

Ethanol Production from Rice Winery Waste - Rice Wine Cake by Simultaneous Saccharification and Fermentation Without Cooking

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Ethanol production by the simultaneous saccharification and fermentation (SSF) of low-value rice wine cake (RWC) without cooking was investigated. RWC is the filtered solid waste of fermented rice wine mash and contains 53% raw starch. For the SSF, the RWC slurry was mixed with the raw-starch-digesting enzyme of *Rhizopus* sp. and yeast, where the yeast strain was selected from 300 strains and identified as *Saccharomyces cerevisiae* KV25. The highest efficiency (94%) of ethanol production was achieved when the uncooked RWC slurry contained 23.03% starch. The optimal SSF conditions were determined as 1.125 units of the raw-starch-digesting enzyme per gram of RWC, a fermentation temperature of 30°C, slurry pH of 4.5, 36-h-old seeding culture, initial yeast cell number of 2×10^7 per ml of slurry, 17 mM of urea as the nitrogen additive, 0.25 mM of Cu^{2+} as the metal ion additive, and a fermentation time of 90 h. Under these optimal conditions, the ethanol production resulting from the SSF of the uncooked RWC slurry was improved to 16.8% (v/v) from 15.1% (v/v) of pre-optimization.

Keywords: Rice wine cake, ethanol fermentation, raw-starch-digesting enzyme, *Saccharomyces cerevisiae* KV25

Bioethanol is an attractive, sustainable energy source for fuel that can contribute to a cleaner environment [31]. Bioethanol manufactured from renewable resources by microbial fermentation is an attractive alternative as it is carbon dioxide neutral, meaning that the amount of CO_2 released from the fermentation is 100% offset by the amount of CO_2 absorbed by the plants grown to make it [31]. The production of ethanol from the starch of wheat, barley, cassava, or maize by fermentation with the traditional yeast *Saccharomyces cerevisiae* is already a well-known

process [8]. By altering the nutritional conditions, it is also possible to increase the ethanol yield and the survival of the yeast at high concentrations of ethanol [8]. Agriculture-based industries generate a large amount of solid waste, such as peels from cassavas, plantains, bananas, and oranges [20]; however, instead of allowing this waste to become solid municipal waste, it is more beneficial to convert it into useful end-products. Thus, it has now been recognized that such waste can be utilized as cheap raw materials for certain industries or as inexpensive substrates for microbiological processes [20]. The use of waste material is also economical, as it is more readily available and much cheaper. So far, agro-waste, such as cassava-peel hydrolysate [1], *Carica papaya* [2], and kitchen waste have already been used for ethanol production [27].

Rice wine cake (RWC) is the filtered solid waste from rice-wine fermentation. Some wineries use uncooked raw rice for rice wine production. After completing the simultaneous saccharification and fermentation (SSF), the mash is filtered and the clear rice wine and RWC are separated. RWC contained 62.2% solid, including 53% raw starch, 5.84% protein, 0.62% lipid, 2.38% cellulose, 4–8.6% (v/w) residual ethanol, 0.35% minerals, 2.08 ppm vitamin B1, 1.39 ppm vitamin B2, 407.45 ppm vitamin C, 3×10^9 /g of total yeast cells, and 4.1×10^3 /g of viable cells [16]. However, the possibility of using RWC for ethanol fermentation has not yet received much attention. The use of RWC in ethanol production can not only reduce the waste material created by wineries, but also lower the cost of ethanol production. Accordingly, this study investigated the use of RWC as a substrate for ethanol production during SSF using yeast and a raw-starch-digesting enzyme (RSDE). The potential industrial applications of RSDEs have already attracted attention, as RSDEs are capable of hydrolyzing complex polymers of large granules of raw starch into glucose and other oligosaccharides at mild temperatures without requiring the conventional two-step process for ethanol fermentation involving liquefaction by cooking at a high temperature

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and saccharification [14, 30]. Matsumoto *et al.* [18] reported that SSF without cooking brings more advantages than acid hydrolysis, bases, or cooking at a high temperature, including higher quality products, energy efficiency, and a safer working environment. The processing equipment also lasts longer, as the milder conditions reduce corrosion. However, the greatest benefit of ethanol fermentation without cooking is the reduction of high heating energy costs for liquefaction and saccharification of the feedstock.

