PRODUCTION OF EXTRACELLULAR EMULSIFIERS FROM YEASTS FOR USING IN DAIRY AND FOOD PRODUCTS

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Abstract

The main objective of this study was to test the ability of a number of yeast strains for production extracellular bioemulsifiers for potential use in food. An emulsifying agent was extracted from each strain of yeast tested, including 9 species of genera other than *Candida* utilis. From the 11 products tested, eight had emulsifying properties that were better than those of commonly used as food emulsifiers' mono-glyceride (Glycerol monoleate). *Candida* utilis EMCC 120 was selected for further study due to the excellent emulsification properties of its extracellular product and the food grade status of the yeast strain. Extracellular bioemulsifiers extracted emulsifier was purified by ultrafiltration. The purified product was evaluated for chemical and physical stability to establish its potential use as a natural emulsifier in processed foods. The yield of purified bioemulsifier was 3.2 g l^{-1} of the original dry cell weight for *Candida* utilis EMCC 120, and 0.85 g l^{-1} for *Saccharomyces cerevisiae* EMCC 69. Carbohydrate content of the purified bioemulsifier was 76%. Protein content was 23.75 % for *Candida* utilis EMCC 120. Butter oil-in-water emulsions were stabilized over a broad range of conditions, from pH 2 to 11, with up to 5% sodium chloride or up to 15% sucrose in the aqueous phase. In the presence of a low concentration of various solutes, emulsions were stable to three cycles of freezing and thawing.

Key words: Yeast, emulsifier, emulsification activity, emulsion stability, dairy product

INTRODUCTION

Glycerol monoleate (GMO) and Glycerol monostearate (GMS) are synthetic emulsifiers widely used in the food industry (Gaonkar, 1991). Although they are very effective in their intended function, these compounds are gradually losing favor due to increased pressure from consumers to reduce the use of artificial or chemically synthesized additives in foods. Thus, an increasing consciousness among consumers is driving a steady increase in demand for more natural food ingredients and additives. Bioemulsifier from microorganisms have a number of advantages over the chemically synthesized counterparts because of their biodegradable nature effectiveness at a

wide range of temperature, pH, salinities, and synthesis, under user-friendly conditions, e.g., low temperatures and pressures. Owing to their diverse biosynthetic capabilities, microorganisms are promising candidates for emulsifier production (Shepherd *et al.*, 1995). Bioemulsifiers are microbial metabolites with a hydrophobic moiety that is a fatty acid, and hydrophilic moiety that is a carbohydrate, amino acid, peptide or phosphate. Some of the most commonly studied microbial emulsifiers have included the polysaccharide-lipid complexes, e.g., emulsan from *Acinetobacter calcoacetics,* and the polysaccharide- mannoprotein complexes, e.g., liposan from *Candida lipolitica* (Cirigliano and Carman, 1984 and 1985, Kaplan and Rosenberg, 1982, Kaplan *et al.*, 1985). The bioemulsifiers are extracellular or bound to the cell surface, and all contained carbohydrate, and peptide material, e.g., liposan from *Candida lipolitica* (Cirigliano and Carman, 1984 and 1985) on an extracellular bioemulsifier from *Candida utilis* (Shepherd *et al.*, 1995). As an emulsifying agent, extracellular bioemulsifier from yeasts may present certain advantages. The difficulty of removing residual hydrocarbons from bioemulsifier from alkane-grown yeasts would preclude their use in certain applications (Cameron *et al* 1988). The present study deals with production of a bioemulsifier, from yeast grown on modified Czapek's yeast broth that could potentially be used in food and dairy industries and many other applications.

MATERIALS AND METHODS

Yeast Strains

Eleven yeast strains were used in this study. *Candida utilis* NCYC 769 (EMCC 120), *Candida kefyr* NCYC 744 (EMCC 68), *Creptococcus marinus* NCYC 784 (EMCC 121), and *Pichia anomala* NCYC 20 (EMCC 121) (National Collection of Yeast Cultures, Research Park Colney, Norwich NR4 7UA, U.K.) *Saccharomyces cerevisiae* ATCC 287 (EMCC 69), *Saccharomyces cerevisiae* ATCC 4126 (EMCC 71), and *Candida bombicola* ATCC 22214 (EMCC 91). *Candida utilis* NRRL Y-900 (EMCC 41). *Candida tropicalis* DSM 70156 (EMCC 2) (Deutsche Sammlung von Mikroorganismen, Grtmany). All strains were obtained from the Egyptian Microbial Culture Collection, Cairo MIRCEN, Faculty of Agriculture, Ain Shams University, Commercial two strains of baker's yeast were obtained from the local market.

