS DNA polymerase was procured from TAKARA Biotechnology (Dalian, China). Protease inhibitor cocktail was from Sangon Biotech (Shanghai, China). *Escherichia coli* XL10 gold was used for all DNA manipulations. Luria–Bertani (LB) medium with 100 μg/mL ampicillin and low-salt luria-bertani (LSLB) containing 25 μg/mL zeocin was used to cultivate *Escherichia coli*. *K. marxianus* YHJ 010 [*Kmura3::Kan*^{r} *Kmleu2::hisG, Kmtrp1::hisG* auxotrophic strain derived from *K. marxianus* NBRC 1777 (NITE Biological Resource Center, Japan)] was cultivated on solid or in liquid YPD media (Yeast extract 10 g/L, peptone 20 g/L, and glucose 20 g/L). YPD plates containing 400 μg/mL zeocin or synthetic dropout medium (SD; Yeast nitrogen base 6.7 g/L, glucose 20 g/L) with appropriate amino acids[20] were used to screen the yeast transformants. YPS medium containing 20 g/L soluble starch was used to measure the starch utilization ability of the strains under aerobic conditions. YPS medium containing 200 g/L soluble starch or raw starch (corn, potato, wheat, or cassava) was used for anaerobic fermentation. 0.5 g/L potassium disulfide was added as a bacteriostatic agent during the raw starch fermentation. All experiments were carried out in triplicates and the standard differences were calculated.

Construction of plasmids and yeast transformation

The α-amylase gene from *A. oryzae* (*Aoamy*) and *D. occidentalis* (*Doamy*) as well as the glucoamylase gene from *D. occidentalis* (*Dogam1*) were amplified using primers AoAMY-EcoRI-F, AoAMY-NotI-R, DoAMY-EcoRI-F, DoAMY-NotI-R, DoGAM1-EcoRI-F, and DoGAM1-NotI-R (Table 1). The genes were inserted into the plasmid YEGAp at the *Eco*R I and *Not* I sites to construct plasmids YEGAp-AoAMY, YEGAp-DoAMY, and YEGAp-DoGAM1 (Table 2). Subsequently, an expression cassette including the GAPDH promoter, *Doamy* and the GAPDH terminator was amplified by PCR using the primers GAP-StuI-F and GAP-StuI-R (Table 1) from YEGAp-DoAMY and inserted into pKMURA3[21] at the *Stu* I site. The *Aoamy* gene expression cassette was also amplified by the primers GAP-StuI-F and GAP-StuI-R from YEGAp-AoAMY and inserted into pKMLEU2[21] at the blunted *Bam*H I site. To construct an integrative expression plasmid of *Dogam1*, the *Km26SrRNA* gene was amplified by the primers Km26sRNA-F and Km26sRNA-R (Table 1) and inserted into the blunted plasmid pGAPZαA, which was digested by *Bgl* I and *Bam*H I to

Table 2. Yeast Strains and Plasmids Used in This Study

	Description	Reference
Plasmids		
YEGAp	*Sctrp1* in pUC19	21
pKMLEU2	*Kmleu2* in pGEMT-easy	21
pKMURA3	*Kmura3* in pUC19	21
pZ-26sRNA	*Km26sRNA* in pGAPZaA	This study
YEGAp-AoAMY	*Aoamy* under GAPDH promoter	This study
YEGAp-DoAMY	*Doamy* under GAPDH promoter	This study
YEGAp-DoGAM1	*Dogam1* under GAPDH promoter	This study
pKMLEU2-AoAMY	*Aoamy* expression	This study
pKMURA3-DoAMY	*Dogam1* expression	This study
pZ-26sRNA-DoGAM1	*Dogam1*expression	This study
Strains		
YHJ 010	Δ*Kmura3::Kan*^{r}Δ*Kmleu2::hisG*, Δ*Kmtrp1::hisG*	21
YRL 006	Δ*Kmura3::Kan*^{r} Δ*Kmleu2::hisG*, Δ*Kmtrp1::hisG Kmleu2::Aoamy*	This study
YRL 007	Δ*Kmura3::Kan*^{r} Δ*Kmleu2::hisG*, Δ*Kmtrp1::hisG Kmleu2::Aoamy, Kmtrp1:: Dogam1*	This study
YRL 008	Δ*Kmura3::Kan*^{r} Δ*Kmleu2::hisG*, Δ*Kmtrp1::hisG Kmleu2::Aoamy, Sctrp1:: Dogam1 Kmura3::Doamy*	This study
YRL 009	Δ*Kmura3::Kan*^{r} Δ*Kmleu2::hisG*, Δ*Kmtrp1::hisG Kmleu2::Aoamy, Kmtrp1:: Dogam1 Kmura3::-Doamy, 26sRNA::zeo^r-Dogam1*	This study

construct pZ-26sRNA. Then, *Dogam1* expression cassette was amplified by the primers GAP-StuI-F and GAP-StuI-R from YEGAp-DoGAM1 and inserted into the *Eco*R I-digested and blunted pZ-26sRNA. The three constructed expression plasmids were named pKMLEU2-AoAMY, pKMURA3-DoAMY, and pZ-26sRNA-DoGAM1 and contained the selection genes *Leu2*, *Ura3,* and *Zeo*^{R}, respectively.

