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Cassava as a cheap source of carbon for rhizobial inoculant production using an amylase-producing fungus and a glycerol-producing yeast

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Summary

The aim of this research was to develop methods to use low-cost carbon compounds for rhizobial inoculant production. Five raw starch materials; steamed cassava, sticky rice, fresh corn, dry corn and sorghum were tested for sugar production by an amylase-producing fungus. Streamed cassava produced the highest amount of reducing sugar after fermentation. *Bradyrhizobium japonicum* USDA110, *Azorhizobium caulinodans* IRBG23, *Rhizobium phaseoli* TAL1383, *Sinorhizobium fredii* HH103, and *Mesorhizobium ciceri* USDA2429 were tested on minimal medium supplemented with reducing sugar obtained from cassava fermentation. All strains, except *B. japonicum* USDA110, could grow in medium containing cassava sugar derived from 100 g steamed cassava per litre, and the growth rates for these strains were similar to those in medium containing 0.5% (w/v) mannitol. The sugar derived from steamed cassava was further used for production of glycerol using yeast. After 1 day of yeast fermentation, the culture containing glycerol and heat-killed yeast cells, was used to formulate media for culturing bradyrhizobia. A formulation medium, FM4, with a glycerol concentration of 0.6 g/l and yeast cells (OD_{600} = 0.1) supported growth of *B. japonicum* USDA110 up to 3.61×10^9 c.f.u./ml in 7 days. These results demonstrate that steamed cassava could be used to provide cheap and effective carbon sources for rhizobial inoculant production.

Introduction

Legume productivity can be increased by inoculation of the seeds with rhizobia, which form effective nitrogen-fixing nodules on leguminous hosts. For effective nodulation of legumes, seeds are generally inoculated with an appropriate rhizobial strain before sowing. For industrial production of rhizobial inoculants, it is important to identify inexpensive and easily available sources of nutrients for culture medium. In such media preparations, a single source of carbon cannot be used for all strains, because rhizobial strains of different genera often differ in carbon utilization. Generally, fast-growing rhizobia can utilize a variety of sugars such as glucose, sucrose, maltose, whereas bradyrhizobia appear to be nutritionally fastidious (Stowers 1985). Bradyrhizobia can use arabinose, gluconate and some sugar alcohol, such as mannitol and glycerol as a preferred carbon source (Stowers 1985; Lie *et al.* 1992). Generally, mannitol is universally used for cultivation both fast- and slow-growing rhizobia (Stowers 1985).

For large-scale production of rhizobial inoculant, it is desirable to identify alternative sources of carbon that are less expensive than mannitol. It is known that raw starch material can be changed to sugar by using a biotechnological process (Chumkhunthod *et al.* 2001). Starch is composed of two α-glucan polymers, amylose and amylopectin, as linear and branched structures, respectively. Some groups of bacteria and fungi produce α-amylase, β-amylase or amyloglucosidase (Zeikus & Johnson 1991) that convert starch to maltose and glucose (saccharification). It should be possible to convert some inexpensive starch material, such as cassava tubers, sticky rice, corn or sorghum, into sugars by microbial fermentation for large-scale inoculant production of fast-growing rhizobia. It is also known from the literature that glycerol can be produced from glucose through yeast fermentation (Radler & Schutz 1982; Parekh & Pandey 1985; Vijaikishore & Karanth 1987; Romano *et al.* 1997). Glycerol production using yeast fermentation can be enhanced by adding sodium sulphite or alkaline reacting salts into medium (James 1928). Based on this information, we hypothesized that the unpurified sugars derived from microbial fermentation of low cost starch material could be used directly for producing glycerol, which could be further used as a carbon source for the large-scale production of a bradyrhizobial inoculant. The objectives of this research

were to select a suitable raw starch material and develop appropriate methods for sugar production using amylase-producing fungi, and glycerol production by yeast fermentation, and then use the unpurified sugar and glycerol to formulate a culture medium for cultivation of rhizobia and bradyrhizobia, respectively.

Materials and Methods

Rhizobial strains

Fifty-two strains of bradyrhizobia (*Bradyrhizobium japonicum* USDA110 as a reference strain), and nine strains of azorhizobia (*Azorhizobium caulinodans* IRBG23 as a reference strain) were received from the Thailand Department of Agriculture, Bangkok, Thailand. Six strains of rhizobia (*Rhizobium phaseoli* TAL1383 as a reference strain), four strains of sinorhizobia (*Sinorhizobium fredii* HH103 as a reference strain) and four strains of mesorhizobia (*Mesorhizobium ciceri* USDA2429 as a reference strain) were received from the University of Minnesota, USA.

