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Production of biosurfactant by *Wickerhamomyces anomalus* PY189 and its application in lemongrass oil encapsulation

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ABSTRACT: In this study, the production and characterization of a biosurfactant from yeast *Wickerhamomyces anomalus* strain PY189 was carried out. The highest efficiency for biosurfactant production was found when the organism was grown in a medium containing 4% (v/v) soya bean oil and 0.4% (w/v) $NaNO_3$ at 30 °C and pH 5.5 for 7 days. After 7 days of cultivation, *W. anomalus* PY189 was able to produce up to 0.57 g/l of biosurfactant as ethyl acetate extracts. The culture supernatant was able to reduce the surface tension of the culture broth from 42.5 mN/m to 36.5 mN/m with a critical micelle concentration of 204 mg/l. The crude extract of biosurfactants was then applied to the encapsulation of lemongrass oil. Emulsions of lemongrass oil in 20 mg/dl maltodextrin solution (oil:maltodextrin solution ratios of 0.2:1, 0.15:1, and 0.1:1) containing 0.8% and 1% (w/v) crude biosurfactant extract were stable for at least 24 h and had an average oil droplet size of less than 10 μm. Lemongrass oil microcapsules were later produced using a spray drying technique. This microcapsule exhibited microbial growth inhibition activity against *E. coli*, *S. aureus*, and *Salmonella* at 5% (w/v) concentration.

KEYWORDS: emulsifier, sophorolipid, microencapsulation

INTRODUCTION

Biosurfactants (or bioemulsifiers) are surface-active compounds from biological sources, usually produced by bacteria, yeast, or fungi. They are amphiphatic molecules consisting of a hydrophilic and a hydrophobic domain and capable of reducing surface and interfacial tension at the interface between liquids, solids, and gases. They have advantages over their chemical counterparts in biodegradability and extreme temperature or pH and in having lower toxicity to animals[1]. Biosurfactants may have one of the following structures: glycolipid, polysaccharide-lipid complex, lipoprotein or lipopeptide, or phospholipid. The most commonly isolated and widely studied group of surfactants produced by microorganism is glycolipids. Glycolipids are carbohydrates in combination with long-chain fatty acid. The best known glycolipids are trehalolipids, sophorolipids and rhamnolipids[2]. They possess good surface active properties and show excellent compatibility with human skin, a property that is very important for cosmetic and personal care applications. Furthermore, they can be used in various other sectors due to their emulsifying, antimicrobial, and other beneficial properties[3]. Sophorolipids can be synthesized in high concentrations by nonpathogenic yeasts. This fact makes sophorolipids particularly attractive for further commercial production and use.

Wickerhamomyces anomalus (*Pichia anomala*, Kurtzman et al)[4], one of the nonpathogenic yeast, has been isolated from various environments and has shown remarkable physiological robustness towards chemical, physical and biological stresses, such as extreme pH or water activity[5]. It produces a number of killer proteins, including beta-1,3-glucanase, with different glycosylation showing antimicrobial activities against not only bacteria but also yeast and filamentous fungi. Killer proteins are also useful matrix for generating and producing anti-idiotypic antibodies killing microorganisms and even viruses[6]. These features make *W. anomalus* a valuable microbial agent for improving feed and food safety during preservation and antimicrobial therapies in human and animals[7]. Moreover, it has been reported that *W. anomalus* has flavour enhancing effect in food and beverage fermentation[8,9].

We have previously reported that *W. anomalus* PY1, a thermo-tolerant strain isolated from fermented food, is a sophorolipid producer which can produce sophorolipids in a wide range of temperature (20–40 °C)[10]. Enhanced production of sophorolipids by PY1 or its derivative strains is thus expected to be useful for improvement of preservation and value of foods.

