УДК 663.12+661.185

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BIOSURFACTANT-PRODUCING YEASTS ISOLATED FROM FLOWERING PLANTS AND BEES

The yeast strains (n=160) have been isolated from various flowering plants and bees Apis mellifera. Oil-spreading method was used to assay the ability of the isolated yeasts to produce biosurfactants. Five most active strains able to synthesize glycolipid biosurfactants produced the oil-spreading zone with diameter 3.66-50 cm. The addition of oleic acid, sunflower oil and octadecane significantly increased biosurfactant activity of the studied strains. Crude biosurfactants produced by the strains Candida sp. 79a and 156a were isolated as ethyl acetate extract and proved to be a mixture of glycolipids by thin-layer chromatography.

Key words: yeasts, biosurfactants, glycolipids

Biosurfactants are amphiphilic molecules that contain both hydrophilic and lipophilic moieties and are produced by various microorganisms both prokaryotes and eukaryotes. They can accumulate between different phases thus reducing surface and interfacial tension. Generally there are two groups of biosurfactants: low-molecular weight compounds that include glycolipids and lipopeptides and high-molecular weight biosurfactants which are lipopolysaccharides, lipoproteins and their combination.

Most yeasts reported to synthesize biosurfactants produce low-molecular weight glycolipid compounds. Among them are sophorolipids which are extracellular glycolipids composed of a glucose dimer sophorose (hydrophilic part) linked glycosidically to a hydroxyl fatty acid moiety (hydrophobic part). Sophorolipids are considered among the most promising biosurfactants being produced by non-pathogenic yeasts in comparatively high amounts and possessing low eco-toxicity and high biodegradability [13]. Most yeasts known to synthesize sophorolipids belong to ascomycetous yeasts namely *Starmerella* clade [5], although a basidiomycete *Rhodotorula bogoriensis* is known to produce small quantities of these biosurfactants [15]. The best known sophorolipid producer is the strain *Candida bombicola* ATCC 22214 reported to synthesize up to 400 g/l sophorolipid [9]. Many yeast strains belonging to *Starmerella* yeast clade are isolated from bees or habitats visited by bees [11].

Therefore the purpose of this work was to isolate novel biosurfactant-producing yeasts from flowering plants and bees.

Materials and Methods. *Isolation and selection of biosurfactant-producing yeasts.* Single flowers of flowering plants have been collected during the period of March-May 2011. The samples were placed in sterile glass tubes and used for yeast isolation on the same day. One gram of each sample was transferred to a tube containing 10 ml of YPD medium (10 g/l yeast extract, 20g/l peptone, 20g/l glucose). The tubes were incubated at 25 ^{0}C at 250 rpm for 3 days. After that 0.1 ml of appropriate dilutions of the suspension were placed on YPD agar medium supplemented with 40 mg/l of streptomycin, pH=4.5, and incubated for a week at 25 ^{0}C. When isolating yeasts from bees, the insects were allowed to walk 15-30 min on the surface of plates with YPD agar medium. Individual colonies on the plate which represented different morphotypes have been examined microscopically and yeast strains have been selected. Each strain was purified by streaking at least 3 times.

The ability of each strain to produce biosurfactant was determined by cultivating in SL medium which was composed of 100g/l glucose, 1.5 g/l yeast extract, 4 g/l NH_4Cl, 1 g/l KH_2PO_4, 0.1 g/l NaCl, 0.5 g/l $MgSO_4$x7H2O, in distilled water [5]. The lipophilic carbon source (oleic acid, tetradecane, octadecane, sunflower oil, pentadecanoic acid) have been added at 5% (v/v) concentration. Cultures were cultivated at 25 ^{0}C at 250 rpm for 6 days.

Oil-spreading method. Fifty milliliters of distilled water have been poured to a large Petri dish (15cm diameter) and 20µl of crude oil have been added to the water surface. 10µl of yeast culture cultivated in SL medium for 6 days at 25^{0}C have been added to the surface of oil. The diameter of the clear zone formed on the surface was measured. The experiment was done in triplicate.

