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Bioethanol production from sweet potato by co-immobilization of saccharolytic molds and *Saccharomyces cerevisiae*

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ABSTRACT

To investigate the bioethanol production from sweet potato, the saccharification and fermentation conditions of co-immobilization of saccharolytic molds (*Aspergillus oryzae* and *Monascus purpureus*) with *Saccharomyces cerevisiae* were analyzed. The immobilized yeast cells showed that at 10% glucose YPD (yeast extract peptone dextrose) the maximum fermentation rate was 80.23%. Viability of yeasts cells were 95.70% at a final ethanol concentration of 6%. Immobilization enhanced the ethanol tolerance of yeast cells. In co-immobilization of *S. cerevisiae* with *A. oryzae* or *M. purpureus*, the optimal hardening time of gel beads was between 15 and 60 min. Bioethanol production was 3.05–3.17% (v v^{-1}) and the $Y_{E/S}$ (yield of ethanol production/starch consumption) was 0.31–0.37 at pH 4, 30 °C and 150 rpm during 13 days fermentation period. Co-immobilization of *S. cerevisiae* with a mixed cultures of *A. oryzae* and *M. purpureus* at a ratio of 2:1, the bioethanol production was 3.84% (v v^{-1}), and the $Y_{E/S}$ was 0.39 for a 11 days incubation. However a ratio of *A. oryzae* and *M. purpureus* at 1:2 resulted a bioethanol production rate of 4.08% (v v^{-1}), and a $Y_{E/S}$ of 0.41 after 9 days of fermentation.

1. Introduction

In most developing countries, energy depends on imports and more than 90% of total energy comes from non-renewable fuel sources. This relationship causes pressure on oil supply, emission of CO_2 to the atmosphere, inducing climate change environmental pollution [1]. Therefore, bioenergy (eg. bioethanol) is considered as one of the key renewable energy resources in the future, with economic and environmental benefits [2–5]. Worldwide bioethanol production is dominated by Brazil and the USA. In recent years, the development and application of bioethanol from sweet potato is the main goal of Taiwan Renewable Energy Policy, as the advantages of sweet potato are its easy growth, adaptation to many farming conditions and prices are more stable than other agricultural major energy crops [6–8].

The ethanol fermentation processes from starchy materials commonly involves two stages [9]: (i) liquefaction of starch by α-amylase and enzymatic saccharification of the low molecular weight liquefaction products such as dextrin to produce glucose; (ii) fermentation of glucose to ethanol. It has been estimated that the energy input of the first stage is about 30–40% of the total energy during bioethanol production from starch for high temperature (around 90 °C) to precook and dissolve the particles [10]. The development a process for simultaneous liquefaction, saccharification and fermentation of starch would reduce the energy input and increase the efficiency of substrate utilization [11]. Many researchers have been attempted to combine the two-stage fermentation process in a single-step [12,13]. Co-culturing methods have also been used, but not on an industrial scale. Because the two strains used in co-cultures do not always have similar culture requirements such as pH, temperature, nutrient, oxygen demand, etc [14], it is very difficult to optimize the conditions for one strain without affecting the other strains. Therefore, co-immobilization different kinds of microorganisms within the same porous matrix by co-immobilization and combination two-stage fermentation process in a single-step can reduce the energy input and resolve the above mentioned problem. The purpose of this study was to develop and evaluate a simultaneous single-step system for bioethanol fermentation from sweet potato starch using a co-immobilization method for the aerobic fungi (*Aspergillus oryzae* or *Monascus purpureus*) and the facultative anaerobic yeast (*Saccharomyces cerevisiae*) under limited aerobic culture conditions without imposing special artificial conditions.

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2. Materials and methods

2.1. Tested organisms and culture media

A. oryzae BCRC 30289, *M. purpureus* BCRC 31615, and *S. cerevisiae* BCRC 21494 were obtained from Bioresource Collection and Research Center, Taiwan. The amylase-producing molds were cultured on media containing (%, w v^{-1}) sweet potato starch, 5.0; $NaNO_3$, 0.3; KH_2PO_4, 0.3; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.1; $Fe_2(SO_4)_3 \cdot 7H_2O$, 0.001; agar, 1.5 at 30 °C and pH 5.0. *S. cerevisiae* BCRC 21494 was cultivated on YPD agar containing (%, w v^{-1}) yeast extract, 1.0; peptone, 2.0; dextrose, 2.0; agar, 1.5 at 30 °C and pH 5.0. The ethanol fermentation medium was made from (%, w v^{-1}) sweet potato starch, 10.0; $NaNO_3$, 0.3; KCl, 0.01; $MgSO_4 \cdot 7H_2O$, 0.1; $Fe_2(SO_4)_3 \cdot 7H_2O$, 0.001.

2.2. Immobilization and co-immobilization

S. cerevisiae was mixed with 2.5% (w v^{-1}) Na-alginate solution and the slurry culture was added dropwise into a 6% (w v^{-1}) $CaCl_2$ solution using a 50 mL syringe [15]. Once the slurry was added to the solution, beads of Ca-alginate with entrapped cells were formed with a mean diameter of 3–4 mm. For co-immobilization, *S. cerevisiae* and *A. oryzae* or *M. purpureus* were mixed together and immobilized as described above.