The yeast was integratively transformed by the lithium acetate method, as described by Zhang et al.[22] pKMLEU2-AoAMY was linearized by *Ale* I and transformed into the *K. marxianus* YHJ 010 strain to construct YRL 006 (Table 2). YEGAp-DoGAM1 was linearized by *Sca* I and transformed into the YRL 006 to construct YRL 007. pKMURA3-DoAMY was linearized by *Alp* I and transformed into the YRL 007 to construct YRL 008. pZ-26sRNA-DoGAM1 was linearized by *Apa* I and transformed into YRL 008 to construct YRL 009. The transformants of YRL 006, YRL 007, and YRL 008 were selected in SD medium, while YRL 009 was obtained on YPD plates containing 1000 μg/mL zeocin for multicopy *Dogam1* integration after the prescreening on YPD plates containing 400 μg/mL zeocin.

Starch assimilation of constructed strains at elevated temperatures

To evaluate the starch assimilation ability, the growth was determined using soluble starch as the carbon source. The yeasts were cultivated aerobically in 250 mL flasks containing 20 g/L soluble starch in 50 mL YPS medium for 36 h with a starting OD_{600} of 0.2 at 40, 42, 45, and 48°C. Samples were taken every 4 h to measure the OD_{600}.

Preparation of Cassava powder and starch composition analysis

Cassava tubers were obtained from the market of Guangxi province in China and dried at 70°C. The tubers were then grounded and passed through 100 mesh sieve (diameter of particle in the powder <152 μm). The starch content in the cassava was determined by the acid hydrolysis method[23] with some modifications. Two-hundred milligrams of dry cassava powder were hydrolyzed with 1 mL of 1 M HCl in boiling water for 3 h and neutralized with 1 mL of 1 M NaOH. The amount of reducing sugar in hydrolyzate was then determined by the dinitrosalicylic acid method.[6]

Ethanol fermentation from soluble starch, raw starch, and cassava tuber powder

K. marxianus YRL 007, YRL 008, or YRL 009 were precultured in 250 mL flasks with 50 mL YPS medium containing 20 g/L soluble starch at 37°C for 24 h and then transferred into 80 mL YPS, which contained 30 g/L or 200 g/L soluble starch, or 200 g/L raw starch (corn, tomato, wheat, and cassava) or 200 g/L raw cassava tuber (containing 178.52 g/L cassava starch) with an initial cell density of 5 g/L wet cells in a 100-mL anaerobic bottle supplied with a butyl rubber stopper (Huakai Glassware, Jiangsu, China). The raw starch did not require pretreatment, such as sterilization, gelatinization, and saccharification, in these series of experiments. Specifically, 200 g/L cassava tuber powder was directly added to the anaerobic bottle at nonsterile conditions without additional nutrient supplements. The fermentations were conducted by shaking the flasks at 250 rpm at 40, 42, 45, or 48°C, and samples were taken every 24 h.

Ethanol, glucose, and total sugar analysis

After the samples were collected and centrifuged, the concentration of ethanol and glucose in the supernatant was detected by HPLC using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex, Torrence) with an Agilent 1200 refractive index detector (Agilent Technologies, CA). The system was operated at 75°C with a flow rate of 0.3 mL/min using 2.5 mM H_2SO_4 as the mobile phase. The presence of residual sugar was determined via the phenol sulfuric acid method.[10] The ethanol yield is defined as the amount of ethanol produced per gram of consumed sugar, which was calculated through subtracting the amount of residual sugar from input starch. The ethanol productivity was defined as the amount of ethanol produced per hour.

Table 3. Comparison of Raw Starch Fermentation Among Recombinant Yeast Strains

Starch (g/L)	Temperature (°C)	Initial cell density*	Ethanol (g/L)	Ethanol productivity (g/L/h)	Ethanol yield (g/g)	Time (h)	α-amylase activity (U/mL)	GAM1 activity (U/mL)	Strains	Reference
200	30	10% (v/v)	80.9	0.56	0.51	144	0.62 ± 0.03	5.35 ± 0.35	*S. cerevisiae* ATCC 9763/YIpdAGSAδ	6
150	30	50 g wet cell/L	70	0.97	0.47	72	1.12 ± 0.07	0.33 ± 0.01	*S. cerevisiae* MNIV/δGS	31
100	30	50 g wet cell/L	39	0.46	0.44	84	0.95	0.29	*S. cerevisiae* MN8140SS	7
50	30	OD_{600}~20	18.5	0.39	0.37	48	NA	~9×10^{-9} U/cell	*S. cerevisiae* GRI-117-UK	32
100†	30	3.33 g dry cell/L	51	0.85	0.49†	60	160 U/g of dry cells	19 U/g of dry cells	*S. cerevisiae* YF237-SystemA	33
200	30	50 g wet cell/L	93.3	0.71	~0.49	132	114 U/g of wet cells	57 U/g of wet cells	*S. cerevisiae* YF207/ pGA11/pUFLA	10
30	48	2 g cell/L	10	0.14	0.33	72	~0.45	~1.8	*H. polymorpha* ##3/4	34
200	40	5 g wet cell/L	70.13 ± 1.08	0.37	0.43	192	11.73 ± 0.26	13.64 ± 0.97	YRL 009	This study
200	42	5 g wet cell/L	72.99 ± 1.84	0.38	0.43	192	12.97 ± 0.97	15.93 ± 0.60	YRL 009	This study
200	45	5 g wet cell/L	58.49 ± 1.61	0.35	0.40	168	9.65 ± 0.13	11.05 ± 0.41	YRL 009	This study
200	48	5 g wet cell/L	36.88 ± 0.93	0.25	0.40	144	5.75 ± 0.38	6.25 ± 0.19	YRL 009	This study
30	48	5 g wet cell/L	11.56 ± 0.35	0.24	0.39	48	3.64 ± 0.28	4.81 ± 0.22	YRL 009	This study
200	42	50 g wet cell/L	79.75 ± 0.42	0.82	0.40	96	15.85 ± 1.11	15.98 ± 0.80	YRL 009	This study

If a reference contained several strains, only the best was shown.
*Quoted from the original article.
†10 g/L glucose was added.
NA: Not available from literature.