Emulsifiers play an important role in forming the desirable consistency and texture as well as in phase dispersion to stabilize emulsions[11]. The quality of a vast number of food products relies on the emulsification ability of synthetic emulsifiers. With an increasing consciousness among consumers, a steady increase in demand for more natural food ingredients and additives is observed. Biosurfactants produced by nonpathogenic yeast are a promising natural ingredient that can be used to produce many food products and additives including microcapsules which are to be used for preservation in food experiencing minimal process. Microbial contamination in minimally processed food is a major problem for public health. If not preserved by refrigeration, other methods must be used to prevent microbial growth in such food product. Essential oils from various edible plants, a natural ingredient, are reported to exhibit various biological activities including bacterial inhibition. It has been reported that the essential oils from herbaceous plant, such as citronella (*Cymbopogon nardus*), lemongrass (*C. citratus*)[12], basil (*Ocimum basilicum*)[13], and plants in the ginger family, are capable of controlling the growth of microorganisms in food[14]

Many active agents present in essential oils are unstable compounds. The activity can reduce over storage time due to oxidation or volatilization. Microencapsulation is a mean to retain their activity. Essential oil microencapsulation can be done using methods such as spray-drying. Studies on lemongrass (*C. citratus*) essential oil in food and pharmaceutical applications have been reported. Lemongrass essential oil, due to its broad-spectrum antimicrobial activity, was microencapsulated by simple coacervation method that did not deteriorate the essential oil[15]

This study is divided into two parts. The first part aims to determine the optimum condition for the production of biosurfactants from yeast *W. anomalus* strain PY189 and to characterize the yielded crude biosurfactant extract. The second part aims to investigate the application of biosurfactant extract in the production of antimicrobial microcapsules containing lemongrass oil.

MATERIALS AND METHODS

Microorganisms

W. anomalus PY189, a biosurfactant hyperproducing mutant strain by EMS from *P. anomala* PY1, was obtained by EMS mutagenesis. The microorganisms were maintained at 5–8 °C on yeast malt agar (YM) slants containing (w/v) 0.3% yeast extract, 0.3% malt extract, 0.5% Bacto peptone, 1% glucose and 2% agar.

Carbon source for biosurfactant production

Cell culture (10% v/v) of *W. anomalus* PY189 was prepared as inoculum in YM broth at pH 4.5 and incubated at 30 °C while shaking at 200 rpm for 18 h. The biosurfactant was produced by cultivation of *W. anomalus* PY189 in 50 ml production medium containing (w/v) 0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.4% $NaNO_3$, 0.1% yeast extract and 4% of various carbon sources, which were glucose (w/v), sucrose (w/v), soya bean oil (v/v), palm oil (v/v) and coconut oil (v/v). The culture was placed in a 250-ml Erlenmeyer flask while shaking at 200 rpm for 7 days at 30 °C. Aliquots of the culture were periodically removed to determine growth, and supernatants were kept to determine biosurfactant activity. Growth was measured in terms of dry cell mass. To determine the optimum concentration of the carbon source, concentrations up to 8% of the selected carbon source were used.

Nitrogen source for biosurfactant production

Cell culture (10% v/v) of PY189 was prepared as inoculum in YM broth at pH 4.5 and incubated at 30 °C while shaking at 200 rpm for 18 h. The biosurfactant was produced by cultivation of *W. anomalus* PY189 in 50 ml production medium containing 0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract, 4% (v/v) soya bean oil and 0.4% (w/v) of various nitrogen sources, which were $NaNO_3$, $(NH_4)_2SO_4$, and NH_4NO_3. The culture was placed in a 250-ml Erlenmeyer flask while shaking at 200 rpm for 7 days at 30 °C. Aliquots of the culture were periodically removed for growth determination, and supernatants were used to determine biosurfactant activity. To determine the optimum concentration of the nitrogen source, 0–0.5% of selected nitrogen source were used.

Determination of biosurfactant activity

Biosurfactant activity was determined using oil displacement test. Fifty millilitres of distilled water was added to a large Petri dish (15 cm diameter)

followed by the addition of 15 µl of synthetic oil to the surface of water followed by 10 µl of culture broth supernatant. The diameter of clear zones from triplicate assays of the same sample was determined[16]. Surface tension was determined by the Du Nouy ring method[17] using a Tensiometer (K6, Kruss, Hamburg, Germany). Both oil displacement activity and surface tension were used as criteria to select the condition for *W. anomalus* PY189 cultivation. Critical micelle concentration (CMC) of the biosurfactant was determined[18].