Thin-layer chromatogramphy. Two milliliters of culture broth were extracted with 2 ml of ethyl acetate and extracts were applied onto TLC ALUGRAM SIL G/UV_{254} plates (MACHEREY-NAGEL, Germany). The developing system: chloroform/methanol/water=65/15/2 (v/v/v). The visualizing reagent α-naphthol/sulfuric acid was applied to TLC plates. Sprayed plates were heated at 125 ^{0}C for 5 min. Pink-colored spots (glycolipid-positive) were observed.

Results and Discussion. A total of 160 yeast strains have been isolated from 165 samples of various flowering plants and bees and their products (Table 1). Fourteen yeast strains were isolated from flowers of *R.canina*, thirteen isolates – from of flowers of *T.farfara*, eleven – from both *Virburnum* sp. and *Trifolium* sp. No yeast strains have been isolated from flowers of *G.lutea*, *C.mas*, *L.album*, *N.vulgaris*, *Vicia* sp., *P.avium*, *P.cerasus*, *P.communis*, *Malus* sp. Yeasts have been isolated from 51.5% of the collected samples which is in a good agreement with other studies where yeasts were present in 28-68% of flower samples depending on the season [2, 10].

As the next step we screened 160 isolated yeast strains for biosurfactant production by using the oil-spreading technique which proved to be an easy and reliable method to detect the surface activity [14]. The diameter of the clearing zone formed by biosurfactant-containing solution has been shown to be directly proportional to the concentration of biosurfactant [7, 14]. Sixteen isolates did not produce any detectable clearing zone and other seventy two strains produced the clearing zone with the diameter less than 1cm (Fig. 1). The diameter of the clearing zone produced by fifty one isolates ranged between 1 and 3 cm and the number of the most active yeast strains formed the clearing zone with the diameter more than 3 cm was twenty one. These active strains have been selected for further studies.

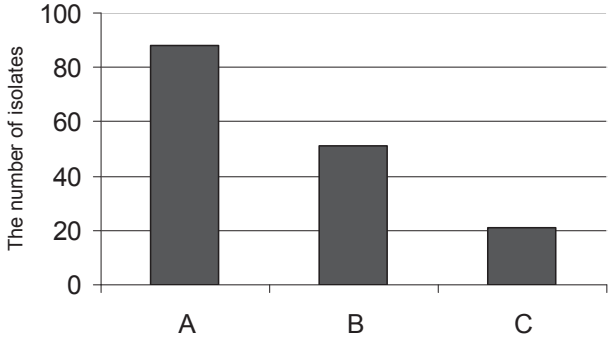

Fig. 1. Oil-spreading activity of the isolated yeast strains. A – the diameter of the clearing zone 0-1cm, B – 1-3cm, C – more than 3 cm

So a high number of yeasts (45%) isolated from flowering plants and bees produced the clearing zone with the diameter more than 1cm. These sugar-rich niches provide a perfect habitat for yeasts which often exhibit osmophilic and lipolytic properties [6]. Biosurfactant-producing yeasts are reported to be isolated from different sources, including oil-contaminated water [3], various plants [8, 12]. Many yeasts which produce sophorolipids have been isolated from such sugar-rich sources as grape juice [4], bee honey [5].

As the purpose of this work is to isolate sophorolipid-producing yeasts crude biosurfactants produced by 21 most active strains (diameter of the clearing zone was more than 3 cm) were analyzed by thin-layer chromatography. The developing system chloroform/methanol/water=65/15/2 currently applied for sophorolipid detection [1] was used. No ά-naphthol-stained glycolipids could be detected when analyzing biosurfactants produced by 10 selected yeast strains and other six yeast strains produced barely detectable spots. Extracts of biosurfactants produced by five yeasts strains 2f, 71a, 79a, 136a and 156a could be easily detected as a mixture of several glycolipids as it could be observed from Fig. 2 where six glycolipids produced by strains 79a and 156a could be distinguished. Such findings could indicate sophorolipid nature of the isolated crude biosurfactants since yeasts produce sophorolipids as a mixture of several structural forms [3, 9]. Nevertheless further analysis is necessary to elucidate the structure of the studied compounds.