Media for testing carbon utilization

The basal medium for rhizobia contained 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl and 0.5 g NH_4Cl/l. Rhizobial strains were grown in basal medium supplemented with various carbon sources, including succinate, malonate, tartrate, pyruvate, fumarate, gluconate, ribose, xylose, mannose, galactose, arabinose, cellobiose, raffinose, fructose, glucose, lactose, sucrose, propanediol, *myo*-inositol, sorbitol, glycerol, and mannitol. Each carbon source was prepared in 10% (w/v), and then filter sterilized and aseptically added to the basal medium to a final concentration of 1% (w/v).

Rhizobial cell preparation and cultivation on testing medium

Rhizobial starter cultures were grown in YEM until they reached 10^8–10^9 c.f.u./ml and were then washed twice with 0.9% (w/v) KCl (Stowers & Elkan 1984). Five μl cells from several dilutions were plated on agar media to determine growth on various carbon sources. The growth on different carbon sources was assessed by comparing growth on basal medium with no added carbon and YEM.

Raw starch materials and sugar production by amylase-producing fungi

Raw starch materials; fresh corn, ground-dry corn, diced cassava tuber, sorghum and sticky rice, were chosen to study sugar production by the amylase-producing fungus, *Chlamydomucor* (SUT1), which was prepared as previously described (Chumkhunthod et al. 2001). Pretreatment of raw starch material was done by steaming. Three grams of dry SUT1 inoculum was added to 50 g of starch material, and fermented at room temperature for 7 days. The reducing sugars were determined daily by using the DNS method (James 1995).

Formulation medium for rhizobia using sugar produced from selected raw starch material as the sole carbon source

Fermented cassava (100, 500 and 1000 g) was mixed thoroughly with 1 l of basal medium and the insoluble material was separated by centrifugation at $16{,}000 \times g$ for 30 min. The supernatant containing the reducing sugar was adjusted to pH 6.8 and sterilized by autoclaving at 110 °C for 40 min. The processed supernatants containing the three levels of reducing sugar were used as formulation media, FM1, FM2, and FM3.

Yeast culture

Yeast (*Saccharomyces cerevisiae*) isolate F, isolated for brewing rice by Thanit and Nantakorn (unpublished), was used for converting saccharified cassava into glycerol. The yeast culture was maintained as an agar slant in a medium modified from Lie *et al.* (1992). This medium contained 1.0 g urea, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.5 g yeast extract and 100 g glucose/l, and its pH was adjusted to 5.0 before autoclaving. To determine optimum conditions for glycerol production, the same medium was used with the following variations: (i) glucose was added as 10, 15, 20 and 40% (w/v) of medium; (ii) $CaCO_3$ was added in powder form as 3, 10, 15, and 20 g/l; and (iii) the pH of fermentation was controlled either at pH 5.0 or 7.0 without $CaCO_3$. All media were sterilized by autoclaving at 110 °C for 40 min.

Preparation of sugar solution from saccharified raw starch material for yeast fermentation

Selected raw starch material was saccharified by the amylase fungus until maximum reducing sugar was produced. One volume of distilled water was mixed well with two volumes of the saccharified raw starch material. The sample was centrifuged at $16{,}000 \times g$ for 30 min. The supernatant was collected and diluted to get optimum concentration of reducing sugar. 1.0 g urea, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 2.5 g yeast extract were added per litre of this supernatant to obtain the yeast medium. The medium was buffered with $CaCO_3$ (3 g/l) and the pH was adjusted to 7.0. Medium was sterilized by autoclaving at 110 °C for 40 min.

Preparation of yeast inoculum

A loop full of yeast collected from agar medium was inoculated into 125-ml Erlenmeyer flask containing 50 ml of normal medium as described above, and

cultured on rotary shaker for 24 h at 30 °C, 200 rev/min. This culture was used as inoculum.

Small scale yeast fermentation

Ten millilitres of yeast inoculum was added to 90 ml of medium in 125-ml Erlenmeyer flask and incubated at 30 °C for 7 days, without shaking. After fermentation, medium was autoclaved at 110 °C, 40 min for breaking the cells then centrifuged at $23{,}000 \times g$ for 5 min. The supernatant was collected for glycerol determination.