Amylolytic enzymes gene copy number and enzyme assay

The copy number of integrated *Aoamy*, *Doamy,* and *Dogam1* genes were determined by Real-time PCR as described by Bubner.[24] Genomic DNAs from YHJ 010, YRL 006, YRL 007, YRL 008, and YRL 009 were quantitatively determined Nanodrop 2000 and used as templates after the concentration was adjusted to almost same. Real-time PCR was conducted on Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA) with THUNDERBIRD SYBR qPCR mix kit (Toyobo, Osaka, Japan). Gene *KmLAC4*[25] was used as an internal control and primers for RT-PCR were shown in Table 1. The α-amylase activity was quantified in 50 mM KH_2PO_4-NaOH buffer at pH 6.0 using the I_2-KI method,[26] and the GAM1 activity was determined by the amount of glucose released in 20 mM sodium acetate at pH 5.5 using soluble starch as the substrate.[6] All assays were carried out at corresponding fermentation temperature (40, 42, 45, or 48°C). The protease activity was determined in 50 mM sodium lactate buffer at pH 4.0, 42°C using casein[27] as the substrate. Every assay was repeated three times and the means were calculated.

Results and Discussion

Gene copy numbers of Aoamy, Doamy, and Dogam1

In this study, four recombinant starch hydrolysis strains were constructed. The starch hydrolase genes and copy number in each strain are shown in Supporting Information Table 1S. The copy number of integrated *Aoamy, Doamy* and *Dogam1* were determined by real-time PCR.[24,25] None of the genes was found in YHJ 010; 1 copy *Aoamy* was detected in YRL 006; 1 copy of *Aoamy* and 1 copy *Dogam1* were detected in YRL 007. YRL 008, which derived from YRL 007, contained 1 more copy of *Doamy* than YRL 007. YRL 009, which derived from YRL 008, contained 1 copy *Aoamy,* 1 copy of *Doamy* and 5 copies *Dogam1* (Supporting Information Table 1S).

Evaluating starch assimilation ability of constructed strains at elevated temperatures using soluble starch

The starch assimilation ability of YHJ 010, YRL 006, YRL 007, YRL 008, and YRL 009 were evaluated through the growth with soluble starch as carbon source at 40, 42, 45, and 48°C before starch fermentation evaluation. As shown in Figure 1 and Supporting Information Table 1S, YRL 007, YRL 008, and YRL 009, which harbored *amy1* and *gam1* gene could grow well with soluble starch at 40, 42, and 45°C. The optimal temperature was 42°C (Figure 1). At all of the temperatures, strain YRL 009 reached highest OD_{600} (YRL 007, 18.76; YRL 008, 22.72; YRL 009, 27.62) with highest growth rate (YRL 007, 0.135/h; YRL 008, 0.149/h; YRL 009, 0.202/h). YRL 008 was the next, and then YRL 007 (Figure 1). The growth of YHJ 010 and YRL 006 (only secreted AoAMY) was weak. In the strains, which could grow with soluble starch, the copy number of amylolytic enzyme genes harbored in strains positively correlated with the growth rate and biomass production at each temperature (Figure 1 and Supporting Information Table 1S). YRL 009 could reach an OD_{600} of 25.12 in 30 h under aerobic conditions with a growth rate of 0.193/h (Supporting Information Table 1S) at exponential phase at 40°C, reach an OD_{600} of 25.87 and 17.76 with a growth rate of 0.202/h and 0.149/h after 24 h culture at 42 and 45°C, respectively