2.3. Culture conditions

The immobilized or free cells of *S. cerevisiae* were cultured in YPD broth with the addition of 10% (w v^{-1}) glucose at pH 5.0, 30 °C, and 150 rpm. Ethanol was added initially to the broth ranging from 0 to 10% (v v^{-1}). For ethanol fermentation, the co-immobilization gel beads (2 or 3 strains of microorganisms) were incubated at pH 4.0, 30 °C, and 150 rpm. To achieve limited aerobic conditions, flasks were fitted with a one-hole silicon stopper into which a cotton-plugged Pasteur pipette was inserted to vent out CO_2 during fermentation [16].

2.4. Analytical methods

Total cell counts and viability of yeast cells were determined by methylene blue method [17]. A 1 mL of yeast suspension was mixed with 9 mL of modified Ringer solution (%, w v^{-1}): NaCl, 0.86; KCl, 0.03; $CaCl_2 \cdot 2H_2O$, 0.044; $Na_2S_2O_3 \cdot 5H_2O$, 0.05; sucrose, 1.0; methylene blue, 0.025. After 5 min incubation, the mixture was shaken and placed in a Thomas counting chamber. Non-viable cells stained blue. The percentage of cell viability was expressed as the number of unstained cells divided by the total number of cells (stained plus unstained). For immobilized yeast cells, 1 g of beads were agitated in 10 mL of 1% (w v^{-1}) sodium citrate buffer for 1 h in order to release the cells [18]. The ethanol concentration in the culture broth was determined by gas chromatography using a Shimadzu Model GC 14A equipped with a flame ionization detector. The column (0.26 cm i.d., 2 m length) was packed with Porapak Q (80–100 mesh) and N_2 was used as carrier gas. Both the injector and detector temperatures were maintained at 230 °C, and the column temperature was adjusted to 175 °C. Total residual sugars were determined by DNS method [19]. The starch concentration was measured colorimetrically using iodine [20]. Sample preparation prior to scanning electron microscopy, involved washing of the gel beads two times with 0.025 M Na-cacodylate buffer (pH 6.8) for 10 min and pre-fixed for 3 h in the same buffer containing 2.5% (v v^{-1}) glutaraldehyde. After washing three times with Na-cacodylate buffer for 10 min, samples were placed for 1.5 h in the same buffer containing 1.0% (w v^{-1}) OsO_4. Dehydration of the samples was carried out through exposure to ethanol (10–100%) for 10 min, and subsequent transfer to a critical point drier. The dry beads were sliced with a razor blade, coated with gold, and examined under a scanning electron microscope [21].

3. Results and discussion

3.1. Effect of initial ethanol concentration on ethanol tolerance of free and immobilized yeast cells

The effects of initial ethanol concentration on cell viability, ethanol production, and fermentation efficiency of free and immobilized yeast cells are shown in Fig. 1. The viability of free cells decreased sharply from 86.67 to 64.36% when the initial ethanol concentration increased from 6 to 8%. In contrast, the viability of immobilized cells declined only from 95.10 to 92.50%. Free cells were more sensitive to ethanol than immobilized cells. Ethanol production of free and immobilized cells were 4.93 and 4.91% (v v^{-1}) without ethanol supplementation and decreased to 0.68 and 1.80% (v v^{-1}) at 10% ethanol supplementation, respectively. The ethanol production rate of free and immobilized cells also decreased from 1.72 and 1.67 g L^{-1} h^{-1} without ethanol supplementation to 0.30 and 0.74 g L^{-1} h^{-1} with 10% ethanol supplementation, respectively. The fermentation efficiencies of immobilized cells were higher than those of free cells, achieving values of 96.48 and 96.09% without the addition of ethanol. However, the addition of 10% ethanol to the medium resulted in a decline to 13.31 and 35.23% for free and immobilized cells. This finding suggests that immobilization increases the ethanol tolerance capacity, enhances the ethanol production, and improves the fermentation efficiency of yeast cells.

However, the accumulation of ethanol during fermentation reduces cell growth, cell viability and ethanol production. These

