Screening of biosurfactant production yeast and yeast-like fungi isolated from the coastal areas of Koh Si Chang

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Abstract

The present work, we have screened biosurfactant producing yeast isolated from the east and the west coastal areas of Koh Si Chang. Sixteen isolates were preliminary screened for the potential biosurfactant producers. The quantities of biosurfactant production, measured as biosurfactant activity: oil displactment test and surfactant tension measurement. Among them, *Aureobasidium pullulans* YTP6-14 was found the highest yield of biosurfactant activity, giving the maximum oil displacement activity of 5.10 cm^2 and was able to reduce the surface tension of medium down to 38.4 mN/m. *A. pullulans* YTP6-14 was further investigated for optimizing the nutritional condition for growth and biosurfactant activity with different carbon and nitrogen sources. The highest biosurfactant activity was obtained when the organism was grown in production medium with 2.5 % (w/v) glucose supplement with 2.5 % (v/v) glycerol as carbon source and peptone as nitrogen source with C/N ratio of 300. This study showed that *A. pullulans* YTP6-14 capable of producing biosurfactant when cultivated at of optimum condition. The biosurfactant obtained was able to reduce surface tension 31.38 mN/m or 38.3 % as well as maximum oil displacement activity of 28.51 cm^2

Keywords: biosurfactant, oil displacement, surface tension, Koh Si Chang

Introduction

Biosurfactants are amphipathic compound consisting of hydrophobic and hydrophilic structure synthesized extracellular or accumulation of cell membrane by microorganisms such as bacteria, yeasts, and fungi (Pal et al., 2009). They are mainly classified into two classes: low molecular weight biosurfactant (lipopeptide, glycolipid) are having a potential in lowering the interfacial and surface tension and high molecular weight biosurfactant or bioemulsifiers are effective stabilizers of oil-in-water emulsions such as amphipathic polysaccharides, proteins, lipopolysaccharides and lipoproteins (Saharan et al., 2011; Ron and Rosenberg, 2001;Van Hamme et al., 2006). The variety of biosurfactant can also be divided based on chemical structure into five groups such as glycolipid, lipopeptides and lipoproteins, fatty acids, neutral lipids and phospholipids, polymeric surfactants and particular biosurfactants (Shafiei et al., 2013; Mukherjee et al., 2006).

In recent years, biosurfactants become more interesting as they have a potential variety for commercial applications such as oil industry and petroleum production, environmental applications, food industries, cleaning products, pharmaceutics and cosmetic industries, agricultural chemicals and the other industries. (Deleu and Paquot 2004; Shafiei et al., 2013; Pal et al., 2009; Banat et al., 2010). These microbial products are low toxicity, biodegradable, specific under activity at extreme condition (Abouseoud et al., 2008) whereas chemical surfactants such as ethoxylates, alkyl benzene sulfonates, alcohol ether sulphates, and alcohol sulphates, were indicated a low rate of biodegradation and the possible toxic

impacts of aquatic organism. (Deleu and Paquot, 2004). However, biosurfactants have been difficulty to compete with chemically synthesized compounds owing to their high production cost and low production yield (Helmy and Kardena, 2011). Therefore, it should be considered for improving the strategies for efficient bioprocesses. Many strategies recommend towards commercialization of economic regarding to biosurfactant production. One of the approaches to enhance production of biosurfactant is the optimization of culture condition that could be investigated for maximum the production of biosurfactant.

In this study we focus on the screening of the potential biosurfactant producing yeast and yeast-like fungi from coastal areas of Koh Si Chang. Sixteen strains were screened to find their potential as biosurfactant producers based on biosurfactant activities. Furthermore, the selected strain was exclusively investigated the optimization of nutritional parameter condition to enhance the biosurfactant production and growth conditions.

Methodology

Sampling and isolation

The samples were collected from seawater and organic material on the east and the west of coastal areas of Koh Si Chang. Two hundred milliliters of the seawater were filtered by 0.45 µm of membrane filters and then the membrane filters were placed on Yeast Malt Agar (YM) agar supplemented with 0.025% sodium propionate and 200 mg/l chloramphenicol. Organic samples were transferred to YM broth supplemented with 0.025% sodium propionate and 200 mg/l chloramphenicol and incubated at room temperature for 24-48 h. then transferred to new YM broth and incubated at room temperature for 24-48 h. The samples were suspended in sterile saline solution [0.85 % (w/v) NaCl]. The serial dilutions were prepared before spread onto YM agar supplemented with 0.025% sodium propionate and 200 mg/l chloramphenicol and incubated at room temperature for 24-48 h. Then the isolated colonies were collected based on morphological characteristics.

