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Dual substrate fermentation using palm oil and glucose for production of eco-friendly biosurfactants using *P. aeruginosa* NITT 6L

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Studies have been conducted to improve biosurfactant (rhamnolipid) production from *Pseudomonas aeruginosa* NITT 6L in liquid state fermentation utilizing palm oil and glucose as substrates. The *p*H and minimal salt media (MSM) compositions such as KH_2PO_4, K_2HPO_4, $NaNO_3$, $MgSO_4$, NaCl and $FeSO_4$ are optimized using design of experiments. One-factor-at-a-time optimization is employed to evaluate the effects of palm oil and its co-substrate glucose on biosurfactant production. The optimal levels of the aforementioned variables are (g/L) glucose 30.0, KH_2PO_4 2.0, K_2HPO_4 5.0, $NaNO_3$ 3.5, $FeSO_4$ 0.003, $MgSO_4$ 0.2, with palm oil concentration of 1.5% (v/v). The fermentation conditions viz. period of fermentation, agitation rate, aeration and concentration of inoculum are optimized by carrying out separate experimentations. The optimum period of fermentation, agitation rate, aeration and concentration of inoculum in the fermentation medium are found to be 7 days, 150 rpm, 60% and 2% (v/v), respectively, for maximum rhamnolipid production of about 3.73 g/RE l.

Keywords: *Pseudomonas aeruginosa*, Biosurfactants, Rhamnolipids, Process optimization, Fermentation

Hydrophobic substrates such as oils are utilized as carbon and energy requirements by many microorganisms. The uptake and growth on these substrates are often associated with the production of biosurfactants which are valuable surface active molecules (Pantazaki *et al*., 2010). Due to their amphipathic nature, these biomolecules are capable of lowering the surface tension, interfacial tension and forming micro emulsion to enable mixing of two immiscible solutions. Such properties exhibit excellent detergency, emulsifying, foaming, and dispersing traits. Some of the features, which make them promising alternatives to chemically synthesized surfactants, are their lower toxicity, higher biodegradability, greater stability at wide range of *p*H and temperature, and better environmental compatibility (Desai and Banat, 1997). Thus, interest towards these biomolecules has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries (Desai and Banat, 1997, Abouseoud *et al*., 2008, Llori *et al*., 2005, Kosaric, 1992, Makkar and Cameotra, 1999 and Raza, 2007). Limited full scale production has been realized for many biosurfactants due to expensive raw material, low production yield and high purification cost. In order to alleviate these problems, many studies had been carried out using cost-free or low-cost feed stocks or agricultural byproducts as substrates for biosurfactant production. Low-cost by-products that had been used as carbon source for biosurfactant production by microbes include olive oil mill effluent, molasses and whey, waste frying oils, orange fruit peeling, potato peel effluent, ground-nut oil refinery with the addition of corn steep liquor, soap stock and waste water from sunflower oil (Babu *et al*., 1996, Daniel *et al*., 1988, Patel and Desai, 1997 Rahman *et al*., 2002 and Makkar and Cameotra, 1999). Despite ongoing research using unconventional sources, selection of appropriate waste substrate is still a challenge. Researchers are facing the problem of finding a waste with the right balance between carbohydrates and lipids to support optimal growth of microorganisms and maximum production of biosurfactant (Finnerty and Singer, 1985 and Fiechter, 1992). Given South-East Asia as one of the major palm oil producer in the world, the management of the ever-increasing organic waste resulting from palm oil mill discharge has been one of the most worrying environmental issues in the country, which requires a practical and economically viable approach to alleviate the problem

In this study, palm oil has been used as a novel substrate for biosurfactant production. Previously most biosurfactant production has been performed using expensive pure forms of sugar like glucose, fructose, and sucrose as carbon sources. However, biosurfactant products obtained using water-soluble carbon sources such as glycerol, glucose, mannitol and ethanol are reported to be inferior to that obtained with water-immiscible substrates such as *n*-alkanes, vegetable oil in terms of little biosurfactant

production when cells are grown on a readily available carbon sources. The production of biosurfactant is triggered only when all the soluble carbon was consumed and when a water-immiscible hydrocarbon is available (Singh and Desai, 1986 and Christofi and Ivshina, 2002). Being water-immiscible, palm oil is not only a favourable choice of carbon source for biosurfactant production but also a cost effective source.