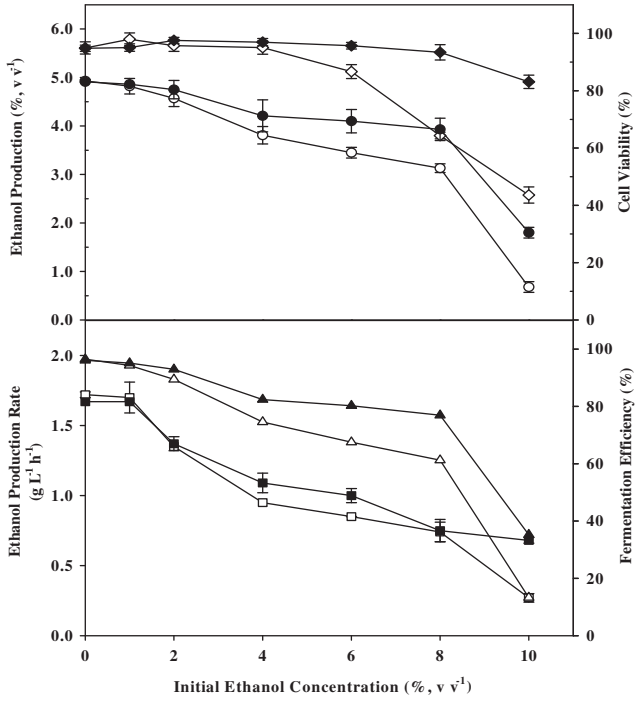

Fig. 1. Effect of initial ethanol concentrations on ethanol tolerance of free (opened) and immobilized cells (closed) of *S. cerevisiae*. Symbol: (○,●) ethanol production; (◇,◆) cell viability; (□,■) ethanol production rate; (△,▲) fermentation efficiency.

phenomena were also observed by Casey and Ingledew [22], Nowak [23], and Cot et al. [3]. These findings highlight the importance in enhancement ethanol tolerance of yeast cells especially for industrial production. Higher cell viabilities in immobilized yeast cells could be explained by the reduction of oxygen diffusion in the carrier, causing a reduction of unsaturated fatty acids in cell membrane of yeasts [24]. However, the presence of higher concentrations of unsaturated fatty acids is a result of the increasing in membrane fluidity and ethanol concentrations [25–27]. Nagar-Legmann and Margalith [28] suggested that high membrane fluidity might be involved in the entrapment of ethanol molecules within the hydrophobic area of the membrane, and therefore interfering with free movement out of the cell.

3.2. Hydrolysis of sweet potato starch by immobilized *A. oryzae* and *M. purpureus*

Sweet potato starch hydrolysis by immobilized *A. oryzae* or *M. purpureus* in Ca-alginate gel beads are shown in Fig. 2. The starch hydrolysis $Y_{p/s}$ (yield of residual sugar/starch consumption) was the highest for a 1 day incubation using 0.3 g dry mycelium of *A. oryzae*, and for a 2 days incubation using 0.1–0.2 g dry mycelium, and then decreased gradually. In immobilized *M. purpureus*, gels with 0.05 g dry mycelium had a maximum $Y_{p/s}$ 0.57 for a 4 days incubation. This suggests that an increase in immobilized mycelium concentration reduces the incubation time and therefore reaches a higher $Y_{p/s}$. In $Y_{E/s}$, the immobilized gels with 0.1 and 0.2 g dry mycelium achieved a higher ethanol production on substrate consumption compared to 0.05 g dry mycelium. In the initial stage, substrate is consumed for biomass production; while in the latter stage, substrate is used for ethanol production. From the $Y_{p/s}$ and $Y_{E/S}$ studies, the optimum mycelium weight of *M. purpureus* was 0.1 g.

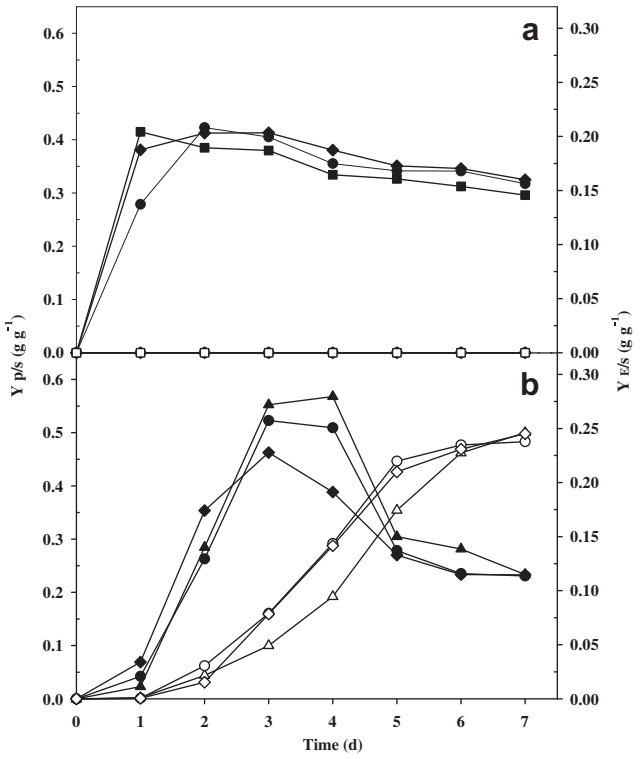

Fig. 2. Effect of mycelium weight on starch saccharification (closed) and ethanol production (opened) in immobilization of *A. oryzae* (a) or *M. purpureus* (b). Symbol: (△) 0.05 g; (○) 0.1 g; (◇) 0.2 g; (□) 0.3 g. $Y_{P/S}$ and $Y_{E/S}$ observed yield of residual sugar and ethanol production on starch consumption, respectively.