Therefore, the objective of this study was high ethanol production from rice winery waste, RWC, using SSF without cooking. Thus, a yeast strain was screened and selected for the raw RWC fermentation and the optimal SSF conditions were then examined.

MATERIALS AND METHODS

Collection and Screening of Yeast

More than 300 yeast strains were collected from domestic and foreign culture collections (KTCC, ATCC, NRRL) and university laboratories. Some yeast strains were also isolated from soils sampled at local distilleries. The yeast strains were then screened based on SSF of uncooked rice slurry (33%, dry solid) at 30°C for 5 days, and the ethanol content in the fermented slurry was then determined. Finally, the best yeast strain, KV25, was selected for the production of ethanol by SSF of RWC.

Identification of Yeast

The DNA of KV25 was isolated and purified using a Wizard Genomic DNA purification kit (Promega, U.S.A.). The identification of the ITS-rDNA was performed according to the methods of Henry *et al.* [11] and White *et al.* [28]. The PCR primers used were ITS1 (5'-TCCGTAGGTGAACCTGCG-3'), ITS3 (5'-GCATCGATGAAGA ACGCAGC-3'), and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [29], and the PCR amplification was performed using a volume of 50 µl. A 5-µl sample containing about 5 ng of DNA was added to the PCR master mixture, which consisted of 5 µl of a 10× PCR buffer, 4 µl of a dNTP mixture (0.1 mM each dNTP), 0.8 µl of each primer (40 pmol of each primer), and 2 units of ExTaq DNA polymerase (Takara Biomedicals, Osaka, Japan), and de-ionized water was added to make a final volume of 50 µl. The amplification consisted of denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and an extension at 72°C for 1 min, followed by a final extension at 72°C for 4 min using a Gene Amp, AB Applied Biosystem (Singapore). The PCR products were then purified and sequenced. The sequence of the ITS-rDNA was aligned with similar well-known sequences from the NCBI database.

Substrates

The RWC used for SSF was obtained from a local rice winery and contained 62.2% solid, including 53% raw starch, 5.84% protein, 0.62% lipid, 2.38% cellulose, 4–8.6% (v/w) residual ethanol, 0.35% minerals, 2.08 ppm vitamin B1, 1.39 ppm vitamin B2, 407.45 ppm vitamin C, 3×10⁹/g of total yeast cells, and 4.1×10³/g of viable cells. The RWC used in this study was the same as the one used by Lim *et al.* [16]

Preparation of Slurry

The RWC was mixed with water at different ratios (1:0.7 to 1:1.7) and homogenized using a blender. Hereinafter, unless otherwise specified, the RWC was mixed with water based on a ratio of 1:1.3 and the resulting slurry contained 23.03% (w/v) raw starch. The slurry was then treated with a raw-starch-digesting enzyme (RSDE), a glucoamylase produced by *Rhizopus* sp., where the proportion of RSDE to substrate was 1.125 U/g as the dry weight base. Unless otherwise mentioned, this proportion of RSDE to substrate was used in all the experiments. To screen the yeast strains, rice, rice-corn starch, and RWC were used, where the mixing ratio of rice to water was 1:2 and rice and corn starch to water was 1:2:5.5.

Preparation of Raw-Starch-Digesting Enzyme (RSDE)

A 10% (v/w) liquid culture of *Rhizopus* sp. was inoculated onto solid wheat bran at 30°C for 3 days, and the moldy wheat bran then dried at 35°C for 12 h. Next, water was added to a ratio of 1:50 (w/v) and stirred for 1 h at room temperature. The extracted liquid was then used as the crude RSDE.

Preparation of Inoculum for SSF

One loopful of yeast cells of 2-day-old yeast grown on a YPD plate was inoculated into a 250-ml Erlenmeyer flask containing 100 ml of YPD broth in a rotary shaking incubator operated at 30°C and 200 rpm for 36 h. The yeast cells harvested by centrifugation were then used to inoculate according to the desired inoculum size.