Identification of microorganism

The isolated colonies were identified the D1/D2 domain of the large subunit (LSU) rRNA gene sequences analysis determined according to the methods described by Kurtzman and Robnett (1998). The purified culture was maintained in YM broth supplemented with 10 % (w/v) glycerol and stored at -80°C.

Screening for potential biosurfactant producer.

Sixteen yeast strains were screened for their biosurfactant activity and the isolated that gave the highest yield of biosurfactant activity after cultivation on modified Hua's medium (4% glucose as carbon source, 0.4% $NaNO_3$, 0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% Yeast extract) (Thaniyavarn et al., 2008) and incubated at 30 °C on a rotary shaker at 200 rpm for 7 days. The culture was harvested by centrifugation at 8,000 rpm, 20 min. The cell free broth of each strain was measured the biosurfactant activity by using following methods.

Oil displacement test

Forty milliliters of distilled water was put into the petri dish. Fifteen microliters of crude oil was added on the surface of the distilled water to generated thin film covered surface. Ten microliters of the supernatant of each sample was dropped into the center of petri dish and observe clearly zone of oil form displaced the water to check the activity of surfactants (Morikawa et al., 2000).

Surface tension activity

Surface tension was measured by using Du Ring method (Lecomte Du Nouy, 1919) at 25 °C, which was performed using a Tensiometer (Ring tensiometer K6, KPUSS, Hamburg, Germany).

Effect of carbon and nitrogen sources on microbial growth and biosurfactant activity

Carbon source

The selected strain was grown in production medium containing 0.06 % peptone, 0.04% yeast extract, 0.5% K_2HPO_4, 0.04% $MgSO_4$.7H2O, and 0.1 % NaCl (Manitchotpisit et al., 2011) using different carbon sources: glucose, sucrose, glycerol, palm oil and soybean oil with keeping initial concentration of carbon in production medium at 5 % (w/v). The concentration of selection of carbon source was tested form 2.5-10 % (w/v).

Furthermore, the production medium with the optimum carbon source was optimized by mixed with another carbon source: glycerol, palm oil and soybean oil. The most appropriate carbon source was selected for further experiment.

Nitrogen source

Various inorganic nitrogen compounds, such as $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl and organic nitrogen compounds, such as peptone and malt extract was added in production medium with optimum carbon source. The initial nitrogen concentration was added in production medium at 0.06 % (w/v). The optimum carbon and nitrogen source was also selected at the C/N ratios of 100, 200, 300 and 400 with keeping concentration of carbon source 5 % (w/v).

Assay for growth and biosurfactant activity

The selected strain of 10% (w/v) cell inoculums was grown in 50 ml production medium supplemented with different carbon sources or nitrogen sources and incubated at 30°C on a rotary shaker 200 rpm. After cultivation for 7 days, cells were harvested by centrifugation (8,000 rpm, 20 min, 4°C) and washed twice with distilled water. Cell biomass was determined after dry cell at 105 °C for constant weights. The optimum concentration of carbon and nitrogen sources were evaluated by cell free broth gave the higher oil displacement activity and could reduce minimum value of the surface tension.

Results

Screening for the potential biosurfactant producer

Sixteen strains of yeast isolated from the east and the west of the coastal areas of Koh Si Chang were screened for the potential biosurfactant producers. All strains were cultivated in modified's Hue medium with glucose as carbon source and incubated at 200 rpm on rotary shaker at 30 °C for 7days. Among them, *Aureobasidium pullulans* YTP6-14 showed the highest biosurfactant activity, giving the maximum oil displacement activity of 5.10 cm^2 and was able to reduce the surface tension of medium down to 38.4 mN/m or 33.22% reduction as shown in Table 1. Therefore, *A.pullulans* YTP6-14 was selected and further investigated for optimizing the nutritional condition for growth and biosurfactant activity with different carbon and nitrogen sources.

Table 1 Screening for the potential biosurfactant producers measured by their biosurfactant activities.