3.3. Effect of co-immobilized gels hardening time on ethanol production

Longer gel hardening time causes the inner structure of gel to become more rigid and impedes the substrate transference [29]. In co-immobilization studies using a 10–15 min hardening time for 0.2 g *A. oryzae* mycelium and 5×10^8 cells mL^{-1} *S. cerevisiae*, starch hydrolysis was the best with residual starch at the end of fermentation being 0.40–0.43% (w v^{-1})(Fig. 3). Residual starch was over 1% (w v^{-1}) with a 30–60 min hardening time. Residual sugar accumulated quickly at the initial stage of fermentation, but it was fermented to ethanol by yeast after 4 days and causing a reduction of the residual sugar. When the gel hardening time was over 30 min, substrate transfer through the bead matrix could have been reduced and therefore negatively impacted the starch hydrolysis. *A. oryzae* with a high growth strain was unable to ferment ethanol immediately. The maximum ethanol production was 2.03, 2.02, 0.87, 0.88 and 0.86% (v v^{-1}) with gel hardening time 10, 15, 30, 60, and 75 min, respectively. Although, the ethanol production was the highest with hardening time 10 min, and the $Y_{E/s}$ was 0.21. However, there is a positive correlation between hardening time and gel strengths [30]. High mechanical strength could make more variances during ethanol fermentation. The optimum gel hardening time was 15 min causing enhanced gel structure.

In co-immobilization studies of 0.1 g *M. purpureus* mycelium and 5×10^8 cells mL^{-1} *S. cerevisiae*, a hardening time of 60 and 75 min improved starch hydrolysis, and the residual starch was 1.51 and 1.33% (w v^{-1}) at the end of fermentation. However, the residual starch was over 2% (w v^{-1}) with a hardening time of 15 and 30 min (Fig. 3b). Residual sugar was the lowest indicting that no inhibition of substrate up-take was present. However, the ethanol production was only between 2 and 3% for a 15 and 40 min hardening time. Because of *M. purpureus* is a slow growing strain, cells do not form a dense layer near the gel surface, causing oxygen restrictions to the inner area of gel. Residual sugar accumulated with hardening times of 60–75 min after a 5 days fermentation, and the ethanol production increased sharply. The maximum ethanol production for a hardening time of 60 and 75 min were 2.81 and 2.90% (v v^{-1}), and the $Y_{E/s}$ were 0.33 and 0.33 for a 10 days incubation, respectively. There was not conspicuously different between two hardening times. The optimum gel hardening time was 60 min.

3.4. Relationship between yeast concentration and ethanol production in co-immobilization

The effects of yeast cell concentrations during co-immobilization on ethanol yield are shown in Fig. 4. Increasing the yeast cell concentration resulted in a decline of residual sugars during a 2–4 days incubation. The maximum ethanol production and $Y_{E/s}$ were 3.05% (v v^{-1}) and 0.31 for a 13 days incubation with yeast concentration 5×10^6 cells mL^{-1}, respectively. These results indicated that the growth of *A. oryzae* affected ethanol production when the yeast concentration was below 5×10^6 cells mL^{-1}. However, most of the sugar was converted to mycelium mass when the yeast concentration was greater than 5×10^6 cells mL^{-1}. The ethanol production was 2.55% (v v^{-1}) and $Y_{E/s}$ was 0.26. Residual sugar was mainly utilized for cell maintenance rather than ethanol production. Growth of yeast was superior to that of the *A. oryzae* impacting starch hydrolysis negatively [31]. Therefore, the co-immobilized gel beads containing 0.2 g *A. oryzae* mycelium and 5×10^6 cells mL^{-1} *S. cereviaise* proved to be the best for ethanol production.

In co-immobilization studies using 0.1 g *M. purpureus* mycelium and 5×10^5–10^7 cells mL^{-1} yeast, residual sugars accumulated at the initial stage of fermentation but decreased after 3 days. The residual sugar was utilized quickly in the fermentation process