Optimization of SSF Conditions for RWC

To determine the optimal SSF conditions for RWC when using the RSDE and *S. cerevisiae* KV25, the following parameters were examined: incubation temperature (20–40°C), pH of RWC slurry (3.0, 3.5, 4.0, 5.0, 6.0, 7.0, and 8.0), fermentation time (24–144 h), initial yeast cell number (1×10⁴, 1×10⁵, 1×10⁶, 1×10⁷, 2×10⁷, 3×10⁷, and 1×10⁸/ml). Studies were also conducted to investigate the effect of various additives in the RWC slurry on the ethanol production by SSF. The additives examined were nitrogen sources [urea, malt extract, casamino acids, yeast extract, tryptone, tryptic soy, soytone, peptone, skim milk, nutrient broth, NH₄NO₃, NH₄Cl, NaNO₃, and KNO₃ at 0.1% (w/v)] surfactants [Tween 80, Tween 20, and Triton X-100 at 0.5% (v/v); EDTA and SDS at 0.4 mM], and metal salts (MgCl₂, MnCl₂, CuSO₄, CoCl₂, FeSO₄, ZnSO₄, KCl, CaCl₂, and NaCl at 0.25 mM). Unless otherwise mentioned, the SSF was carried out in a 250-ml Erlenmeyer flask containing 120 ml of the RWC slurry at pH 3.5 with an initial cell number (2.5×10⁷/ml) of a 36-h-old seeding culture and RSDE of 1.125 units/g RWC at 30°C for 5 days. Each experiment was performed in triplicate.

Ethanol Production at Optimal Conditions

The ethanol production by SSF was conducted at 30°C in a 3-l flask containing 1.5 l of the RWC slurry, pH 4.5, treated with the RSDE. The slurry was supplemented with urea at 17 mM and Cu²⁺ at 0.25 mM. The yeast cells were inoculated into the slurry to make a final concentration of 2×10⁷ cells/ml.

Determination of RSDE Activity

A 0.05 M sodium acetate buffer (pH 5.0) was used to dilute aliquots of the enzyme and dissolve the raw rice starch. The blank contained a 0.5-ml aliquot of the enzyme, 1 ml of DNS, and 0.5 ml of a 2% raw rice starch solution. The reaction mixture contained 0.5 ml of the

2% raw rice starch solution and a 0.5-ml aliquot of the enzyme at 30°C for 30 min. The reaction was then stopped by the addition of 1 ml of 3,5-dinitrosalicylic acid (DNS). The blank and reaction mixture were both boiled at 100°C for 5 min, and then cooled, 5 ml of distilled water was added, and the absorbance measured at 540 nm. A 0.1% glucose solution (Sigma) was used to make the standard curve. One unit (U) of RSDE activity was defined as the amount of enzyme in 1 ml that liberated 1 μ mole of glucose per minute from the raw rice starch [19]. The reducing sugar was measured using a previously reported method [19].

Ethanol Analysis

The ethanol content was analyzed using HPLC. The column was a Shodex SH1011 (8.0 mm ID \times 300 mm) and the mobile phase was 0.01 N sulfuric acid. The temperature was kept at 50°C with a flow rate of 0.6 ml/min and sample volume of 10 μ l. The effluent from the column was monitored using an RI detector (Shodex RI-101).

Statistical Program

The data was analyzed using one- and two-way analyses of variance (ANOVA) ($\alpha=0.05$), followed by a comparison of the means using Duncan's multiple range test (SAS Institute, Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

Screening and Selection of Yeast Strain

The ethanol produced from the uncooked rice slurry by the various yeast strains varied from 4% to 18.4% (v/v), and Table 1 shows the top 18 ethanol-producing yeast strains with significantly different levels of production ($P<0.0024$). Among these strains, six strains, KV25, KT26, K111, KT76, KS53, and VN11, producing a relatively high amount of ethanol, were selected for a second screening. As shown in Table 2, strain KV25 produced the highest concentration of ethanol from the uncooked rice-corn and RWC slurries at 17.32% and 17.73% (v/v), respectively, which was significantly higher than the amounts produced by the other yeast strains ($P<0.0001$). Strain KV25 was subsequently identified as *Saccharomyces cerevisiae* according to the sequencing results of the ITS1 and ITS4 from the yeast rDNA.

Optimal SSF Conditions

Effect of mixing ratio of RWC to water. The effects of different mixing ratios of RWC and water on the ethanol production were examined and the results are shown in Table 3, where RWC mixed with water at a ratio of 1:1.3 produced the highest amount of ethanol per gram of RWC, 0.369 ml/g RWC. Therefore, this proportion was used in all the following experiments.

Effect of fermentation temperature. The fermentation temperature was found to have a significant effect on the ethanol production from the RWC slurry (Fig. 1), where the optimal temperature range for high ethanol production was 25–30°C with 15.5% and 15.7% (v/v) at 25°C and

Table 1. Ethanol production from uncooked rice slurry^a.