In the present work, we have considered rhamnolipids which are typical biosurfactants produced by *Pseudomonas aeruginosa*. Rhamnolipids constitute one of the most interesting classes of biosurfactants because of their advantageous characteristics. With respect to their production, they show high concentrations as compared to other biosurfactants. Rhamnolipids are able to reduce the water/air surface tension from 72mN/m to values close to 30mN/m, as well as the water/oil interface tension from 43mN/m to values around 1mN/m (Costa *et al*., 2010).

Experimental Section

Micro-organism

The *Pseudomonas aeruginosa* NITT 6L used in this study has been previously isolated in the laboratory from air (Vanavil *et al*., 2013). The isolated strain is maintained on nutrient agar slants at 4°C to minimize biological activity, and is sub-cultured every week.

Preparation of seed culture

Nutrient Broth is used to prepare inoculum using a loop of bacterial colony from agar plate and the culture is grown on a rotary incubator shaker for 16 h at 35°C and 150 rpm. This primary inoculum is used to inoculate the production media in shake flasks.

Production media

From our previous study using Plackett-Burman design, palm oil is found to be second significant carbon source for production of rhamnolipids from this strain (Vanavil *et al*., 2013). Production media is optimized previously in our laboratory using Central Composite Design and used for rhamnolipid production. It consists of 40g/L glucose, 3.5g/L sodium nitrate, 0.2g/L magnesium sulphate and 3mg/L $FeSO_4$, KH_2PO_4, K_2HPO_4, NaCl.

In present study palm oil is used as dual substrate along with glucose. Glucose, palm oil and magnesium sulphateis autoclaved separately and added to medium. Ferrous sulphateis sterilized through filter sterilization. Initial *p*H of the media is adjusted to 7.00.

Extraction and estimation of rhamnolipid

Supernatant sample (0.5 mL) is extracted with 1 mL chloroform: methanol (2:1, v/v). The organic phase is evaporated and 0.5 mL of deionized H_2O is added. Rhamnolipid yield is expressed in terms of rhamnose concentration in g/L. The rhamnose concentration is calculated from standard curves prepared with L-rhamnose (0-100 mg/L) using phenol- sulphuric acid method (Wang *et al*., 2007).

Optimization of palm oil and glucose concentrations for rhamnolipid production

In order to evaluate the most appropriate dual substrate system for the production of rhamnolipids, influence of various concentrations of palm oil and glucoseare investigated. Palm oil is employed at the following concentrations: 0.5, 1.0, 1.5 and 2.0% (v/v). Here glucose is fixed at 4.0% (w/v). Glucose is employed at the following concentrations: 0, 1.0, 2.0, 3.0 and 4.0% (w/v) while palm oil is fixed at 1.5%. These studies are carried in 250 mL flasks containing 100 mL production media and inoculated with 2% overnight seed culture, initial *p*H 7 and incubated at 37°C for 72 h in rotary shaker. Rhamnose concentration in the supernatant is estimated as before.

Effect of agitation rate on production of rhamnolipids

Effect of agitation is studied at three different agitation speed 100, 150 and 200 rpm in 250 mL flasks containing 100 mL production media. These flasks are inoculated with 2% overnight seed culture, initial *p*H 7 and incubated at 37°C for 7 days in rotary shaker. Rhamnose concentration in the supernatant is estimated as before.

Effect of aeration on production of rhamnolipids

To study the effect of aeration, the volume of production media is varied at different volumes ranging from 25, 50, 75, 100, 125, 150 and 175 mL in 250 mL conical flasks corresponding to volumetric oxygen percentage of 90, 80, 70, 60, 50, 40 and 30%, respectively. These flasks are inoculated with 2% overnight seed culture, initial *p*H 7 and incubated at 37°C, 150 rpm for 7 days. Rhamnose concentration in the supernatant is estimated as before.

Effect of inoculum concentration on the production of rhamnolipids

Different concentrations of inoculums (v/v) ranging as 1, 2 and 4% are tested. Overnight seed culture is used as inoculum and is inoculated in 250 mL flasks containing 100 mL production media, thereby maintaining 60% aeration level. The cultures are incubated at 37°C; 150 rpm for 7 days. Rhamnose concentration in the supernatant is estimated as before.