Large scale yeast fermentation

Yeast inoculum (100 ml) was added to 900 ml of medium in a 1.5-l fermentor. The temperature of fermentation was controlled at 30 °C, and agitator speed was kept at 200 rev/min. In experiment to study the effect of pH on glycerol production (without $CaCO_3$), the pH was controlled at 5.0 and 7.0 during fermentation by an automatic pH controller with addition of 3 M NaOH and 1 M Phosphoric acid. For glycerol production using saccharified cassava, $CaCO_3$ (3 g/l) was added to the medium and pH was adjusted to 7.0. The fermentation was operated as described above without automatic pH control.

Formulation medium for bradyrhizobial cultivation by using medium prefermented by yeast

The medium for bradyrhizobial cultivation composed of (in 1 l) 0.5 g NH_4Cl, 1.0 g KH_2PO_4, 0.2 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2$ and 0.04 g $FeCl_3$, supplemented with 5 g glycerol and 1 g yeast extract (Lie *et al*. 1992). In formulation experiments, sugar prepared from saccharified raw starch material was used for yeast fermentation, which fermented until a high concentration of glycerol was produced. The fermented medium was then diluted to 5 and 10% (v/v) with basal medium (without glycerol and yeast extract), and sterilized by autoclaving at 110 °C, 40 min. These processed media were used as formulation media, FM4 and FM5. All growth experiment media were adjusted to pH 6.8. The experiment was carried out in 125-ml Erlenmeyer flasks containing 50 ml of medium. 0.5 ml of starter culture was inoculated into the medium and cultured on a rotary shaker for 7 days at 28 °C, 200 rev/min. The final cell concentration was determined by using total plate count method. The formulated medium was used for cultivation bradyrhizobia in a 2000-ml Erlenmeyer flask containing 1000 ml of fermented medium.

Glycerol determination

Samples for glycerol determination were centrifuged at $23{,}000 \times g$ for 5 min. Supernatant was collected and determined the glycerol content by using glycerol determination kits (Boehringer Mannhem).

Results

Sources of carbon for different rhizobia

To identify the sources of carbon that could be utilized by strains of different fast- and slow-growing rhizobia, 22 carbon sources (see Materials and methods) were tested. Most strains of *Rhizobium* and *Sinorhizobium* could utilize most of these carbon compounds, whereas *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium* strains were fastidious carbon utilizers. Mannitol was the most favoured carbon source for all fast- and slow-growing rhizobia. Glucose and maltose, which were the breakdown products of starch, were well utilized by the most of strains in genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, but these sugars were utilized by only a few strains of *Azorhizobium* and *Bradyrhizobium*. However, all *Azorhizobium* and *Bradyrhizobium* strains could catabolize glycerol, which was produced from glucose during anaerobic fermentation by yeast.

Cassava as a cheap source for producing glucose and maltose for Rhizobium *inoculant production*

As an alternative to using glucose and maltose in the growth medium for *Rhizobium* inoculant production, we used starch, fermented with an amylase-producing fungus, as a source of glucose and maltose. Five low-cost raw starch materials; steamed cassava, sticky rice, fresh corn, dry corn and sorghum, were tested for sugar production by amylase-producing fungus SUT1 (Figure 1a). Among these, steamed cassava produced the highest amount of sugar (83.63 mg/g), which was fourfold higher than the amount of sugar obtained from fresh corn (< 20 mg/g) after 4 days fermentation. Therefore, steamed cassava was selected as the source of starch for fermentation with amylase-producing fungus to obtain reducing sugars for rhizobial cultivation. The highest yield of sugars from steamed cassava was obtained after 4 days of fermentation (Figure 1b).

Different amounts of saccharified cassava were mixed well with basal medium in order to get different concentrations of sugar. This solution was used for the rhizobial growth experiment. The results showed that the *Rhizobium, Sinorhizobium, Mesorhizobium* and *Azorhizobium* strains could grow in medium containing cassava sugar derived from 100 g steamed cassava/l, and the growth rates for these strains were similar to those in medium containing 0.5% (w/v) mannitol (Figure 2). HPLC analysis of this medium before inoculation with rhizobia showed that it contained 0.56% (w/v) glucose, 0.20% (w/v) maltose. When the amount of cassava sugar in the medium was increased to an equivalent of 500 g of steamed cassava per litre, these strains did not grow. The *Bradyrhizobium* strain did not utilize cassava sugar as a carbon source.