Production and extraction of biosurfactant

Biosurfactant production was analysed in 2 l of production medium with 4% soya bean oil as the carbon source, pH 5.5, shaken at 200 rpm for 7 days at 30 °C. After 7 days, cells were removed by centrifugation at 10 000*g* for 15 min. The supernatants were kept at 4 °C overnight. The oil was removed by hexane extraction. The aqueous phase was then extracted 3 times with ethyl acetate. The solvent layer was evaporated to dryness in a vacuum at 40 °C. The crude extracts were obtained and the structural characteristics and CMC were determined[18]. Crude extract was dissolved in 50 mM Tris-HCl buffer pH 8.0, serially diluted within the range of 0.1–1000 mg/l, and the surface tension was then measured. The CMC values were determined by plotting the surface tension values against the log of biosurfactant concentrations.

Thin layer chromatography of biosurfactants

Crude extracts, prepared as described above, were dissolved in ethyl acetate and analysed by TLC on gel plates. The samples were then resolved in chloroform:methanol:water (65:25:4 v/v), and visualized with iodine vapour and Molisch reagent for lipid and carbohydrate detection, respectively.

Emulsification of lemongrass oil

Solutions of biosurfactant in maltodextrin solution (20 mg/dl) were prepared at various concentrations of biosurfactant (0.6%, 0.8% and 1% by weight). Lemongrass (*C. citratus*) oil was added into the solutions at oil:maltodextrin solution ratios of 0.2:1, 0.15:1, and 0.1:1. The solution was then homogenized with a hand homogenizer at 19 000 rpm for 5 min. Then homogenized solution (10 ml) was pipetted into a test tube and let stand for 24 h at room temperature. The emulsion that showed no separation was chosen for the production of microcapsule using spray drying technique.

Microencapsulation

Total oil content of the powder was determined by the method modified from that of Hogan et al[19]. Total oil was determined by gently shaking 2 g of powder with 250 ml petroleum ether in a sealed 500 ml glass bottle at 30 °C, 200 rpm for 6 h. The solvent was filtered through Whatman filter paper. The extracted oil in the filtrate was determined gravimetrically following the removal of solvent by rotary evaporation and oven drying at 50 °C for 2 h. The extractable oil was determined by gently shaking 2 g of powder with 250 ml petroleum ether at 30 °C, and 200 rpm for 15 min. Microencapsulation efficiency (ME) was calculated as follows:

$$\mathrm{ME} = 1 - (\text{extractable oil})/(\text{total oil}).$$

One thousand millilitres of lemongrass oil emulsion was sprayed dried at 25 ± 5 ml/min with the inlet air temperature of 180 °C. The dried microcapsules were packaged in an aluminium foil laminated pouch for further analyses.

A small amount of microcapsules were observed using a scanning electron microscope in order to determine the particle size and to observe particle morphology.

Minimum inhibitory concentration of microcapsule

MIC of lemongrass oil and microcapsule were determined using the agar dilution method. *S. aureus*, *E. coli*, and *Salmonella* sp. were grown in nutrient agar at 37 °C for 24 h. The microorganisms were inoculated in Müller-Hinton broth (w/v, 30% beef infusion, 2% casein hydrolysate and 0.15% starch, pH adjusted to neutral at 25 °C) and incubated at 37 °C for 18–24 h (density 10^8 CFU/ml). Lemongrass oil and microcapsule were prepared at different concentrations and then transferred into Müller-Hinton agar (MHA) contained 0.5% Tween 20. MHA was allowed to solidify in a Petri dish. Two microlitres of microorganism was dropped on the surface of MHA and incubated at 37 °C for 24 h.

RESULTS AND DISCUSSION

Biosurfactant production in carbon sources

In this experiment, glucose, sucrose, soya bean oil, palm oil and coconut oil were used as carbon sources in culture medium. Biosurfactant production was determined by biosurfactant activities, both in terms of surface-tension reduction and by oil displacement test. The results in Table 1 indicate

Table 1 Various carbon sources on biosurfactant production by *W. anomalus* PY189.

Carbon source (4%)	Cell dry weight (g/l)	Surface tension (mN/m)	ΔST (mN/m)	Oil displacement (cm^2)
Glucose	14.9 ± 0.7	44.3 ± 0.5	15.23	3.14 ± 0.29
Sucrose	10.4 ± 0.8	47.8 ± 0.8	13.67	2.72 ± 0.26
Soya bean oil	15.2 ± 0.3	38.9 ± 0.6	16.56	12.33 ± 0.29
Palm oil	14.8 ± 0.3	40.3 ± 1.0	10.33	6.3 ± 1.0
Coconut oil	6.8 ± 0.7	54.9 ± 0.3	9.14	0.25 ± 0.14

Table 2 Various concentrations of soya bean oil on biosurfactant production by *W. anomalus* PY189.