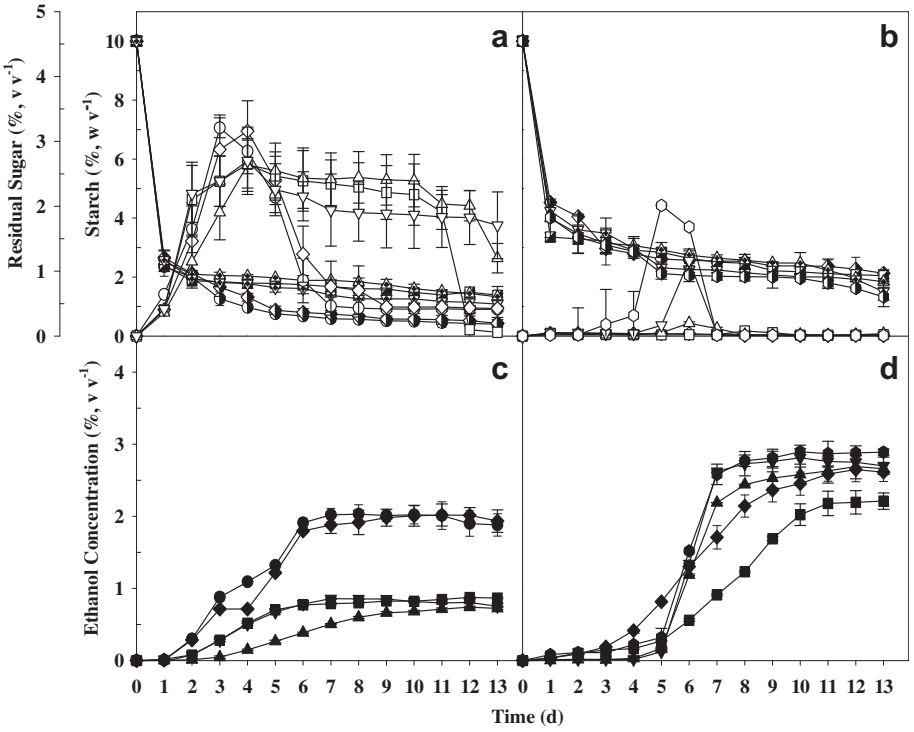

Fig. 3. Effect of gel hardening time on ethanol production in co-immobilization of *A. oryzae* (a,c); *M. purpureus* (b,d) and *S. cerevisiae*. Semi-filled, starch; opened, residual sugar; closed, ethanol concentration. Symbol: (○) 10 min; (◇) 15 min; (□) 30 min; (△) 45 min; (▽) 60 min; (⬡) 75 min.

when the yeast concentration was increased to 5×10^9 cells mL^{-1}. However, the ethanol production was only 0.92% (v v^{-1}), with the sugars being utilized for cell maintenance of *M. prupureus*, therefore limiting the available carbon for growth and ethanol production. The same phenomenon was also described by Tanaka et al. [31]. In addition, the rate of starch hydrolysis was slower than the consumption of sugar. Residual starch was therefore 2.14% (w v^{-1}). The maximum ethanol production was 3.17% (v v^{-1}) and $Y_{E/S}$ was 0.37 after 13 days fermentation for a yeast concentration of 5×10^6 cells mL^{-1}.

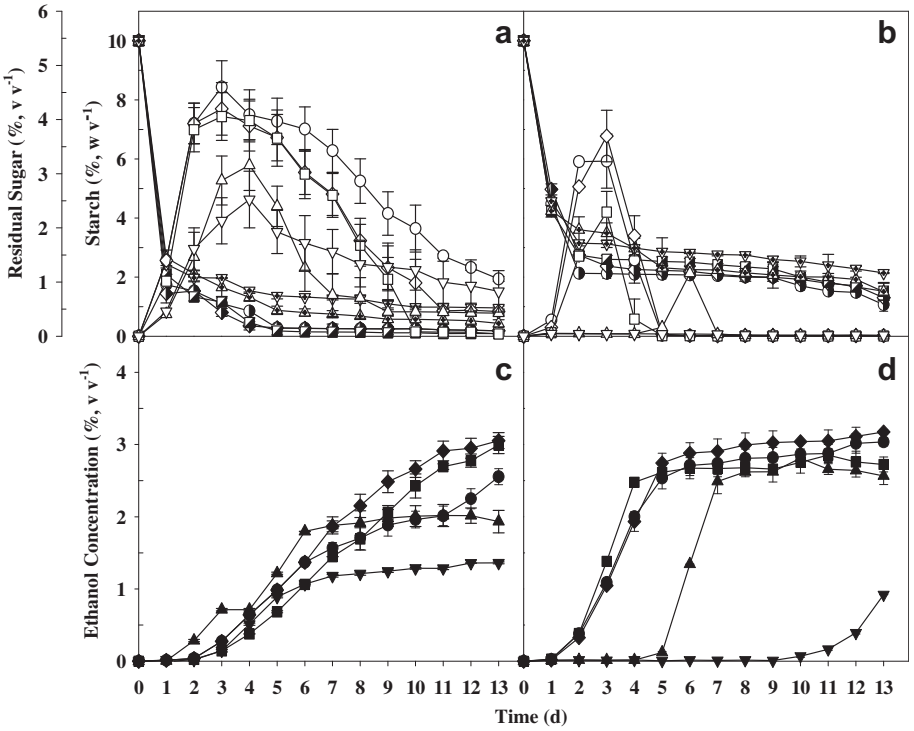

Fig. 4. Relationship between yeast concentration and ethanol production in co-immobilization of *S. cerevisiae* and *A. oryzae* (a,c); *M. purpureus* (b,d). Semi-filled, starch; opened, residual sugar; closed, ethanol concentration. Symbol: (○) 5×10^5 cells mL^{-1}; (◇) 5×10^6 cells mL^{-1}; (□) 5×10^7 cells mL^{-1}; (△) 5×10^8 cells mL^{-1}; (▽) 5×10^9 cells mL^{-1}.