Yeast strain	Ethanol (% v/v)
KV25	18.41 ^a
KT76	18.39 ^a
K112	18.03 ^{ba}
KA4	16.51 ^e
KA5	16.62 ^{de}
KS53	18.03 ^{ba}
KA3.2	16.78 ^{de}
KR7	17.10 ^{dec}
VN11	17.47 ^{bdac}
KS7	17.31 ^{bdcc}
KS6	17.01 ^{dec}
KS2	16.89 ^{de}
VN10	17.31 ^{bcde}
K111	18.09 ^{ba}
KT26	18.03 ^{ba}
KR3.2	16.94 ^{dec}
KC6	16.73 ^{de}
KV14	17.81 ^{bac}
Statistical analysis	$P<0.0024$

^aThe ethanol production was conducted in a 250-ml Erlenmeyer flask containing 120 ml of rice slurry [about 35% (w/v) of solid], pH-3.5, initial cell number of 2.5×10^7 /ml from a 36-h-old seeding culture, and RSDE (1.125 U/g rice) at 30°C for 5 days.

^bThe means in the same column followed by the same letters were not significantly different in a *t*-test.

30°C, respectively. Meanwhile, 37°C and 45°C produced the lowest ethanol yield of 13.4% (v/v) and 6.9 % (v/v), respectively. Therefore, since 30°C was suitable for both the saccharification process of the raw starch by the RSDE of *Rhizopus* sp. (data not shown) and the ethanol fermentation in SSF by *S. cerevisiae* KV25, an incubation

Table 2. Ethanol production in uncooked slurries with different yeast strains^a.

Yeast strain	Ethanol (% v/v)	
	Rice-corn	RWC
KV25	17.32 ^a	17.73 ^{a^b}
KT26	16.56 ^{cd}	16.23 ^f
KS53	15.92 ^f	17.06 ^{cd}
K111	16.24 ^{def}	17.36 ^{bc}
KT76	15.16 ^g	17.26 ^{cde}
VN11	16.94 ^b	15.82 ^g
Statistical analysis	$P<0.0001$	$P<0.0001$

^aThe ethanol production was conducted in a 3-l glass jar containing 1.5 l of slurry, pH-3.5, initial cell number of 2.5×10^7 /ml from a 36-h-old seeding culture, and RSDE (1.125 U/gds) at 30°C for 5 days. At the beginning of the fermentation, the rice-corn slurry contained 35% solid, whereas the RWC slurry contained 23.03% (w/v) raw starch and 3.3% (v/v) residual ethanol.

^bThe means in the same column followed by the same letters were not significantly different in a *t*-test.

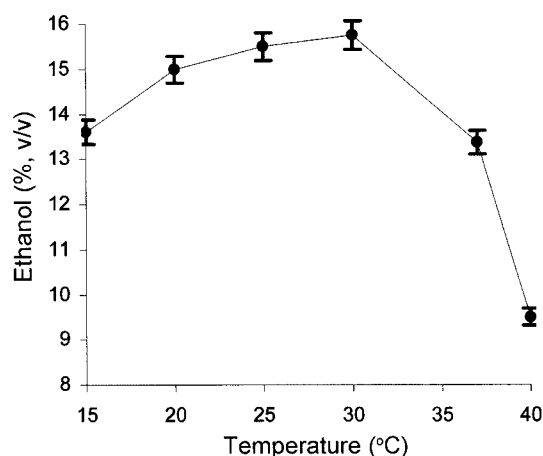
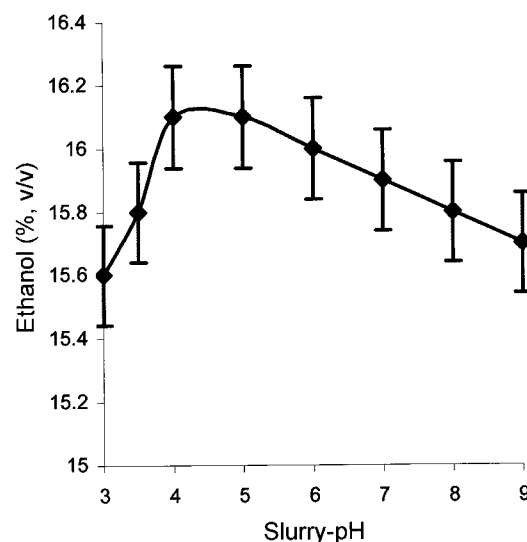
Table 3. Ethanol production from RWC mixed with different amounts of water^a.