Soya bean oil concentration (%)	Cell dry weight (g/l)	Surface tension (mN/m)	ΔST (mN/m)	Oil displacement (cm^2)
0	2.2 ± 0.6	55.8 ± 0.6	1.25	1.13 ± 0.24
1	6.5 ± 0.7	49.2 ± 0.8	1.7	1.79 ± 0.36
2	10.8 ± 0.6	46.2 ± 0.5	4.76	8.17 ± 0.32
4	14.0 ± 0.4	37.6 ± 0.7	18.5	12.6 ± 1.0
8	10.5 ± 0.7	38.8 ± 0.3	16.12	11.23 ± 0.22

that the carbon source promoting the best biosurfactant production was soya bean oil. Using this carbon source, *W. anomalus* PY189 could produce 12.33 cm^2 of oil displacement activity and reduced surface tension activity to 38.9 mN/m at 7 days of cultivation. The result in Table 2 suggested that 4% (v/v) soya bean oil was the most appropriate carbon source for biosurfactant production by PY189.

Biosurfactant production nitrogen sources

$NaNO_3$ supported growth and biosurfactant production of the microorganism (Table 3). *W. anomalus* PY189 could produce the biosurfactant with the highest activity in the presence of $NaNO_3$. Acidic nitrogen sources such as NH_4NO_3 and $(NH_4)_2SO_4$ reduced the culture pH from the initial value of 5.5 to about 3–4, and yielded low biosurfactant production. The results in Table 4 suggest that 0.4% (w/v) $NaNO_3$ was the most effective nitrogen source for biosurfactant production by *W. anomalus* PY189.

Characterization of biosurfactants

The dry crude extract was obtained at 0.57 g/l from soy bean oil medium. The crude biosurfactant extract showed a CMC of 204 mg/l. TLC analysis

Table 3 Various nitrogen sources on biosurfactant production by *W. anomalus* PY189.

Nitrogen source (0.4%)	Cell dry weight (g/l)	Surface tension (mN/m)	ΔST (mN/m)	Oil displacement (cm^2)
$NaNO_3$	12.8 ± 0.3	37.7 ± 0.7	16.7	11.8 ± 0.4
$(NH_4)_2SO_4$	10.7 ± 0.3	42.5 ± 1.0	12.5	9.8 ± 0.5
NH_4NO_3	11.8 ± 0.7	40.8 ± 0.4	11.6	8.9 ± 0.9

Table 4 Various nitrogen sources on biosurfactant production by *W. anomalus* PY189.

$NaNO_3$ concentration (%)	Oil displacement (cm^2)	Cell dry weight (g/l)	Surface tension (mN/m)	ΔST (mN/m)
0	2.3 ± 0.4	42.5 ± 0.5	10.57	0.53 ± 0.29
0.1	4.2 ± 0.5	40.2 ± 0.6	10.95	4.68 ± 0.25
0.2	9.7 ± 0.3	39.6 ± 0.8	14.8	4.92 ± 0.32
0.3	10.8 ± 0.4	40.3 ± 1.1	13.06	9.2 ± 1.2
0.4	14.5 ± 0.6	36.5 ± 0.3	18.45	10.17 ± 0.23
0.5	10.20 ± 0.13	38.8 ± 0.6	16.24	8.5 ± 0.5

showed that all the iodine positive spots but F2 and F3 were positive for Molisch reagent staining test (Fig. 1). The positive spots obtained from the iodine and Molisch reagent tests for crude extract indicated that biosurfactant produced by PY189 is of glycolipid type.

Emulsification of lemongrass oil

Emulsions prepared from various ratios of lemongrass oil in 20 mg/dl maltodextrin solution containing 0.6–1% (w/v) crude biosurfactant were used to determine emulsion stability and average volume of the oil droplet. Only the emulsion containing 0.6% crude biosurfactant was unstable and separation of oil was observed after homogenization. All the resulting oil-in-water (O/W) emulsion appeared milky white-yellow colour and had low viscosity. Volume average diameter, $D[4,3]$, of lemongrass oil droplets in emulsion with different biosurfactant concentrations and oil:maltodextrin ratios are compared in Table 5. The results suggested that an increase in biosurfactant concentration or a reduction in oil:maltodextrin ratio led to a decrease in oil droplet size in emulsion. The optimal concentration of biosurfactant for spray-drying was therefore determined to be 1% for oil:maltodextrin 0.15:1 and 0.2:1 levels.