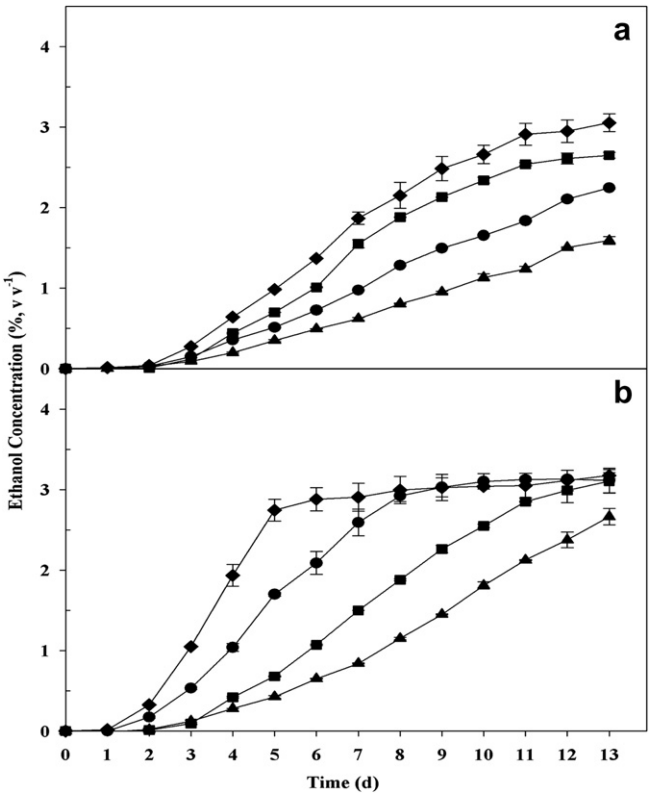

Fig. 5. Effect of initial pH on ethanol fermentation process in co-immobilization of *A. oryzae* (a); *M. purpureus* (b) and *S. cerevisiae*. Symbol: (●) pH 3.0; (◆) pH 4.0; (■) pH 5.0; (▲) pH 6.0.

3.5. Effect of initial pH on ethanol fermentation in co-immobilization

The pH value of media is a very important factor for amylolytic enzymes as well as ethanol production [32]. The co-immobilization of *A. oryzae* and *S. cerevisiae* showed that a maximum ethanol production of 3.05% (v v^{-1}) could be achieved at an initial pH 4.0, followed by 2.65% (v v^{-1}) when the initial pH was 5.0 (Fig. 5). The ethanol production reduced gradually when the pH value was lower than 4.0 or higher than 5.0. The ethanol production at pH 3.0 and pH 6.0 were 2.37 and 1.59% (v v^{-1}) and the residual sugars were 2.38 and 3.21% (w v^{-1}) (data not shown), respectively. These results indicated that yeast cells could not ferment sugar to ethanol at these pH ranges, and the sugars were mainly of non-fermentable nature. The optimum pH of α-amylase is 5.0–8.0 [33], and that of glucoamylase is 4.0–5.0 [34]. Activities of such enzymes involved in the cleavage of polysaccharide chains are reduced at pH 3.0 and 6.0. Starch was only hydrolyzed by acid at an initial pH of 3.0. The co-immobilization of *M. purpureus* and *S. cerevisiae* resulted in enhanced consumption of residual sugars at all tested pH, and the ethanol production rates showed a different picture. The initial pH 4.0 gave the highest ethanol production of 3.17% (v v^{-1}), followed by 3.11% (v v^{-1}) at pH 3.0, and 2.66% (v v^{-1}) at pH 6.0. The highest ethanol yield and production rate were achieved at an initial pH 4.0.

3.6. Scanning electron micrograph of co-immobilized gel beads

In co-immobilization gel beads of *S. cerevisiae* and *A. oryzae* or *M. purpureus*, the gel beads surface (Figs. 6a and 7a) as well as the inner bead surface (Figs. 6c and 7c) were smooth before the incubation with tested microbes. Irregular growth of microorganisms was observed on the outer gel beads surface (Figs. 6b and 7b) or the inner surface (Figs. 6d and 7d) after 13 days of fermentation. No overgrown of felt-