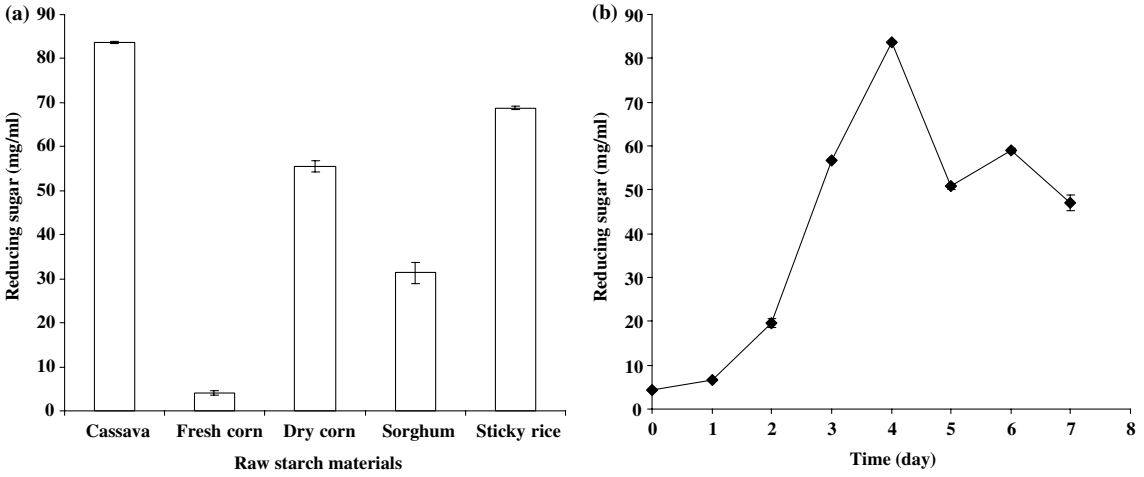

Figure 1. Reducing sugar produced from various raw starch materials (a), and time-course of reducing sugar production from cassava (b), by using amylase-producing fungus SUT1.

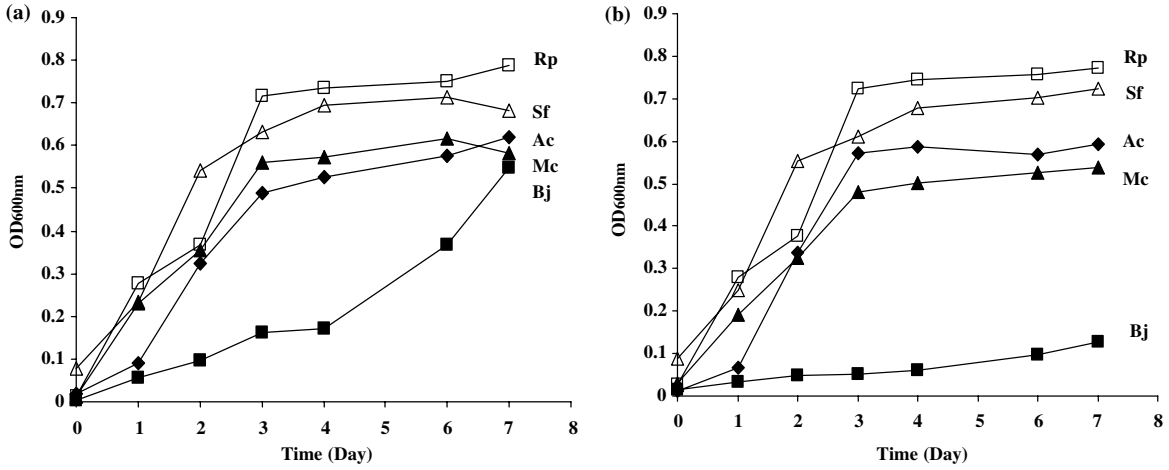

Figure 2. Growth of rhizobia in basal medium containing 0.5% (w/v) mannitol (a), and formulated medium made from 100 g of cassava per litre (b). Symbols used for different genera: *Azorhizobium* (closed diamonds); *Bradyrhizobium* (closed squares); *Mesorhizobium* (closed triangles); *Rhizobium* (open squares) and *Sinorhizobium* (open triangles).