Yeast species	**ODA[a] (cm^2)**	**min ST[b] (mN/m)**	**ΔST[c]**
Aureobasidium pullulans YTP6-14	5.10±0.63	38.4±0.40	33.22
Metschnikowia sp. YTW5-4	0.13±0.03	46.7±0.20	18.73
Candida glabrata YTP6-5	0.07±0.00	48.5±0.30	15.61
Rhodotorula mucilaginase YTD8-10	0.04±0.13	54.0±0.78	6.12
Saccharomyces cerevisiae YTU9-27	0.05±0.00	55.3±0.29	3.87
Wickernamomyces anomalus YTD8-2	0.56±0.07	42.3±0.49	23.48
Candida rugasa OTP9-2	0.19±0.01	48.6±0.55	14.75
Issatchekia terricola YTP11-68	0.14±0.01	50.3±0.53	12.51
Pichia kudravarii OTP12-2	0.11±0.02	52.7±0.12	8.29
Wickernamomyces siamensis SHN1	0.28±0.03	46.5±0.33	17.99
Candida metasilosis YTP11-8	0.07±0.03	53.3±0.55	6.39
Myerozyma caribbica LEN1	0.09±0.02	54.8±0.23	5.52
Hanseniaspora opuntiae YTU9-6	0.11±0.02	51.2±029	10.23
Debaryomyces nepalensis YTY7-1	0.32±0.06	54.6±0.36	5.04
Candida troplicalis TP1-1N5	0.05±0.02	52.3±0.43	9.13
Rhodotorula azeoricum TP1-2N10	0.87±0.07	44.2±0.12	21.19

[a] oil displacement activity
[b] minimum of surface tension
[c]% reduction of surface tension, percentage of initial surface tension of culture medium minus individual surface tension and divide by initial surface tension.

Effect of carbon sources on growth and biosurfactant production.

The carbon sources were important source on microbial growth and also production of biosurfactant. Therefore, in this study demonstrated the various carbon sources were supplemented to production medium with initial concentration of 5% (w/v). The culture was incubated at 30°C on rotary shaker 200 rpm. After cultivation for7 days, the maximum growth was obtained when *A. pullulans* YTP6-14 was cultivated on production medium supplement with glucose (8.79 g/l). Regarding the biosurfactant activity, the cell free broths were carried out. *A. pullulans* YTP6-14 cultivated in medium supplemented with glucose and glycerol gave the minimum surface tension of 35.7 and 35.42 mN/m, respectively. However, oil displacement value of medium supplemented with glycerol was only 2.59 cm^2 while the present of glucose gave the highest of oil displacement value of 6.30 cm^2 as shown in Table 2. Therefore, the medium supplemented with glucose was used for biosurfactant production by *A. pullulans* YTP6-14 and selected to further experiment.

Table 2 The effect of various carbon sources on growth and biosurfactant activities by *A. pullulans* YTP6-14 incubated at 30°C, 200 rpm for 7 days.

Carbon source	DCW[a] (g/l)	ODA[b] (cm^2)	Min ST[c] (mN/m)	ΔST[d]
Sucrose	5.84±0.02	3.57±0.29	38.48±0.8	24.8
Glucose	8.79±0.07	6.30±0.31	35.7 ±0.2	32.9
Glycerol	4.97±0.03	2.59±0.15	35.42±0.4	32.3
Soybean oil	5.54±0.01	3.49±0.22	41.49±0.7	17.6
Palm oil	5.59±0.01	5.72±0.24	40.96±0.5	22.7

[a]dry cell weight
[b]oil displacement activity
[c]minimum of surface tension
[d]% reduction of surface tension, percentage of initial surface tension of culture medium minus individual surface tension and divide by initial surface tension.

The biosurfactant production was determined by the biosurfactant activity the reduction of surfactant tension and the oil displacement test. 5% glucose was the effective concentration for the biosurfactant production. At 7 days, the surface tension and oil displacement activity of the culture broth were found to be 35.43 mN/m and 7.04 cm^2, respectively (Table 3.)

Table 3 The effect of different carbon concentrations on growth and biosurfactant activities by *A. pullulans* YTP6-14 incubated at 30°C, 200 rpm for 7 days.

Glucose concentration (%)	DCW[a] (g/l)	ODA[b] (cm^2)	Min ST[c] (mN/m)	ΔST[d]	pH
2.5	4.10±0.02	4.09±0.85	37.00±0.5	29.5	3.69
5	8.98±0.00	7.04±0.61	35.43±0.3	31.7	3.80
7.5	8.28±0.01	5.66±0.38	36.02±0.1	30.3	3.73
10	8.00±0.03	3.58±0.47	36.23±0.2	29.3	3.73

[a] dry cell weight
[b] oil displacement activity
[c] minimum of surface tension
[d] % reduction of surface tension, percentage of initial surface tension of culture medium minus individual surface tension and divide by initial surface tension.