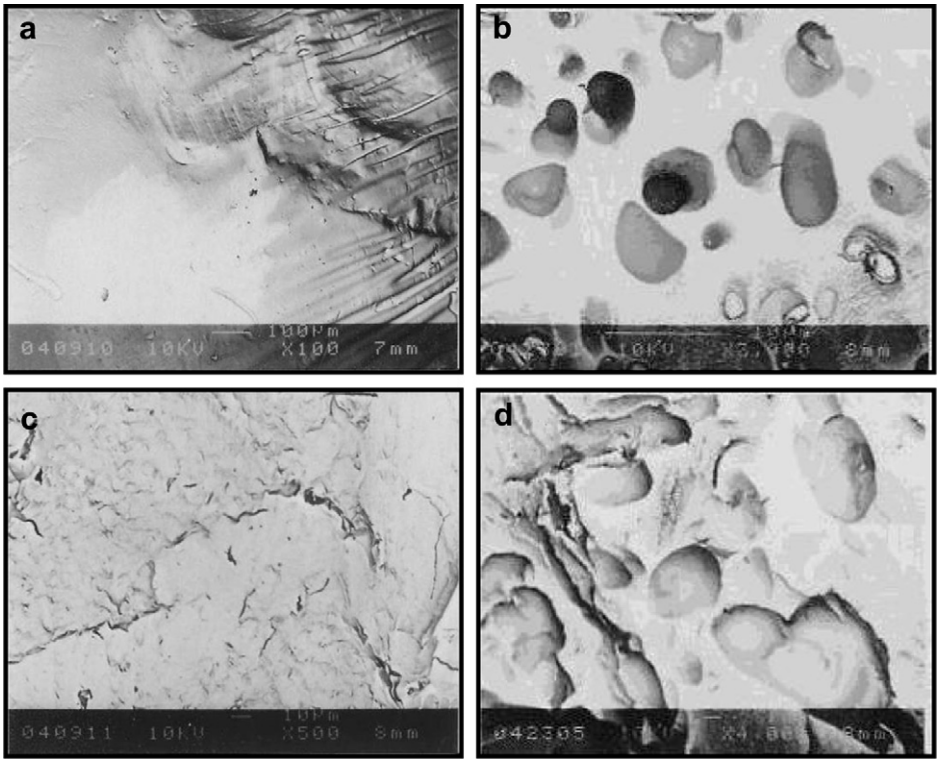

Fig. 6. Scanning electron microscopic images of the surface and inner co-immobilized gel beads of *A. oryzae* and *S. cerevisiae*. (a) surface of the fresh beads (100×); (b) surface of the used beads after 13 days incubation (3000×); (c) inner of the fresh beads (100×); (d) inner of the used beads after 13 days incubation (4000×).

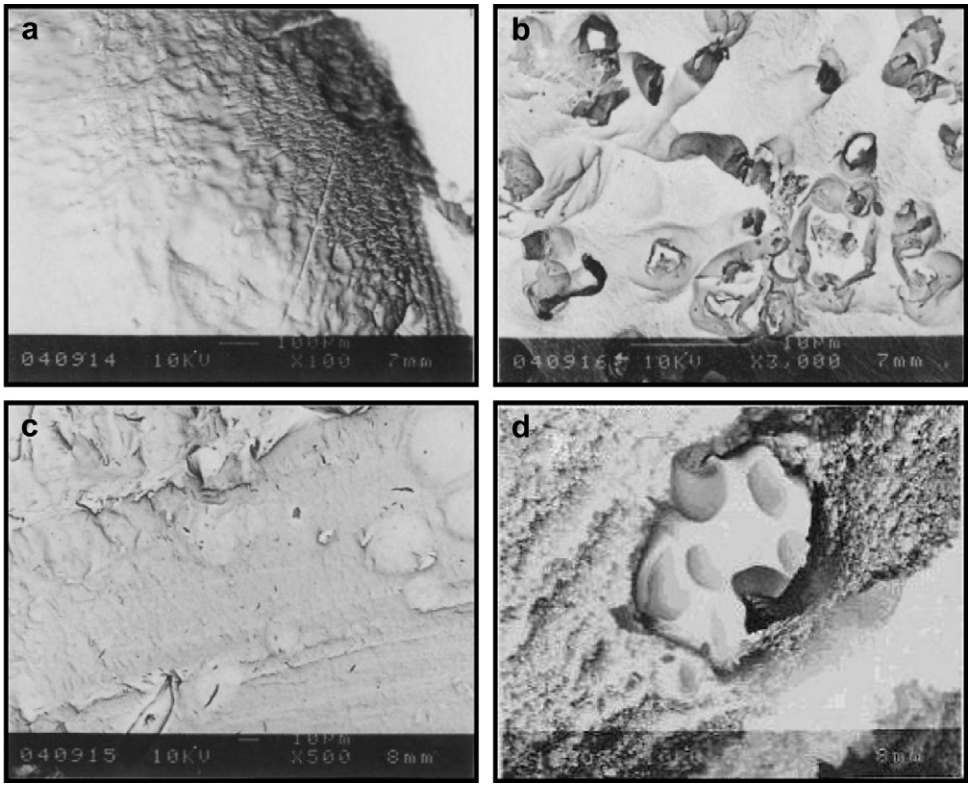

Fig. 7. Scanning electron microscopic images of the surface and inner co-immobilized gel beads of *M. purpureus* and *S. cerevisiae*. (a) surface of the fresh beads (100×); (b) surface of the used beads after 13 days incubation (3000×); (c) inner of the fresh beads (100×); (d) inner of the used beads after 13 days incubation (4000×).