Cassava as a cheap source for glycerol production for Bradyrhizobium inoculant production

B. japonicum USDA 110 utilizes glycerol but not glucose. To use saccharified cassava for USDA110 inoculant production, it would be necessary to convert it into glycerol. We conducted a series of experiments to determine suitable conditions for conversion of the saccharified cassava into glycerol. We determined the effect of initial glucose concentrations on glycerol yield,

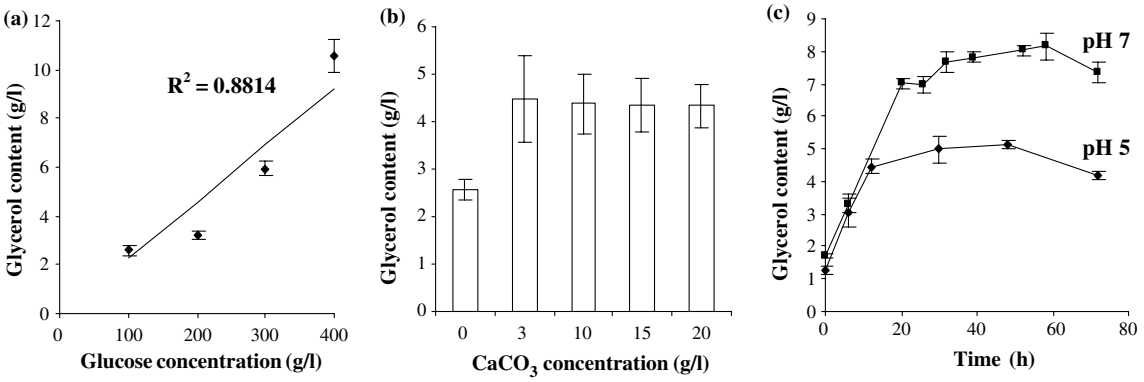

Figure 3. Glycerol production by yeast isolate *F* at different concentrations of glucose at pH 5.0 without addition of $CaCO_3$ (a), at different concentrations of $CaCO_3$ at pH 5.0 (b), and without addition of $CaCO_3$ at pH 5.0 and pH 7.0 (c).

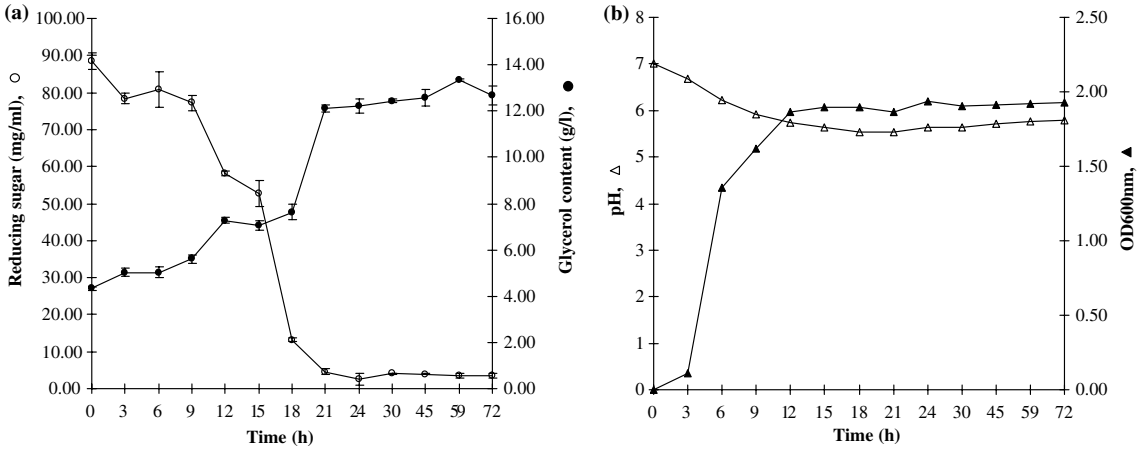

Figure 4. Time-course of sugar utilization and glycerol production (a), and the growth and pH changing by yeast isolate F in medium containing sugar made from cassava at pH 7.0 (b). Symbols: reducing sugar (open circles); glycerol (closed circles); pH (open triangles) and OD_{600} nm (closed triangles).