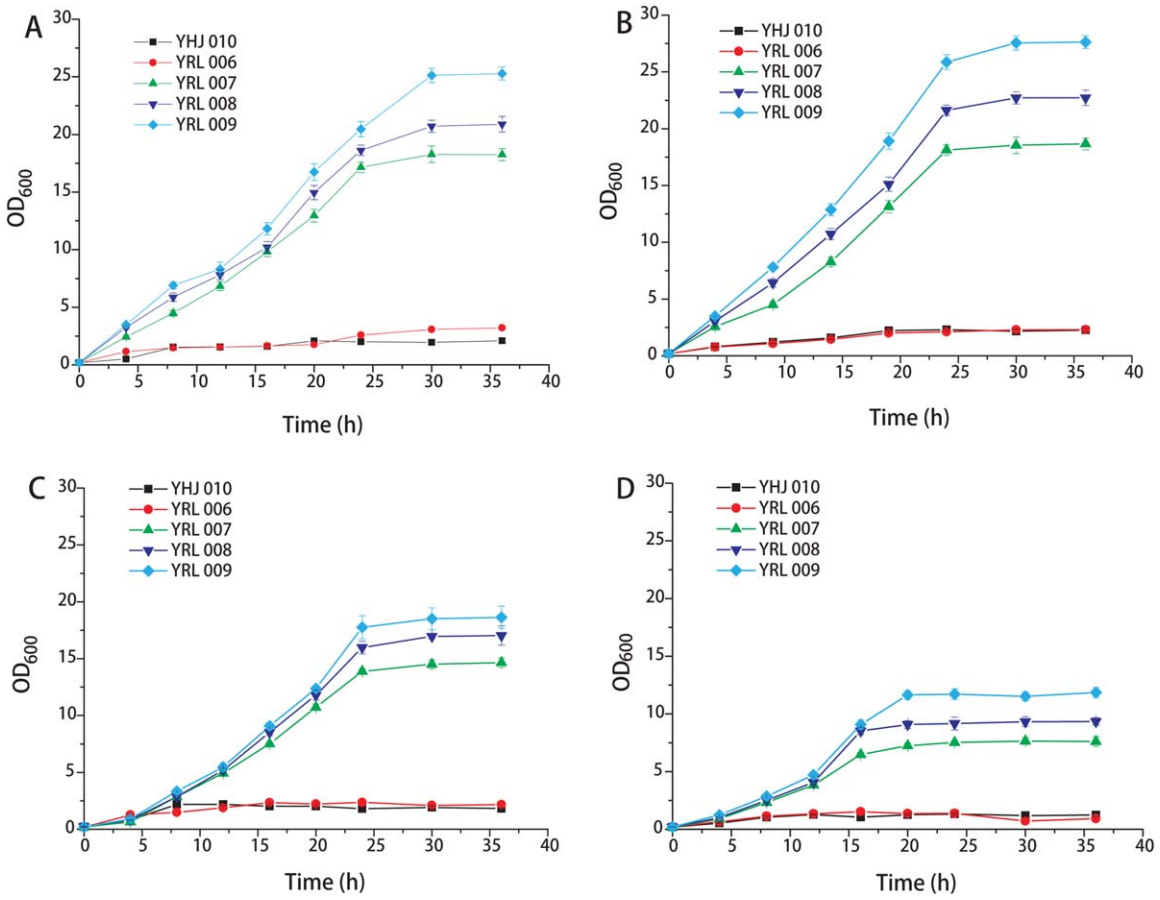

Figure 1. Growth curves of *K. marxianus* YHJ 010, YRL 006, YRL 007, YRL 008, and YRL 009 with 20 g/L soluble starch as carbon source under aerobic conditions at 40°C (A), 42°C (B), 45°C (C), and 48°C (D).

(Figure 1B,C, Supporting Information Table 1S). However, increasing the temperature further weakened the growth rate. At 48°C, the growth rates of all recombinant strains decreased (Figure 1D). YRL 009 could only reach an OD_{600} of 11.65 and its growth rate decreased to 0.094/h (Figure 1D, Supporting Information Table 1S).

Fermentation with soluble starch and raw starch

To determine their starch fermentation abilities, *K. marxianus* YRL 007, YRL 008, and YRL 009 were cultured in anaerobic bottles using YPS containing 200 g/L soluble starch at 42°C. YRL 007, which contained one copy of *Aoamy* and one copy of *Dogam1*, produced 51.42 g/L ethanol in 8 days (Figure 2A). YRL 008, which was derived from YRL 007 and contained one more copy of *Doamy*, resulted in improved ethanol production (59.95 g/L at eighth day; Figure 2B). YRL 009, which was derived from YRL 008 containing 5 copies of *Dogam1*, produced the highest amount of ethanol (72.99 g/L), with an ethanol productivity of 0.38 g/L/h. and 30.55 g/L sugar remained after 8 days fermentation (Figure 2C).

As mentioned by Laluce and Mattoon,[28] the difference in ethanol productivity mainly depended on the activity of GAM1. The Andriy group also reported that increasing the GAM1 gene copy numbers improved the starch hydrolysis capability and ethanol production.[11] The results in this study were consistent with their conclusions. *K. marxianus* YRL 007, YRL 008, and YRL 009 harbored both *Aoamy* and *Doamy*, but their starch hydrolysis enzymatic activities positively correlated with the *Dogam1* copy number (Table 2 and Supporting Information Table 1S). In the fermentation using 200 g/L soluble starch, the maximum activity of α-amylase and GAM1 during the fermentation at 42°C were 7.16 and 7.25 U/mL for YRL 007, 10.72 and 7.22 U/mL for YRL 008, and 12.97 and 15.93 U/mL for YRL 009. The maximum α-amylase and GAM1 activities of YRL 009, which harbored the most copy number of amylolytic enzyme genes were 1.8 times and 2.2 times of that of YRL 007, respectively (Figure 2). YRL 009 showed the highest ethanol productivity. As shown in Table 3, the α-amylase activities of the strains constructed in this study were the highest in all reported recombinant strains. It is possible that the activity of the α-amylase expressed in this study is high enough for starch hydrolysis, and then, the saccharification process turns to be a limiting step. So, with the increase of GAM1 activity, the ethanol fermentation was promoted.