Powder morphology and particle size analysis

After the spray drying process, the spray-dried microcapsules became dry powder with light yellow colour. The outer topographies of spray-dried lemongrass oil microcapsule were determined by SEM (Fig. 2). The spray-dried powder was almost spherical and had small holes dispersing on their wall. The particle surface was smooth. When the microcapsules were cross-sectioned, it could be observed that the core material was in the form of small droplets embedded in the wall matrix layer (Fig. 3). The average particle sizes and microencapsulation efficiency of lemon grass oil microcapsule prepared with oil:maltodextrin ratios of 0.2:1 and

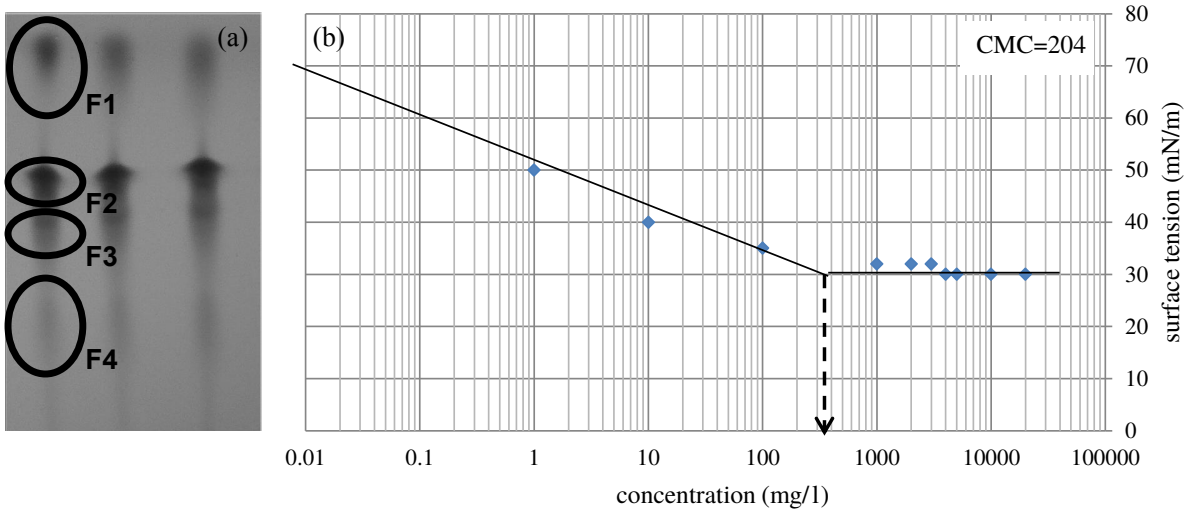

Fig. 1 (a) TLC analysis visualized with iodine vapour of biosurfactant produced by *W. anomalus* PY189. (b) CMC of biosurfactant produced by *W. anomalus* PY189.

Table 5 Volume average diameter (μm) of lemongrass oil emulsion.

Biosurfactant concentration (%)	Oil:maltodextrin		
	0.1:1	0.15:1	0.2:1
0.6	ND	ND	ND
0.8	10.2	11.5	13.7
1.0	4.1	6.0	8.0

ND: not determined due to instability.

Table 6 MIC of lemongrass oil and microcapsules.

Samples	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella* sp.
Lemongrass oil (% v/v)	0.0625	0.0625	0.125
Microcapsules (% w/v)	5	5	5