Table 1

Isolation of yeast strains from flowering plants and bees

Isolation source	Location	Number of isolated strains	Number of biosurfactant-producing strains	Isolation source	Location	Number of isolated strains	Number of biosurfactant-producing strains
Flowers of *Tussilágo fárfara*	Park "Feofania", Kiev	13	12	Flowers of *Crataegus submollis*	Territory of the Institute of Microbiology and Virology, Kiev	8	7
Flowers of *Gágea lútea*	Park "Feofania", Kiev	-	-	Flowers of *Aesculus hippocastanum*	As above	6	6
Flowers of *Viola odorata*	Territory of Institute of microbiology and virology, Kiev	11	10	Flowers of *Veronica officinalis*	As above	10	8
Flowers of *Anemone ranunculoides*	As above	3	3	Flowers of *Erysimum cheiranthoides*	As above	1	1
Flowers of *Taraxacum officinale*	As above	7	6	Flowers of *Vicia* sp.	As above	-	-
Flowers of *Leonurus quinquelobatus*	As above	3	3	Flowers of *Sorbus aucuparia*	As above	5	5
Flowers of *Forsythia europae*	As above	10	9	Flowers of *Trifolium* sp.	As above	11	11
Flowers of *Armeniaca vulgaris*	As above	6	5	Flowers of *Rosa canina*	As above	14	12
Flowers of *Prunus avium*	As above	-	-	Flowers of *Magnolia* sp.	Territory of Military clinical hospital, Kiev	2	1
Flowers of *Prunus cerasus*	As above	-	-	Flowers of *Malus* sp.	Territory of Military clinical hospital, Kiev	-	-
Flowers of *Pyrus communis*	As above	-	-	Flowers of *Robinia pseudoacacia*	Goloseevskiy park, Kiev	7	7
Flowers of *Chelidonium majus*	As above	1	1	Flowers of *Tulipa gesneriana*	Private household, Kiev region	1	1
Flowers of *Lamium album*	As above	-	-	Flowers of *Narcissus vulgaris*	As above	-	-
Flowers of *Spiraea* sp.	As above	3	3	Flowers of *Cornus mas*	As above	-	-
Flowers of *Viburnum* sp.	As above	11	11	*Apis mellifera*	As above	8	6
Flowers of *Syringa vulgaris*	As above	7	5	Beebread	As above	12	11

Fig. 2. TLC analysis of crude biosurfactants produced by strains 79a (1) and 156a (2) in the medium SL supplemented with 5% sunflower oil

The lipophilic carbon substrate plays a crucial role in the production of sophorolipids by yeasts [13]. It has been shown that the addition of such a substrate to the cultivation medium considerably increased the oil-spreading activity of the selected strains (Table 2). The most effective ones were sunflower oil, octadecane and oleic acid. The diameter of the clearing zone increased 2-3 times for the strains 79a and 156a when compared to the control (medium SL without lipophilic carbon substrate). The oil-spreading activity of the strains 2f, 71a and 136a went up by more than 10 times when these compounds were added: the diameter of the clearing zone produced by the strain 71a, for example, increased 15.2-24.5 times compared to the control. The addition of pentadecanoic acid did not have any considerable effect on the oil-spreading activity of the strains 71a, 79a and 156a and even resulted in the decrease of the clearing zone produced by the strains 2f and 136a by more than 30%. Tetradecane increased the oil-spreading activity of the strains 2f, 71a, 136a and 156a by 100% and more.

The strains 79a and 156a have been the most active even when cultivated in the medium SL without lipophilic carbon source and therefore have been selected for further studies.

The strains 79a and 156a were phenotypically identified as *Candida* sp., belonging to *Starmerella* clade (data not shown). Colonies are smooth, cream-colored, glistening. No pseudohyphae were present. Cells are ovoidal to elongate (1.5-2.5x2.5-4) μm, single and in pairs (Fig. 3). No ascospores have been detected.