Since *K. marxianus* YRL 009 had the strongest fermentation ability, its fermentation ability was evaluated further at various temperatures. At 40°C, 70.13 g/L ethanol was produced from 165.08 g/L sugar consumed with a productivity of 0.37 g/L/h in 8 days (Figure 3A). At 42°C, 72.99 g/L ethanol was produced from 167.86 g/L sugar consumed with a productivity of 0.38 g/L/h in 8 days (Figure 3B), while only 58.49 g/L ethanol (Figure 3C) and 36.88 g/L ethanol (Figure 3D) were produced at 45 and 48°C, respectively. The ethanol productivity decreased from 0.38 g/L/h (42°C) to 0.31 g/L/h (45°C) and 0.19 g/L/h (48°C) when the

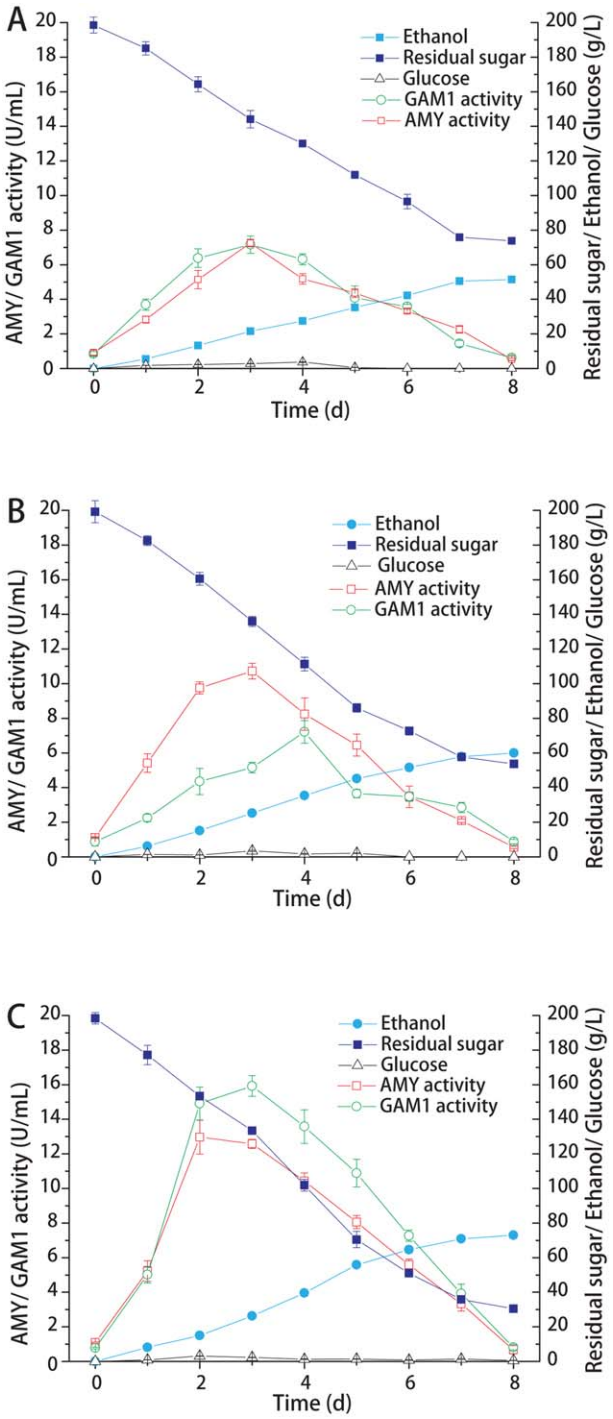

Figure 2. Time course of anaerobic fermentation with YPS containing 200 g/L soluble starch at 42°C by YRL 007 (A), YRL 008 (B), and YRL 009 (C).

fermentation temperature increased. The maximum activity of α- amylase during the fermentation increased from 11.73 U/mL (40°C) to 12.97 U/mL (42°C) and then declined to 9.65 U/mL (45°C) and 5.75 U/mL (48°C), and the maximum activity of GAM1 also increased from 13.64 U/mL (40°C) to 15.93 U/mL (42°C), then decreased to 11.05 U/mL (45°C) and 6.25 U/mL (48°C; Figure 3). The amylolytic enzyme activities were consistent with the fermentation results at each temperature.

Because the optimal temperature of amylolytic enzymes involved in CBP is ~50°C, fermentation with thermotolerant yeast at elevated temperatures should promote ethanol production. However, obtaining an efficient fermentation at elevated temperatures is difficult. The recombinant thermotolerant yeast *Hansenula polymorpha* fermented 30 g/L soluble starch at 48°C, which was the highest temperature reported to date, and produced only 10 g/L ethanol in 72 h with a productivity of 0.14 g/L/h.[11] In this study, fermentation with YRL 009 using 30 g/L soluble starch at 48°C produced 11.56 g/L ethanol in 48 h with a higher productivity of 0.24 g/L/h (Table 3). This result constituted a clear improvement over previously reported fermentation processes at elevated temperatures. However, both the results in this study and the previous results showed that the efficiency was decreased at higher temperatures. This decrease could possibly be attributed to the cell viability and the decreased expression level of enzymes.