RWC:water ratio	Dried weight (%, w/w)	Ethanol produced	
		% (v/v)	ml/g RWC
1:0.7	33.87	15.30	0.260
1:0.9	30.13	16.75	0.318
1:1.1	26.98	16.40	0.344
1:1.3	25.28	16.05	0.369
1:1.5	22.98	14.70	0.368
1:1.7	21.20	13.55	0.366

^aThe ethanol fermentation was carried out in a 3-l glass jar containing 1.5 l of slurry, pH-3.5, initial cell number of 2.5×10^7 /ml, and RSDE (1.125 U/gds) at 30°C for 5 days. The RWC slurry at the beginning of the fermentation contained 23.03% raw starch and 3.3% (v/v) residual ethanol.

temperature of 30°C was used in all the following experiments. Generally, the optimal temperature for maximum ethanol yield varies according to the yeast strain, such as 30°C for *S. cerevisiae* 21 in the fermentation of glucoamylase-treated starch [28], 38°C for *S. cerevisiae* [24], and 37°C for *S. diastolicus* using acid-solubilized starch [5]. In addition, the time required to complete the fermentation process decreases as the temperature increases from 17°C to 33°C [3]. This was the same in the present study, where the time required to complete the fermentation decreased from 144 h at 20°C to 114 h at 30°C (data not shown).

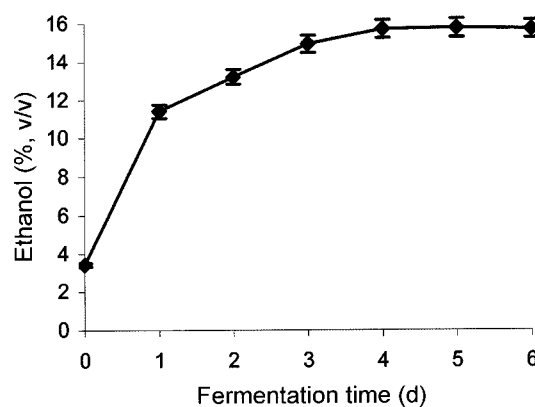
Effect of initial pH of medium. The initial pH of the RWC slurry had a marked effect on the ethanol production by *S. cerevisiae* KV25 ($P < 0.05$) (Fig. 2). Whereas the maximal ethanol production was 16.1% (v/v) at pH 4.5 after 114 h of fermentation, a pH below 3.0 or above 9.0 produced a sharp decrease in the ethanol production. The optimal pH also varied depending on the substrate used for the fermentation: pH 4.0–4.5 for sucrose [13] and pH 2.8–3.4 for sugar cane [10]. A low pH is already known to prevent microbial contamination, while promoting relatively high

**Fig. 1.** Effect of culture temperature on SSF of uncooked RWC.**Fig. 2.** Effect of initial slurry-pH on SSF of uncooked RWC.

ethanol productivity [7]. Therefore, as the RWC slurry with a pH of 4.5 produced a relatively high ethanol concentration, a pH 4.5 slurry was used in all the following experiments.

Effect of fermentation time. The fermentation time had a significant effect on the ethanol production ($P < 0.05$), where the ethanol yield continued to increase from day 1 to day 4 (Fig. 3). A longer fermentation time beyond 4 days produced no further increase. After 4 days, the ethanol produced was 15.7% (v/v).

Effect of initial cell number. Fig. 4 shows that the initial yeast cell number had a marked effect on the ethanol production ($P < 0.05$), when more ethanol was produced when increasing the initial cell number from 1×10^4 to 1×10^7 cells/ml. In contrast, the ethanol produced in the slurry inoculated with 2×10^7 , 3×10^7 , and 10^8 cells/ml was not significantly different ($P > 0.05$). Thus, since a lower initial cell number causes a lower ethanol yield and requires a longer time to complete the fermentation, whereas