using yeast fermentation. Results showed that the yield of glycerol was directly correlated with the glucose concentrations (Figure 3a). A concentration of 10% (w/v) glucose in the medium, which produced a glycerol yield of 2.58 g/l, was selected for development of glycerol production in further studies, because such a concentration of glucose could be easily achieved through steamed cassava fermentation. We also determined the effect of $CaCO_3$ on the yield of glycerol from glucose fermentation, since it was known from the literature that addition of $CaCO_3$ to the medium enhanced glycerol production (Lie *et al.* 1992). The results showed that the addition of 3 g $CaCO_3$/l to the medium during yeast fermentation resulted in 67% increase in glycerol production, compared to yeast fermentation without $CaCO_3$ (Figure 3b). However, increasing the $CaCO_3$ concentration to 10 g/l or higher did not enhance glycerol production further. It is known from the literature that alkaline conditions can also enhance glycerol production from glucose fermentation (James 1928; Vijaikishore & Karanth 1987; Lie *et al.* 1992). Therefore, we conducted an experiment to determine the optimum pH condition for production of glycerol from glucose fermentation using yeast. The results showed the yield of glycerol was highest at pH 7.0 (Figure 3c).

Based on the results of the above three experiments on the amounts of glucose and $CaCO_3$, and the pH of the medium, another experiment was conducted using sugar extracted from saccharified cassava. The cassava sugar solution was added to the growth medium such that the final concentration of sugar is equivalent to 100 g/l, which is obtained from 1.2 kg of steamed cassava. $CaCO_3$ was added into this medium at a concentration of 3 g/l and the pH was adjusted to 7.0. Although the growth of *S. cerevisiae* reached a stationary phase around 12 h (Figure 4a), the conversion of sugar to glycerol continued until about 21 h (Figure 4b). The amount of reducing sugar was less than 5 g/l, and the glycerol content in medium remained at 12–13 g/l after 21 h. The pH of the medium decreased slowly from 7.0 to 5.8 during the initial 12 h, which remained unchanged till the end of the experiment (Figure 4a).

Formulation media for bradyrhizobial cultivation

The fermented medium containing 1.2–1.3% (w/v) glycerol and yeast cells was autoclaved and used as a source of nutrients for *Bradyrhizobium* inoculant production. Two formulation media, FM4 and FM5, were prepared by supplementing *Bradyrhizobium* basal medium with the fermented medium so that the final glycerol concentration became 0.6 g/l (FM4) and 1.2 g/l (FM5). *B. japonicum* USDA110 could grow well in both FM4 and FM5. After 7 days, the number of bradyrhizobial c.f.u./ml in FM4 and FM5 reached 3.61×10^9 and 4.67×10^9, respectively, compared to 1.82×10^9 c.f.u./ml in the basal medium supplemented with 5 g/l glycerol and 1 g/l of yeast extract, and 6.35×10^6 c.f.u./ml in the

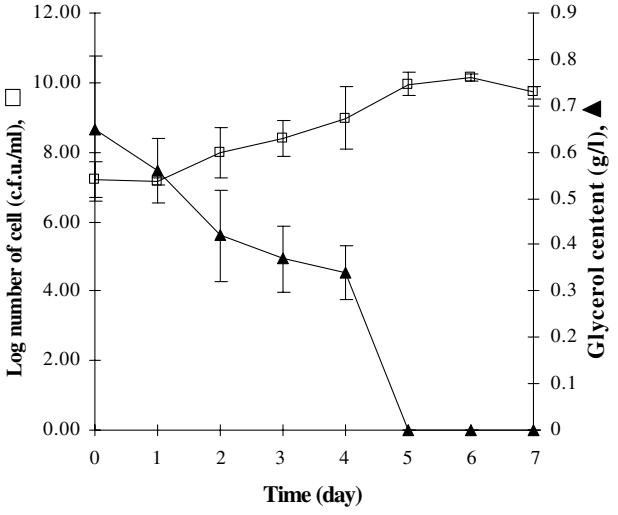

Figure 5. Time-course of bradyrhizobial growth and glycerol production in formulated medium FM4. Symbols: viable cells (open squares) and glycerol content (closed triangles).

basal medium without glycerol and yeast extract. Therefore, FM4 was further tested for bradyrhizobial cultivation using 1 l culture volume. The number of cells and glycerol consumption by bradyrhizobia were determined. The glycerol concentration dropped from 0.65 g/l at the beginning of cultivation to zero after 5 days, indicating that glycerol was completely utilized by bradyrhizobia (Figure 5). The number of bradyrhizobial cells reached 10^{10} c.f.u./ml after 6 days and decreased to 10^9 c.f.u./ml after 7 days of cultivation.