The direct utilization of raw starch without pretreatment can significantly reduce the cost. In this study, raw starch (from corn, potato, wheat, and cassava) was also used to evaluate the direct fermentation ability of *K. marxianus* YRL 009 (Table 4). At 42°C, fermentation stopped at the seventh day (cassava) or eighth day (corn, potato, and wheat). The fermentation of cassava starch produced 66.52 g/L ethanol from 200 g/L starch with 162.46 g/L sugar consumed and an ethanol productivity of 0.40 g/L/h, which was the highest compared with other raw starches (Table 4). Other starches (corn, potato, and wheat) seemed to be less effective as substrates than cassava starch. With 200 g/L of raw corn, potato, and wheat starch, 56.82, 50.73, and 52.78 g/L of ethanol were produced at a rate of 0.30, 0.26, and 0.28 g/L/h, respectively. The raw starches were also fermented at 40, 45, and 48°C (Table 4). YRL 009 produced 53.45 g/L (corn), 51.06 g/L (potato), 56.66 g/L (wheat), and 65.88 g/L (cassava) ethanol at 40°C, 44.68 g/L (corn), 43.24 g/L (potato), 43.64 g/L (wheat) and 55.36 g/L (cassava) ethanol at 45°C in 8 days and 29.57 g/L (corn), 27.31 g/L (potato), 27.45 g/L (wheat) and 32.16 g/L (cassava) ethanol at 48°C in 5 days. The fermentation temperature negatively correlated with the ethanol production, similar to the results in Table 3. This effect may also be due to the reduced cell viability and expression of enzymes.

Fermentation with cassava tuber powder

To evaluate the ability of *K. marxianus* YRL 009 to ferment raw material, cassava tuber powder was fermented at 40, 42, 45, and 48°C. Even without additional nutritional supplements and under nonsterile conditions, 69.73 g/L ethanol (Figure 4B) was produced from 166.07 g/L cassava tuber powder, and a productivity of 0.36 g/L/h was achieved in 8 days at 42°C, which was the optimal fermentation temperature. At 40, 45, and 48°C, 66.03 g/L ethanol (Figure 4A), 57.83 g/L ethanol (Figure 4C), and 30.58 g/L ethanol (Figure 4D) were produced, respectively.

Determination of the limitation factor in the fermentation

Firstly, the pH of the supernatant during the fermentation was measured to determine the cause of the decreased amylolytic activity during the fermentation (Figures 2–4). The pH decreased from 5.4 to 3.1 during the fermentation of raw cassava starch. Although the optimal pH values for DoGAM1, AoAMy, and DoAMY are 3.5–5.0, 5.6, and 6.0, respectively,[26,29] AoAMY and DoAMY only showed ~10 and 50% of their optimal pH activity at pH 3.8. Because the

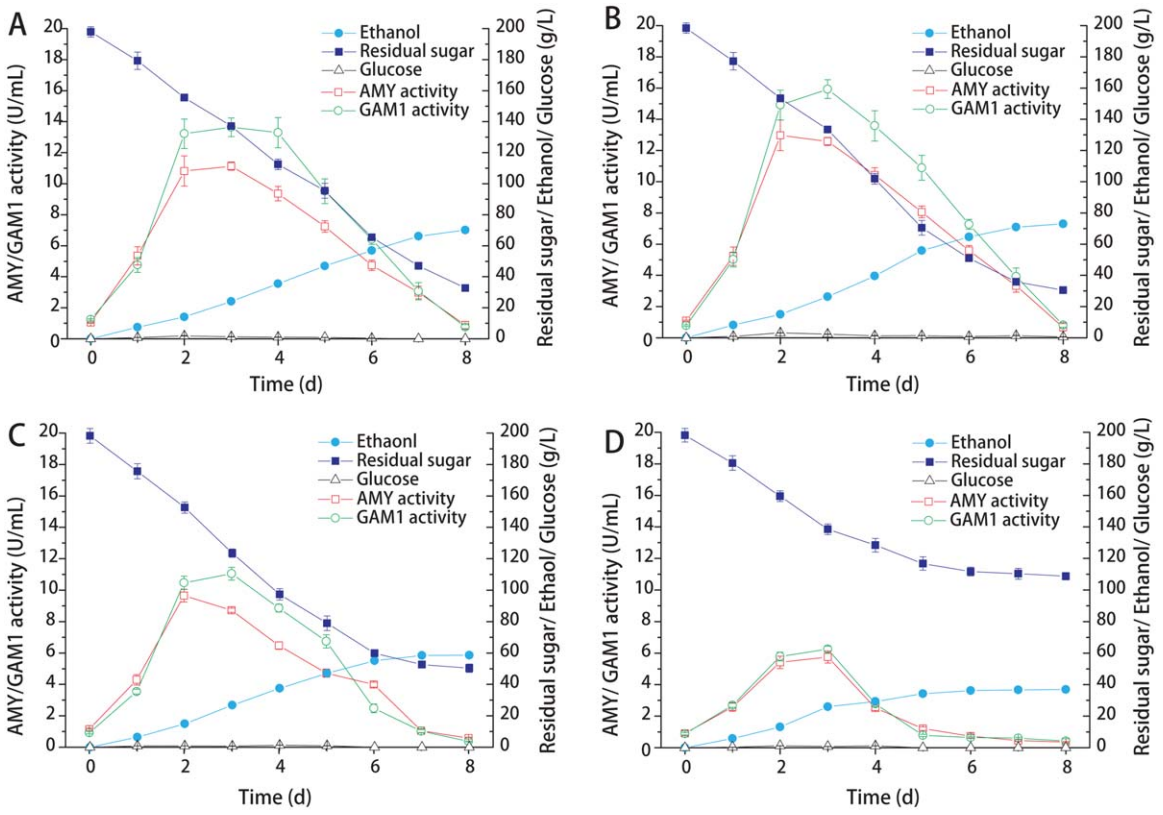

Figure 3. Anaerobic fermentation with 200 g/L (w/v) soluble starch using YRL 009 at 40°C (A), 42°C (B), 45°C (C), and 48°C (D).