**Fig. 3.** Effect of fermentation time on SSF of uncooked RWC.

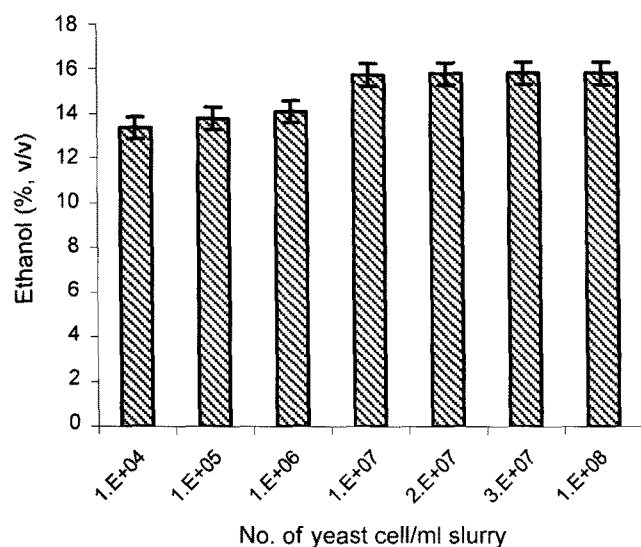


Fig. 4. Effect of initial yeast cell number on SSF of uncooked RWC.

a higher initial cell number costs more, an inoculum size of 2×10^7 was used in all the following experiments, as the ethanol produced with an inoculum size of 2×10^7 yeast cells/ml was similar to that with 3×10^7 and 1×10^8 yeast cells/ml.

Effects of Additives in Medium

Nitrogen additives. As nutrient supplementation is already known to improve the fermentation process [3, 8, 21, 26], this study examined the effect of 14 different nitrogen sources on the ethanol production in the RWC slurry. Fig. 5A shows that tryptone, urea, and yeast extract at a level of 0.1% (w/w) enhanced the ethanol production the most when compared with the unsupplemented control and other nitrogen additives. Within 90 h of fermentation, the highest concentration of ethanol was produced in the tryptone-supplemented RWC (16.27%, v/v), followed by the urea-supplemented RWC (16.22%, v/v) and the yeast extract-supplemented RWC (16.17%, v/v) when compared with the unsupplemented control slurry (15.7%). In a medium supplemented with proper nitrogen, the time required to complete the fermentation is decreased from 114 to 90 h, plus nitrogen additives, such as yeast extract and glutamic acid, in wheat mashes are known to stimulate the growth of the yeast and reduce the fermentation time [26]. Conversely, a medium without proper nutrients results in sluggish fermentation, where the rate of sugar utilization is extremely slow [13]. In this study, the supplementation of NaNO_3 and KNO_3 inhibited the fermentation, resulting in an ethanol production of 12.2% and 13.5% (v/v), respectively, which was lower than that produced with proper nitrogen. The same trend was also reported by Isono and Hoshino [13], where nitrate salts inhibited ethanol production and were not utilized by *Saccharomyces* sp. It has also been proven