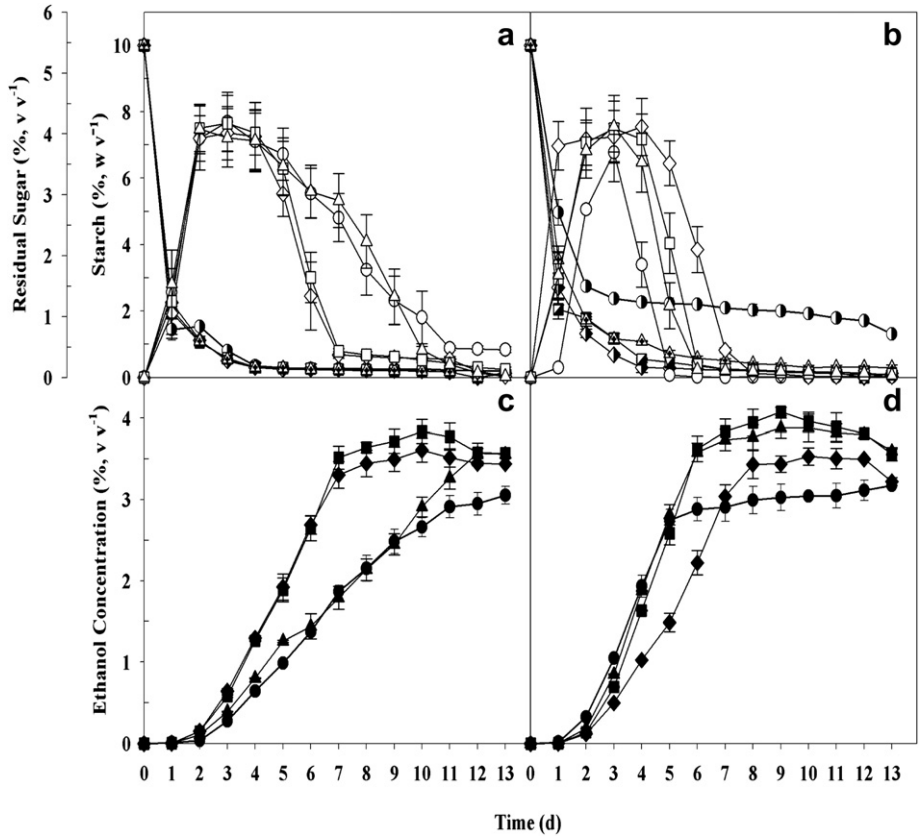

Fig. 8. Effect of mixed molds ratio co-immobilized with *S. cerevisiae* on ethanol fermentation. (a,c) total mycelium weight was 0.2 g and the ratio of *A. oryzae* and *M. purpureus* was (◇) 1:1; (□) 2:1; (△) 3:1; (○) only *A. oryzae*; (b,d) total mycelium weight was 0.1 g and the ratio of *A. oryzae* and *M. purpureus* was (◇) 1:1; (□) 1:2; (△) 1:3; (○) only *M. purpureus*. Semi-filled, starch; opened, residual sugar; closed, ethanol concentration.

like mold mycelium layer was seen in co-immobilized gel beads. The results showed that *A. oryzae* or *M. purpureus* mainly grew on the oxygen-rich surface area of gel beads, whereas *S. cerevisiae* grew mainly in the oxygen-deficient inner part and to a lesser extent also on the oxygen-rich surface area. Kurosawa et al. [12] also described that microorganisms in gel beads during ethanol fermentation formed a similar pattern as described above.

3.7. Co-immobilization of mixed molds and S. cerevisiae

The above results showed that co-immobilization of 0.2 g *A. oryzae* mycelium with 5×10^6 cells mL^{-1} *S. cerevisiae* had high starch hydrolysis with residual starch concentrations being 0.19% (w v^{-1}). However ethanol production was only 3.05% (v v^{-1}) due to higher cell number of *A. oryzae* on the gel beads surface causing a restriction of residual sugar in the inner part of the cell beads and therefore reducing ethanol production. As a result of this most sugars were used for cell growth and maintenance rather than ethanol production. In order to improve the ethanol fermentation yields, mixed cultures of *A. oryzae* and *M. purpureus* at a ratio of 1:1, 2:1 and 3:1 were used. Fig. 8a showed the residual starch to be 0.13, 0.13 and 0.15% (w v^{-1}) for the ratio 1:1, 2:1 and 3:1, respectively. Similar results were found in the co-immobilization of *A. oryzae* and *S. cerevisiae*. The mixed cultures with *M. purpureus* did not affect the starch hydrolysis. The maximum ethanol production was 3.84% (v v^{-1}) and $Y_{E/S}$ was 0.39 when the A:M (*A. oryzae*:*M. purpureus*) mixed ratio was 2:1 for a 11 days fermentation. The ethanol yield was higher than for a co-immobilization of *A. oryzae* and *S. cerevisiae*.

Co-immobilization of 0.1 g *M. purpureus* mycelium with 5×10^6 cells mL^{-1} *S. cerevisiae* had high ethanol production. However, the starch hydrolysis rate was slower than the sugar consumption. The residual starch concentration was 1.30% (w v^{-1}) and the ethanol yield was low. To increase the ethanol production, the starch hydrolysis rate was adjusted with a A:M ratio of a total mycelium weight of 0.1 g. This caused the residual starch drop to 0.13, 0.13 and 0.30% (w v^{-1}) with the A:M ratio of 1:1, 1:2 and 1:3, respectively (Fig. 8b). The starch hydrolysis increased with the mixing culture of *A. oryzae*. The maximum ethanol production was 4.08% (v v^{-1}) and $Y_{E/S}$ was 0.41 when the A:M ratio was 1:2 after 9 days fermentation. This result was better than for a co-immobilization of *M. purpureus* only.

From this investigation, the simultaneous fermentation of sweet potato starch to ethanol can be achieved through the co-immobilization of *S. cerevisiae* and *A. oryzae*, *M. purpureus* or both. The mixed blends of molds and *S. cerevisiae* gave the highest ethanol yields and the shortest fermentation times. It is a potential process for ethanol production from sweet potato starch.

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