Table 4. Ethanol Produced at 40, 42, 45, and 48°C with 200 g/L Various Raw Starch and Cassava Tuber Powder by YRL 009

	Ethanol (g/L)			
Raw starches	40°C	42°C	45°C	48°C
Corn	53.45 ± 2.87	56.82 ± 1.42	44.68 ± 1.21	29.57 ± 0.98
Potato	51.06 ± 1.66	50.73 ± 2.27	43.24 ± 1.74	27.31 ± 1.67
Wheat	56.66 ± 2.03	52.78 ± 1.56	43.64 ± 1.39	27.45 ± 1.26
Cassava	65.88 ± 1.67	66.52 ± 0.94	55.36 ± 1.95	32.16 ± 0.86
Cassava tuber	66.03 ± 0.54	69.73 ± 1.98	57.83 ± 1.62	30.58 ± 1.02

change in pH might reduce the amylolytic activity, a buffered YPS medium containing 20% (w/v) raw cassava starch with 0.1 M sodium phosphate buffer (pH 5.6) was used in the fermentation with YRL 009. Cassava starch produced 72.61 g/L ethanol in 7 days (Supporting Information Figure 1S). Compared with the fermentation in buffered medium containing 200 g/L cassava starch, the pH was relatively stable (between 5.6 and 4.8) during the fermentation. The concentration of produced ethanol increased from 66.52 to 72.61 g/L, and the ethanol productivity increased from 0.40 to 0.43 g/L/h. However, the amylolytic activity still decreased after 3 days of fermentation as a result of the buffered medium.

Second, the protease activities during the fermentation were determined. Although during the fermentation without protease inhibitor, protease activity increased from 0.16 to 2.56 U/mL (Figure 5A) and no protease activity was detected when protease inhibitor was added (Figure 5B), the decrease of the amylolytic activities after 3 days fermentation was not avoided by inhibiting protease (Figure 5B). The amylolytic activities were improved little (maximum α-amylase activity was 14.63 and 15.52 U/mL, and the maximum GAM1 was 15.03 and 16.25 U/mL (Figure 5) during the fermentation without or with protease inhibitor, respectively. Therefore, the protease activity during the fermentation was not the main reason, which led to the decrease of amylolytic activity.

Third, the ratio of viable yeast cells to total cells during the fermentation was analyzed using a methylene blue stain (Figure 5). During the first 3 days, the number of viable cells increased and accounted for more than 85% of the total cells. Subsequently, the ratio decreased and only 5% of the total cells remained active on the eighth day when the fermentation stopped in all groups. This result indicated that it was possible that the amylolytic activity decreased due to the death or reduced viability of yeast cells. The decrease of cell viability is possibly due to the increased concentration of ethanol and the accumulation of metabolic side products. In previous report, the decrease of viability of cell could lead to an increase of the protease activity in the fermentation of yeast,[30] so it is possible that the increase of protease