In addition, the optimum carbon source mixed with another carbon sources such as glycerol, palm oil, soybean oil were studied. The medium that present 2.5% glucose and 2.5% glycerol gave the maximum oil displacement value and minimum surface tension. It showed the highest biosurfactant activity than the other supplemented carbon as shown in Table 4. Therefore, the study of the effect of another carbon sources and concentration on growth and biosurfactant activity by *A. pullulans* YTP6-14 was appropriate when cultivation in the production medium with 2.5% glucose and 2.5% glycerol and this medium was chosen for further experiment.

Table 4 The effect of mixed carbon and its concentration on growth and biosurfactant activity by *A. pullulans* YTP6-14 incubated at 30°C, 200 rpm for 7 days.

Ratio (%)	Mixed carbon source	DCW [a] (g/l)	ODA[b] (cm^2)	Min ST[c] (mN/m)	Δ ST[d]	pH
2.5:2.5	Glucose: Glycerol	6.84±0.01	12.67±1.08	33.62±0.4	36	3.71
	Glucose: Palm oil	6.24±0.02	0.68±0.24	41.97±0.8	18.6	3.58
	Glucose: Soybean oil	7.10±0.02	0.86±0.11	43.44±0.4	17.7	3.55
5:5	Glucose: Glycerol	7.89±0.04	3.49±0.17	34.89±0.1	33.6	3.84
	Glucose: Palm oil	5.24±0.03	1.76±0.25	42.67±0.3	17.0	3.64
	Glucose: Soybean oil	5.64±0.08	0.66±0.11	43.93±0.6	17.4	3.63

[a] dry cell weight
[b] oil displacement activity
[c] minimum of surface tension
[d] % reduction of surface tension, percentage of initial surface tension of culture medium minus individual surface tension and divide by initial surface tension.

Effect of nitrogen sources on biosurfactant activity

Regarding the nitrogen sources, various organic and inorganic nitrogen sources were used for growth and biosurfactant production in the medium with optimum carbon source. The initial nitrogen concentration was added in the production medium at 0.06 % (w/v). The results were showed in Table 5. $(NH_4)_2SO_4$ and NH_4NO_3 gave the maximum growth (9.05 g/l and 9.01 g/l, respectively). However, the biosurfactant activity was lower than peptone. Thus, peptone was selected as the appropriated nitrogen source for further experiment.

Table 5 The effect of different nitrogen sources on growth and biosurfactant activity by *A. pullulans* YTP6-14 incubated at 30°C, 200 rpm for 7 days.

Nitrogen source	DCW[a] (g/l)	ODA[b] (cm^2)	min ST[c] (mN/m)	ΔST[d]	pH
Peptone	8.09±0.02	11.39±0.8	33.7±0.0	35.6	3.87
Malt extract	4.12±0.03	7.48±0.17	35.23±0.3	31.3	3.98
$NaNO_3$	8.92±0.01	7.57±0.95	35.11±0.4	32.6	3.99
NH_4NO_3	9.01±0.02	3.81±0.37	36.48±0.4	29.4	3.8
NH_4Cl	8.84±0.00	0.65±0.02	39.60±0.8	23.9	2.57
$(NH_4)_2SO_4$	9.05±0.01	0.65±0.07	40.40±0.5	21.4	2.66

[a] dry cell weight
[b] oil displacement activity
[c] minimum of surface tension
[d] % reduction of surface tension, percentage of initial surface tension of culture medium minus individual surface tension and divide by initial surface tension.

The ratios between carbon and nitrogen sources (C:N) were investigated in this study. For optimization of growth and biosurfactant whilst keeping a constant carbon source concentration (2.5 % glucose and 2.5 % glycerol). The best biosurfactant activity was obtains by C: N ratio of 300 as shown in the Table 6. A reduction of surface tension in the medium was 31.38 mN/m or 38.3 % and the maximum oil displacement activity 28.51 cm^2

Table 6 The effect of C:N radios on growth and biosurfactant activity by *A. pullulans* YTP6-14 incubated at 30°C, 200 rpm for 7 days.