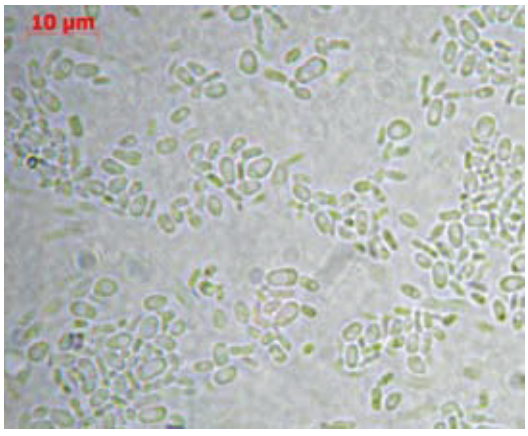

Figure 3. Cells of the strain *Candida* sp. 79a, 2 weeks on YM agar at 25^{0}C

Table 2

The effect of carbon lipophilic substrate on oil-spreading activity of yeast strains

Substrate / Strain	Control (without lipiphilic substrate)		Sunflower oil		Tetradecane		octadecane		Pentadecanoic acid		Oleic acid	
	Zone, d, cm		Zone, d, cm		Zone, d, cm		Zone, d, cm		Zone, d, cm		Zone, d, cm	
	Non-diluted	10-fold diluted	Non-diluted	10-fold diluted	Non-diluted	10-fold diluted	Non-diluted	10-fold diluted	Non-diluted	10-fold diluted	Non-diluted	10-fold diluted
2f	3.66±0.2	-	>14	8.6±0.36	6.37±0.15	-	>14	4.53±0.15	2.53±0.06	-	>14	5.47±0.2
71a	4.17±0.15	-	>14	6.76±0.25	9.27±0.4	-	>14	6.37±0.4	6.23±0.25	-	>14	10.23±0.46
79a	>14	5.0±0.2	>14	11.8±0.36	>14	7.27±0.3	>14	12.77±0.25	>14	5.03±0.25	>14	10.53±0.15
136a	8.3±0.26	-	>14	5.96±0.38	>14	2.7±0.26	7.7±0.17	-	5.77±0.25	-	>14	10.5±0.3
156a	>14	3.83±0.29	>14	13.43±0.32	>14	6.77±0.25	>14	13.9±0.11	>14	5.7±0.2	>14	12.27±0.21

In this work 160 yeast strains have been isolated from various flowering plants and bees. Two strains identified as *Candida* sp. which produced glycolipid biosurfactants have been selected. Further studies on the structure of these biosurfactants and their production by the selected strains will be performed.

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ДРІЖДЖІ-ПРОДУЦЕНТИ БІОСУРФАКТАНТІВ, ІЗОЛЬОВАНІ З КВІТКОВИХ РОСЛИН ТА БДЖІЛ

Р е з ю м е

Сто шістдесят штамів дріжджів були ізольовані від різних квіткових рослин та бджіл *Apis mellifera*. Задля визначення здатності ізольованих штамів продукувати поверхнево-активні речовини був використаний метод «розтікання нафти». П'ять найбільш активних штамів, що синтезували біосурфактанти гліколіпідної природи, формували зону «розтікання нафти» діаметром 3,66-50см. Додавання в середовище олеїнової кислоти, соняшникового масла та октадекана суттєво підвищували активність досліджених штамів. Біосурфактанти, які утворювали штами *Candida* sp.79a та 156a, були екстраговані етилацетатом та показані як суміш гліколіпідів за допомогою тонкошарової хроматографії.

К л ю ч о в і с л о в а: дріжджі, біосурфактанти, гліколіпіди

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ДРОЖЖИ-ПРОДУЦЕНТЫ БИОСУРФАКТАНТОВ, ВЫДЕЛЕННЫЕ ИЗ ЦВЕТКОВЫХ РАСТЕНИЙ И ПЧЕЛ

Р е з ю м е

Сто шестьдесят штаммов дрожжей были выделены из различных цветковых растений и пчел *Apis mellifera*. Для определения способности выделенных штаммов продуцировать биосурфактанты использовали метод «растекания нефти». Пять самых активных штаммов, синтезирующих поверхностно-активные вещества гликолипидной природы, образовывали зону «растекания нефти» диаметром 3,66-50 см. Наличие в среде олеиновой кислоты, подсолнечного масла и октадекана значительно повышало активность исследуемых штаммов. Биосурфактанты, продуцируемые штаммами *Candida* sp. 79a и 156a, были экстрагированы этилацетатом и представляли собой смесь гликолипидов тонкослойной хроматографии.

К л ю ч е в ы е с л о в а: дрожжи, биосурфактанты, гликолипиды.

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