Discussion

Twenty-two carbon sources, including C_3, C_4, C_5, C_6, C_{12}, C_{18} compounds and some sugar alcohols were tested in culture media for different rhizobia. Rhizobia in genus *Rhizobium* and *Sinorhizobium* could use many kinds of carbon compounds. It has been reported that the key enzyme, invertase was detected only in cell extracts of *S. meliloti*, *R. leguminosarum* and *R. trifolii* when cells were grown with sucrose, lactose or maltose (Martinez-Drets *et al.* 1974). Similarly, C_4 compounds, such as succinate is known to support fast growth of *S. meliloti* (Ucker & Signer 1978). However, succinate is not widely used as a carbon source by bradyrhizobia (Stowers 1985). *Mesorhizobium, Azorhizobium* and *Bradyrhizobium* could use C_3, C_5 and some sugar alcohols, but the ability to use these carbon sources varied from strain to strain. Our results confirmed that sugar (glucose, maltose) and glycerol produced from raw starch material can be used as a sole carbon source for rhizobial cultivation.

Cassava was found to be a suitable source of starch material for production of sugar and glycerol, which could be directly used for rhizobial cultivation. Cassava was saccharified better than other raw starch materials. Pretreatment of raw starch material by steaming may facilitate the growth of the fungus SUT1, thereby enhancing amylase activity for saccharification. Steaming may also kill some contaminating microbes, thus reducing secondary decomposition. Therefore, the concentrations of reducing sugar increased during the growth of the fungus. However, the amount of reducing sugar was decreased after 4 days of fermentation because other contaminating microorganisms might use the sugar produced by the fungus (Chumkhunthod *et al.* 2001).

Glucose from saccharified raw starch material was used for glycerol production by yeast fermentation. Normally, ethanol and glycerol are formed in high amounts, when higher sugar concentration is used for fermentation (Radler & Schutz 1982). The fermentation of yeast at neutral pH was applied for improving the glycerol production from low concentration of glucose. $CaCO_3$ was added to maintain the pH of system, since $CaCO_3$ acts as alkaline-reacting salt, which could be dissolved more easily when acid molecules were produced during fermentation. More dissolution of $CaCO_3$ resulted in increasing the pH of medium, which allowed a high amount of glycerol production. However, addition of an excess amount of $CaCO_3$ to the medium did not increase the glycerol production further. Selection of pH for fermentation also depends on the tolerance of the yeast strain (James 1928). We observed that *S. cerevisiae* isolate F produced a low amount of glycerol at pH 8.0 because the cell density was also low at this pH. Therefore, pH 7 was selected for glycerol production, although the optimum pH for growth for *S. cerevisiae* isolate F is 5.0. The pH of system was kept constant during fermentation by automatic pH controller. The amount of glycerol produced was slightly higher in pH 7.0 than in pH 5.0.

The glycerol produced from yeast fermentation was formulated for use in the culture of bradyrhizobia. The glycerol concentration was 0.6 and 1.2 g/l in medium made from 5 and 10% (v/v) fermented stock solution, respectively. In medium made from 5% (v/v) fermented stock solution (FM4) bradyrhizobia could grow as well as in medium containing 5 g glycerol/l and 1 g yeast extract/l. Although the amount of glycerol in FM4 was only 0.6 g/l, FM4 also contained lysed yeast cells, which provided additional source of nutrients, including vitamins and amino acids. Thus, yeast cells from fermented medium could be used as a replacement of yeast extract in the medium for bradyrhizobial cultivation. Bradyrhizobia could grow up to a cell density of 10^{10} c.f.u./ml in FM4 medium.

In conclusion, the results revealed that cassava, a low cost starch material, could be used for rhizobial inoculant production. The first step of starch saccharification using amylase-fungi produced glucose and maltose, which were used by rhizobia in the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Azorhizobium* as carbon sources. Further fermentation of the saccharified starch using yeast produced glycerol, which was used as the source of carbon for bradyrhizobia. The disrupted yeast cells in the medium provided an additional source of nutrients for bradyrhizobia. Thus cassava could be used as a cheap source of carbon for rhizobial inoculant production using amylase-producing fungus and glycerol-producing yeast.

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