C:N ratio	DCW[a] (g/l)	ODA[b] (cm^2)	min ST[c] (mN/m)	ΔST[d]	pH
100	12.43±0.05	7.60±0.85	33.42±0.2	33.2	3.89
200	8.91±0.00	12.14±0.22	32.36±0.5	37.0	4.08
300	5.40±0.01	28.51±1.02	31.38±0.3	38.3	4.16
400	3.337±0.02	16.48±2.54	32.93±0.1	35.3	4.21

[a] dry cell weight
[b] oil displacement activity
[c] minimum of surface tension
[d] % reduction of surface tension, percentage of initial surface tension of culture medium minus individual surface tension and divide by initial surface tension.

Discussions

Sixteen strains of yeast isolated from the coastal areas of Koh Si Chang were screened for the potential biosurfactant producer in modified's Hue medium (Thaniyavarn el at., 2008). The results of this study showed that *Aureobasidium pullulans* YTP6-14 was considerably reduced the surface tension in the medium from 57.5 to 38.4±0.40 mN/m, representing 33.22% reduction and oil displacement test of 5.10±0.63cm^2 (Table 1.). Hamzah et al. (2013) suggested the criterion used for selecting biosurfactant producer that it could reduce the surface tension in the medium below 40 mN/m.

Many reports on the field of biosurfactant have illustrated that the type and concentration of the biosurfactant depend especially on the composition of important nutrients and growth cultivation conditions (Silva el at., 2010). In this study, the optimization condition of the biosurfactant production of *A. pullulans* YTP6-14 were evaluated. According to the quantities of biosurfactant production, measured as biosurfactant activity, the best of the carbon source was obtained when cultivation on the production medium supplemented with2.5 % (w/v) glucose and 2.5% (w/v) glycerol which shown the reduction of surface tension to 36%. Fontes et al. (2010) reported the best medium for biosurfactant production by *Yarrowia lipolytica* IMUFRJ 50682 was contained both glucose 4% (w/v) and glycerol 2% (w/v) which can be reduce the surface tension of 19.5%. Bhardwaj et al. (2013) indicated that when only one carbon sources from glucose and vegetable oil was supplemented for the biosurfactant production by *Torulopsis bombicala*, a very low yield of biosurfactant was obtaind but when both carbon sources were used a high yield was obtained. Glycerol is a byproduct of the upcoming biodiesel industry and can be used as alternative carbon source due to it is a simple fatty acid precursor with high water-soluble substrate. So it is easily utilized by microorganism for growth and biosurfactant production. (Hamzah et al. 2013).

In term of nitrogen source both inorganic and organic nature, we found that peptone is the best nitrogen source for biosurfactant in production by *A. pullulans* YTP6-14 gave minimum surface tension of 35.6% while inorganic nitrogen in form of ammonium salts were not as good as peptone (Table 5). According to earlier reports on nitrogen source, some reported that organic nitrogen was good N source such as Maria et al. (2007) reported the use of peptone (5.0 g/l) supplemented into cashew apple juice (CAJ) for the cultivation of *P. aeruginosa* could reduce surface tension down to 41%. On the contrary various groups reported the preferential of using inorganic N sources for the same purpose. Silva et al. (2010) showed that *P. aeruginosa* 44T1 fail to give good biosurfactant yield with ammonium salts but instead gave good yield when $NaNO_3$ was used in place of ammonium salts. Thus, at present a clear cut conclusion regarding the use of organic or inorganic nitrogen source still

cannot draw from these. Regarding the optimum C:N ratio, we observed that at C:N ratio of 300 is the optimum ratio for biosurfactant production by *A. pullulans* YTP6-14 suggesting the enhancement of surfactant production under nitrogen-limit condition (Pacheco et al., 2010; Abouseoud et al., 2008).

Conclusions

Screening yeast strains for the potential biosurfactant producer from the coastal areas of Koh Si Chang. *A. pullulans* YTP6-14 was selected and further studied for the optimum production of biosurfactant. The optimization of the biosurfactant production condition was observed when the culture strain was grown in the production medium supplemented with 2.5 % (w/v) glucose supplemented with 2.5 % (v/v) glycerol as carbon sources and peptone as a nitrogen source with C:N ratio of 300. Future work, we will focus on the optimum environmental conditions for biosurfactant production and the physiochemical properties of biosurfactants of *A. pullulans* YTP6-14 will be carried out for further applications.

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