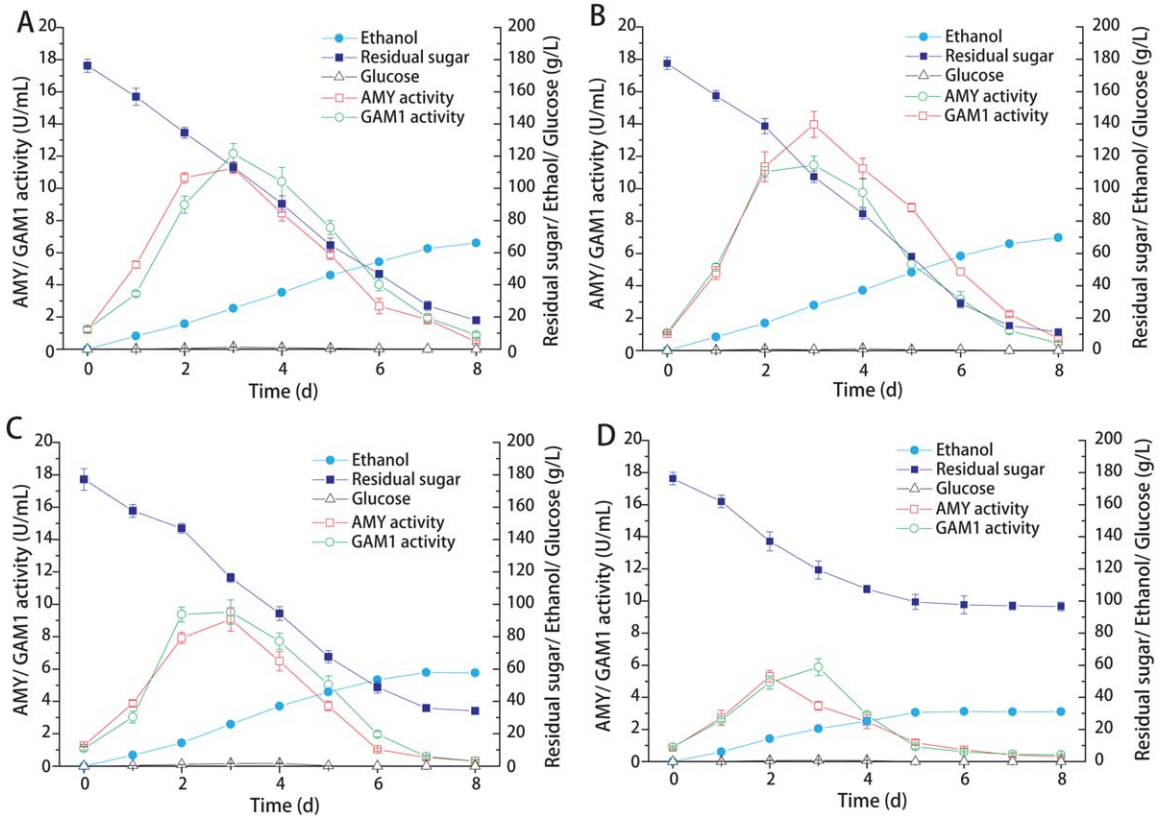

Figure 4. Direct fermentation with 200 g/L (w/v) nonsterile cassava tuber powder using YRL 009 at 40°C (A), 42°C (B), 45°C (C), and 48°C (D).

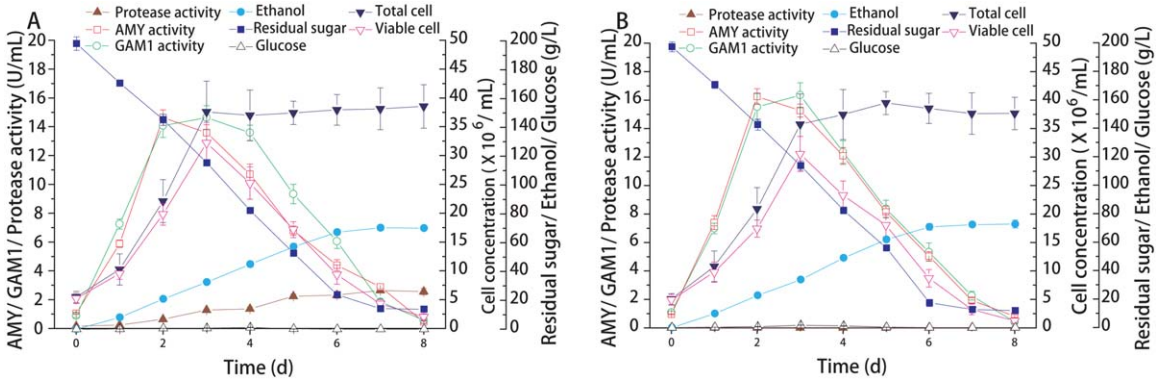

Figure 5. Determination of the effect of protease in fermentation. Fermentation of 200 g/L raw cassava starch without protease inhibitor (A) or with protease inhibitor (B) was analyzed.

activity was released along with the cell death or loss of viability in this study.

Effect of initial cell density in the fermentation

Table 3 shows a comparison of the ethanol formation by recombinant yeasts from previous reports. The highest ethanol productivity reported by Yamada et al. at 0.97 g/L/h, with 70 g/L ethanol produced in 72 h at 30°C. They used a higher wet cell density of 50 g/L. The direct fermentation using YRL 009 was also examined for 50 g/L wet cells and 200 g/L raw cassava starch at 42°C. All of the starch was consumed to produce 79.75 g/L ethanol (Figure 6, Table 3) in 96 h. Compared with the fermentation of 200 g/L cassava starch at 42°C using a lower initial cell density (5 g/L wet cells), the ethanol productivity increased from 0.38 to 0.82 g/L/h. However, a substantial increase in the ethanol productivity due to a higher initial cell density results in higher preculture costs, which is an adverse factor in industrial fermentation. A reasonable initial cell density should be carefully considered in future studies.

In conclusion, a thermotolerant yeast strain, *K. marxianus* YRL 009, that secretes both α-amylase and GAM1, was constructed. This strain could produce ethanol from raw starch without pretreatment and showed a high productivity at temperatures above 40°C. Notably, YRL 009 could also directly ferment cassava tuber powder under non-sterile conditions without additional nutritional supplements at elevated

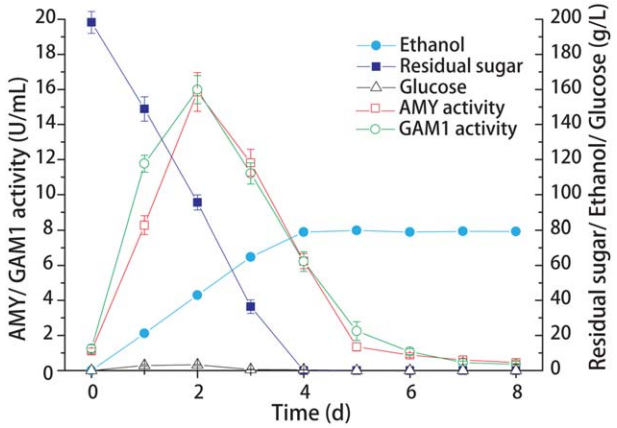

Figure 6. Fermentation with 200 g/L cassava starch using YRL 009 at 42°C with 50 g/L wet cell.

temperatures. Therefore, YRL 009, which has CBP ability, is the best strain for fermenting starches at high temperatures that has been reported to date.

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