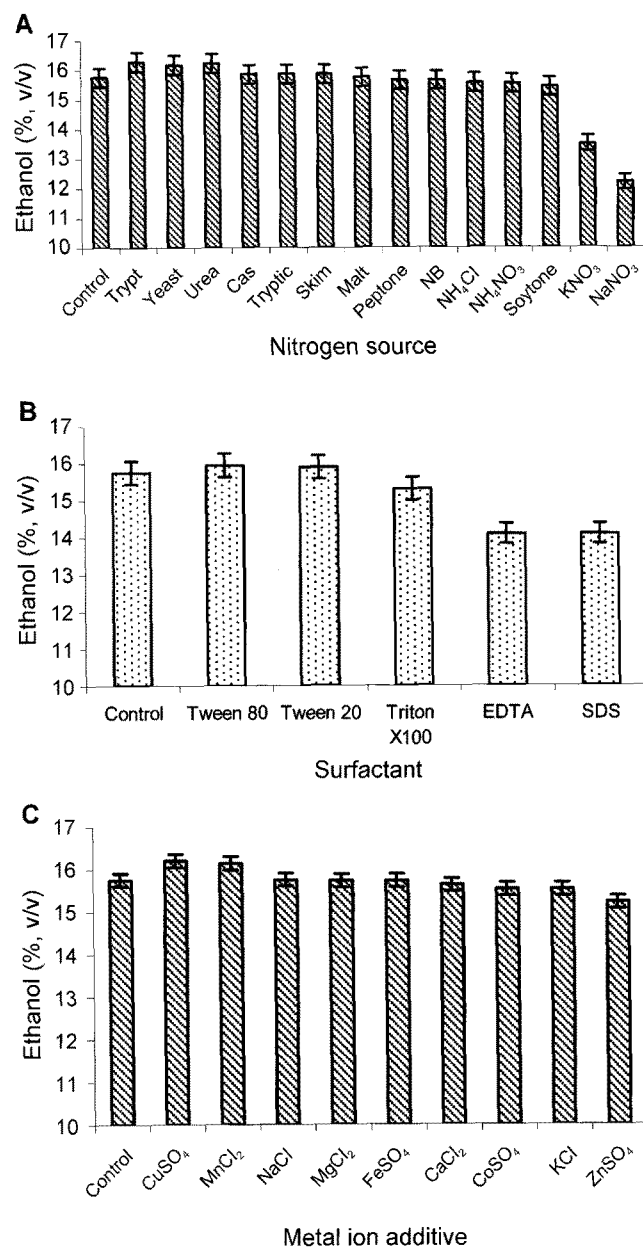


Fig. 5. Effects of medium additives on SSF of uncooked RWC.

A. Nitrogen additives; **B.** Surfactant; **C.** Metal salts. In the control cases, the SSF was carried out in a 250-ml Erlenmeyer flask containing 120 ml of the RWC slurry at pH 3.5 with an initial cell number of 2.5×10^7 /ml from a 36-h-old seeding culture and RSDE of 1.125 units/g RWC, without additives, at 30°C for 5 days. Tryt: tryptone; Yeast: yeast extract; Cas: casamino acids; Tryptic: tryptic soy; Skim: skim milk; Malt: malt extract; NB: nutrient broth.

that, at pH values below 6.0, nitrous acid is formed from nitrate salts and is toxic to yeast [23].

Urea concentration. Since urea enhances ethanol production and is a more economical source of nitrogen, the effect of the urea concentration on the SSF was further examined. When supplementing the RWC slurry with urea at a final

concentration of 15–20 mM, the rate of the fermentation process was accelerated and the time required to complete the fermentation reduced to within 90 h when compared with 114 h for the unsupplemented slurry. In addition, the urea had a marked effect on enhancing the ethanol production ($P < 0.05$) (data not shown). In the RWC slurry supplemented with urea at a final concentration of 17 mM (same as 0.1%), the ethanol production increased to 16.3% (v/v) from 15.1% (v/v) in the unsupplemented slurry, yet the addition of a concentration higher than 20 mM caused a lower ethanol production. This finding was similar to the results of Ingledew [12], where adding a urea concentration of 8 to 16 mM to the medium produced the maximum fermentation rate. In another study, at an incubation temperature of 17–33°C, a wheat mash slurry supplemented with urea at 16 mM decreased the fermentation time and produced more ethanol than the control [3].

Surfactants. The ethanol fermentation was also affected by a surfactant supplement in the medium. Fig. 5B shows that Tween 20 and Tween 80 at a level of 0.05–0.5% slightly enhanced the ethanol yield, whereas the addition of Triton X100 at a level of 0.5% inhibited the ethanol production. Moreover, the addition of Tween 20 and Tween 80 at a concentration more or less than 0.05–0.5% caused a decrease in the ethanol production. It has already been reported that Tween 20 and Tween 80 slightly enhance ethanol fermentation [15]. The addition of Tween 20 at 2.5 g/l is known to have several positive effects on SSF, such as increasing the hydrolysis rate of the substrate and improving the yeast fermentation [17]. However, in this study, the addition of EDTA or SDS at a level of 0.25–2 mM did not enhance the ethanol production by much, whereas all the other concentrations caused a decrease in the ethanol yield.

Metal salts. In a study by Akin-osanaiye [2], ethanol fermentation was conducted in waste *Carica papaya* by coculturing yeast and *A. niger*, where the resulting ethanol yield in a medium supplemented with 0.1% KH_2PO_4 , 0.4% CaCl_2 , 0.05% MgSO_4 , 0.1% Na_2SO_4 , or 0.1% $(\text{NH}_4)_2\text{SO}_4$ was higher than that in the unsupplemented slurry [2]. In this study, different metal salts, such as MgCl_2 , MnCl_2 , CuSO_4 , CoCl_2 , FeSO_4 , ZnSO_4 , KCl , CaCl_2 , and NaCl , were individually used to supplement the RWC slurry at a final concentration of 0.25 mM. Fig. 5C shows that both MnCl_2 and CuSO_4 stimulated the ethanol production rate and enhanced ethanol production, whereas the other metal salts had either no effect or a negative effect on the ethanol fermentation. Since CuSO_4 had the most effect on improving the ethanol production, the effect of different concentrations of 0.15–2.5 mM of CuSO_4 were examined. Whereas the addition of CuSO_4 at a final concentration of 0.15–0.5 mM enhanced the ethanol production, all other concentrations had either no effect or a negative effect on the ethanol production. It has been previously suggested