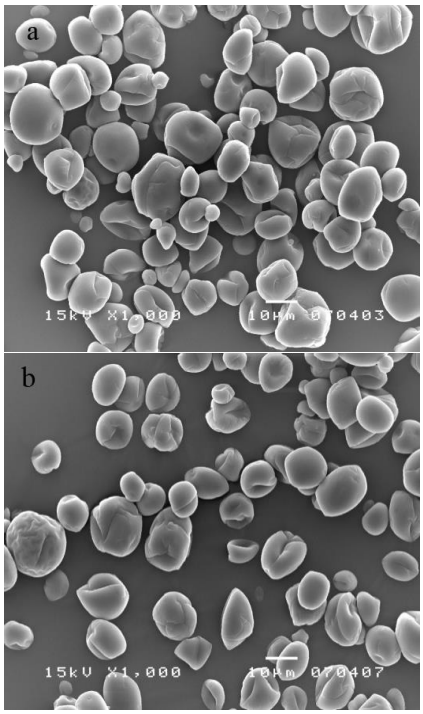

Fig. 2 SEM images of spray-dried lemongrass oil microcapsules prepared from emulsion containing oil:maltodextrin ratios of (a) 0.2:1 and (b) 0.15:1. Emulsion was prepared using 1% biosurfactant in each case.

0.15:1 were 9.2 ± 0.8 μm and 10.8 ± 0.9 μm, and 48% and 52%, respectively.

Minimum inhibitory concentration

MIC of lemongrass oil and its microcapsules prepared from 0.2:1 oil-to-maltodextrin ratio are compared in Table 6. The results from agar dilution method suggested that the microcapsule containing lemongrass oil was capable of inhibiting growth of all three pathogens *E. coli*, *S. aureus* and *Salmonella* sp. at 5% (w/v).

CONCLUSIONS

W. anomalus PY189 could produce biosurfactant when cultured in production medium containing (w/v): 0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract, 0.4% $NaNO_3$ and 4% (v/v) soya bean oil. The PY189 could produce the biosurfactant having 10.17 cm^2 of oil displacement activity and

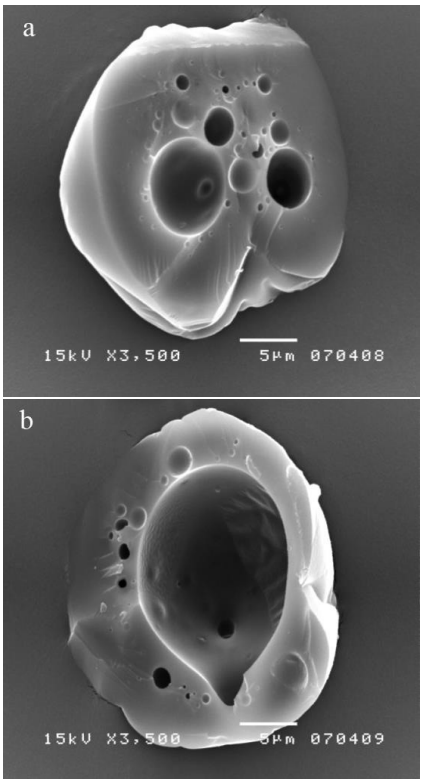

Fig. 3 SEM cross-sectioned images of spray-dried lemongrass oil microcapsules prepared from emulsion containing oil:maltodextrin ratio of (a) 0.2:1 and (b) 0.15:1.

reducing surface tension activity to 36.5 mN/m at 7 days of cultivation and in its crude form. The crude biosurfactant showed a CMC of 204 mg/l. TLC analysis revealed that biosurfactant produced by *W. anomalus* PY189 is of glycolipid type. The emulsions prepared from lemongrass oil in 20 mg/dl maltodextrin solution and 0.8% and 1% biosurfactant concentrations were stable for at least 24 h after preparation. Lemongrass oil microcapsules produced by spray drying technique exhibited microbial inhibition activity against *E. coli*, *S. aureus* and *Salmonella* sp. at 5% (w/v) concentration.

Future perspective

Biosurfactants alter microbial environments by their surface activity and antimicrobial activity that could help ensuring dominance of the habitat in nature by species such as *W. anomalus*[20]. Further understanding of this observation may lead to new means to enhance production of biosurfactants; for instance, a co-culture of *W. anomalus* with some ally or competitor microbe that triggers enhanced production of antimicrobial biosurfactant[21,22]. *W. anomalus* is known to have robust stress biology, giving rise to the possibility that the various activities of solutes, such as osmotic stress, water-activity reduction, and chaotropicity might enhance production of biosurfactant[5]. Microbial stress biology could be utilized to enhance microbial behaviour, and the production of microbial metabolites, for biotechnological purposes[23–26]. Further work is needed to explore additional ways in which biosurfactant production by *W. anomalus* PY189 can be better optimized.

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