Results and Discussion

Effect of palm oil concentration on the production of rhamnolipids

To find the suitable concentration of palm oil that ensures the best rhamnolipid production by the isolated strain, production media containing palm oil at concentrations varying as 0.5, 1.0, 1.5 and 2.0% (v/v), respectively with glucose present at 4% (w/v). The shake flask experiment is carried out for 72 h at 37°C. It can be observed that rhamnolipid production in terms of rhamnose units is high (1.24 g/L) in the presence of 1.5% (v/v) palm oil with 4% (w/v) glucose. Higher concentrations of palm oil resulted in lower rhamnolipid production.

The tendency of *Pseudomonas* strains to utilize oils depends on its lipase activity that enables assimilation of fatty acids and glycerol contained in vegetable oil fractions. Fatty acids are then β-oxidized to maintain cellular growth and glycerol is transformed into lipidic precursor for rhamnolipid biosynthesis via de novo fatty acid synthesis (Marsudi *et al*., 2008). Figure 1 shows the effect of various palm oil concentrations in biomass production. It is evident from the figure that good cell growth is achieved with all concentrations of palm oil.

Effect of glucose concentration on the production of rhamnolipids

To study the effect of glucose concentration in the presence of palm oil as an additional carbon source, next set of experiments are carried out by using the optimized concentration of palm oil from the previous run and by varying concentration of glucose as 0, 1, 2, 3 and 4% (w/v). The shake flasks runs are carried out for 8 days so as to optimize the cultivation time (number of days of incubation). Highest rhamnolipid production in terms of rhamnose units, 3.73 g/L is obtained (Fig. 2) at a concentration of 3% (w/v) glucose along with 1.5% (v/v) palm oil on day 7 (168 h).

Figure 3 shows the effect of various glucose concentrations with 1.5% palm oil on biomass production. Growth is observed even in the absence of glucose, indicating the ability of the strain to utilize palm oil as sole carbon source but the yield is comparatively lower than when dual substrate combination is employed. When the concentration of glucose rose above 3% (w/v), there is an inhibitory effect on microbial growth and the production of rhamnolipids.

Effect of agitation rate on production of rhamnolipids

From Fig. 4, both 150 rpm and 200 rpm favoured the maximum accumulation of rhamnolipids (3.73 g/L rhamnose). There is not much difference in the results obtained at the rotation velocity of 150 and 200 rpm and hence the former can be preferred as the optimal operating factor for enhanced biosurfactant yield.

Effect of aeration on production of rhamnolipids

It was observed that a moderate volume of fermentation medium in comparison to others i.e. 100 mL (60%) showed maximum biosurfactant production. Hence this volume is considered optimum for further course of studies. Increase in aeration leads