that the uptake of metal ions by yeast can be influenced by a number of environmental and experimental factors [6]. In a study by Azenha *et al.* [4], the addition of 0.5 to 1 mM of Cu^{2+} to a yeast nitrogen base medium doubled the yield of ethanol when compared with the unsupplemented medium. It has also been reported that although Cu^{2+} is essential to all organisms and is a constituent of some enzymes, such as cytochrome oxidase in the mitochondria, superoxide-dismutase in the cytosol, and ferroxidase in the plasma membrane, Cu^{2+} has toxic effects at higher concentrations [25]. Moreover, high concentrations of the metal ions Na^+ , K^+ , Ca^{2+} , and Mg^{2+} are known to inhibit the growth of yeast cells [9]. In the present study, with 0.15–0.5 mM of K^+ , Zn^{2+} had a negative effect on the ethanol production.

Ethanol Fermentation in Uncooked RWC Under Optimal Conditions

Since the present results found that urea, yeast extract, and tryptone were equally effective in improving the ethanol production, urea was used as the nitrogen additive in the final experiments, as it is a more economical nitrogen source than the others. In addition, CuSO_4 was selected as the metal salt additive to improve the ethanol production. Fig. 6 shows that the RWC slurry supplemented with both urea and CuSO_4 produced 16.8% (v/v) ethanol, which was

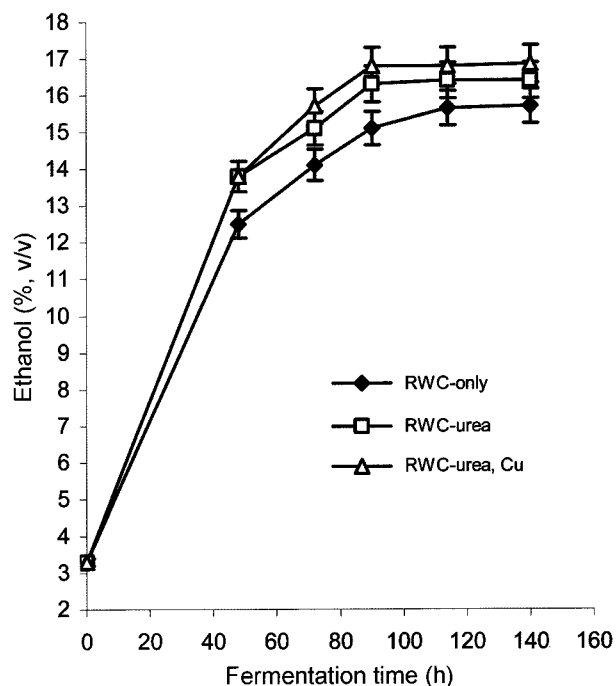


Fig. 6. Ethanol production from uncooked RWC supplemented with urea and Cu^{2+} by SSF.

RWC only: SSF of uncooked RWC slurry without additives; RWC+urea: SSF of uncooked RWC slurry supplemented with 17 mM urea; RWC+urea+ Cu^{2+} : SSF of uncooked RWC slurry supplemented with 17 mM urea and 0.25 mM Cu^{2+} .

significantly higher than the amounts produced in the urea-supplemented slurry (16.3%, v/v) and unsupplemented slurry (15.1%, v/v) after 90 h of fermentation ($P < 0.05$). The time required to complete the fermentation also decreased markedly to 90 h from 114 h with the urea-supplemented medium and the urea- and metal-salts-supplemented medium. It would seem that a medium supplemented with a combination of urea and other additives for maximal ethanol production in a short fermentation time depends on both the medium and the yeast strain [22].

In conclusion, the current results demonstrated that ethanol could be directly produced from the raw starch of RWC by SSF when using the RSDE of *Rhizopus* sp. and *S. cerevisiae* KV25. Under optimal SSF conditions for the uncooked RWC, the ethanol production was improved to 16.8% from 15.1% (v/v) pre-optimization within 90 h of fermentation.

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