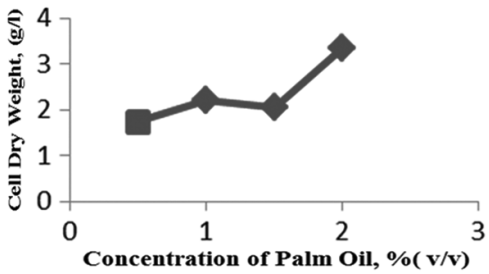

Fig. 1 — Effect of palm oil concentration on biomass production

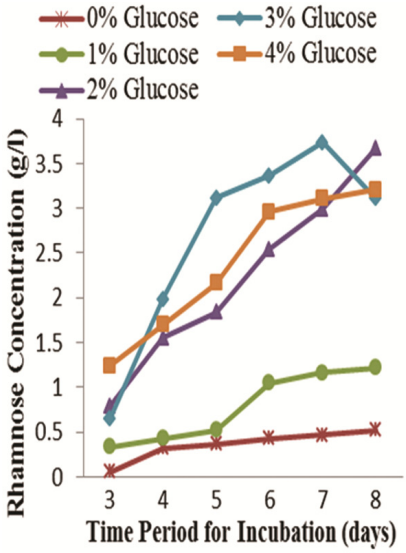

Fig. 2 — Effect of glucose concentration on rhamnolipid production

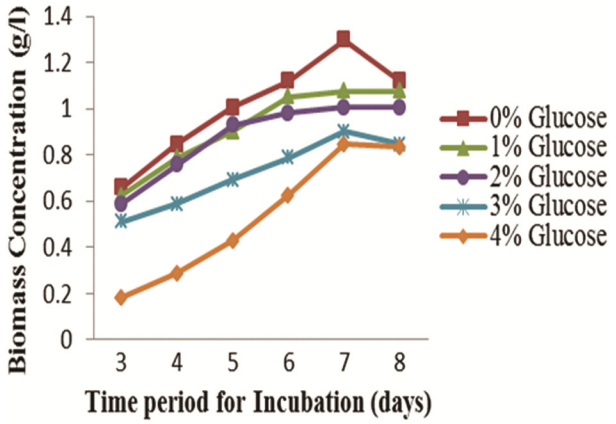

Fig. 3 — Effect of glucose concentration on biomass production

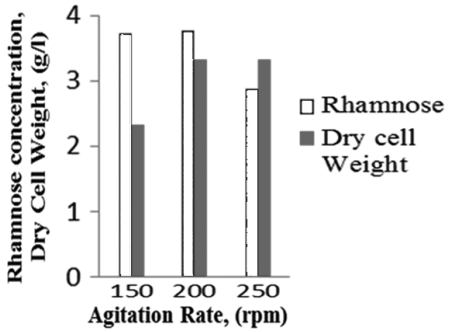

Fig. 4 — Effect of agitation rate on rhamnolipid and biomass production

to high biomass productivity but has less effect on the production of rhamnolipids. Figure 5 displays the effect of various aeration rates on biomass production. Here, biomass production increases with increasing aeration rates.

Effect of inoculum concentration on the production of rhamnolipids

From Fig. 6, it can be observed that highest rhamnose production of 3.73 (g/L) occurred with 2% and 4% inoculum. So it will be most appropriate to use 2% (v/v) as the concentration of inoculum in the production media. Maximum biomass production is obtained with 1% inoculum.

Conclusion

The results of this study show that palm oil can be a good carbon source for the production of rhamnolipids using *P.aeruginosa* NITT 6L. Moreover, palm oil is a typical triacylglycerol and the most produced and traded plant oil in the world, capturing 40 % of global trade (Marsudi *et al*., 2008). South-East Asia is being a major producer of palm oil. Rhamnolipids biosynthesis using cheaper oils like

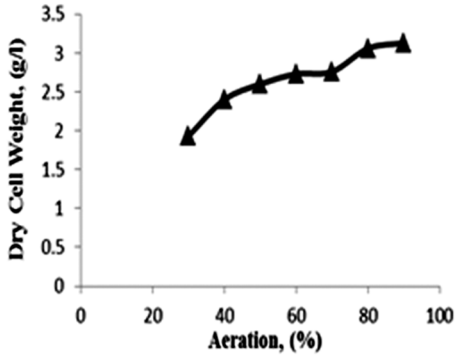

Fig. 5 — Effect of aeration on biomass production

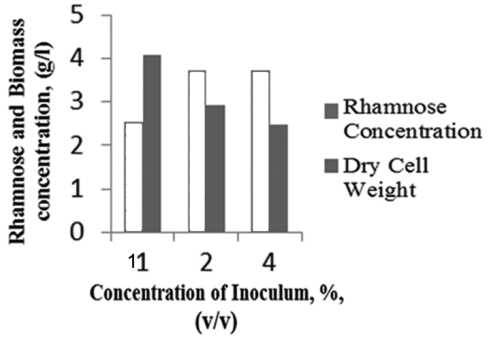

Fig. 6 — Effect of inoculum concentration on rhamnolipid and biomass production

palm oil along with glucose as a co-substrate is carried out to enhance and economize the production. The dual substrate combination produced far better yields of rhamnolipids as compared to using either one of them in the production media. Thus, the optimal levels of the nutrients are decided as (g/L) glucose -30.0, KH_2PO_4 - 2.0, K_2HPO_4 -5.0, $NaNO_3$ - 3.5, $FeSO_4$- 0.003, $MgSO_4$ -0.2, with 1.5% (v/v) palm oil. The fermentation conditions viz. period of fermentation, agitation rate, aeration (volume of the fermentation medium) and concentration of inoculum are optimized. The optimum period of fermentation, agitation rate, aeration and concentration of inoculum in the fermentation medium are found to be 7 days, 150 rpm, 60% and 2% (v/v) for the maximum production of rhamnolipids up to 3.73 g/RE l. This yield is relatively higher when compared to related work using only palm oil as carbon source (Thaniyavarn *et al.*, 2006).

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