All strains were used throughout this work were maintained on malt extract medium and stored at 4 °C.

Media used and growth conditions

Yeast strains were grown on either Czapek's yeast broth medium previously sterilized at 121 °C for 15 min. The media were inoculated with 2% volume of

precultured strains grown on malt extract medium for 72h then incubated at 25°C in a shaking water bath (JULABO LABORTE CHNIK GMBH D.7633 Seelbach, Germany) with agitation rate 200 rpm for 72h. Experiments were carried out in triplicate. Samples were taken every 24h. The samples were analyzed for total viable yeast count, total Carbohydrate, sucrose, glucose and pH.

Screening of strains

Elenmeyer flasks (500 ml) containing 100 ml of Modified Czapek's yeast broth were inoculated with 2% of 24 hr active culture and incubated for 3 days at 25°C in a shaking water bath with agitation at 200 rpm. The extracellular emulsifying agent was extracted from cell free extract using recirculate over modified cellulose ultrafiltration disc with magnetic sterrier, filters with a cut-off of 10000 Dalton to collect the extracellular yield with a molecular mass over 5000 Dalton.

Production of Emulsifier

Candida utilis EMCC 120 was tested on one liter Modular fermentor (Mini Bioreactor, A. Gallen Kamp & Co. Ltd.) operated at 30°C and 400 rpm for 60 h for examining the strain ability to the production of significant amounts of the emulsifying agent.

One extraction method was used to obtain bioemulsifiers from cell free extract of *C. utilis* EMCC 120. In this method, the extracellular bioemulsifier extracted from cell free extract by centrifugation at $10000x_g$ for 10 min. at 5 °C and cell free extract was recirculated over modified cellulose ultrafiltration membrane with magnetic sterrier, It was pressured under 70 bar (95 psi) through a 90 mm diameter cell ultrafiltriation Unit (Millipore corporation, USA) using cellulose membrane with molecular weight (Mw) cut off 10.000 Dalton to collect the extracellular yield with a molecular mass over 5000 Dalton .The concentrated retentate was freeze-dried in a freez-dryer

Biomass Dry Weight

At the end of fermentation period (60 hr), yeast cells in fermentation medium were killed by thermal treatment (75 °C for 15 min.) as suggested by Vendrusculo *et al.* (1994). Yeast cells were harvested by centrifugation at 16000 g for 20 min and then dried at 105 °C until the change of weight was negligible (Ming-Lo, 1997).

Emulsification activity determination

Emulsification activity was evaluated as described by Cameron *et al.*, (1988). Extract was dissolved (0.2 %) in 4 ml of distilled water and 6 ml of kerosene or butter oil. The tube homogeneity is achieved by vortex. After one hour, the emulsified proportion of kerosene or butter oil was compared with the total volume of kerosene

or butter oil added. Emulsion generated by vigorous mixing would separate completely within 1 hr, in absence of any emulsifying agents.

Evaluation of emulsification stability

Effect of some technological parameters, namely, pH range of 2-11, sodium chloride concentrations up to 5% sucrose concentrations up to 15% heat treatment at 63°C / 30 min., freezing at -18°C for 16 hr followed by thawing at 25°C for 8h and refrigeration at 4°C for 30 days, on the stability of purified intracellular emulsifier was evaluated as mentioned by Cameron *et. al.*, (1988).

Analytical Methods

Czapek's yeast broth medium was used to determine the yeast viable cell count as recommended by American Public Health Association (1992). Protein content of extract was determined by the microKjeldahl method according to the AOAC, 1995. Total carbohydrate content was determined using the phenol / sulfuric acid method described by Dubois *et. al.*, (1956), it was using glucose as standard. Determination of glucose or sucrose content of medium was carried out according to the method described by Trinder (1969). Bioemulsifier yield (%) expressed as gram bioemulsifier x 100/ gram biomass wet weight.

RESULTS AND DISCUSSION

All yeast strains examined showed active growth in modified Czapek's yeast broth. It could be noticed that *Candida utilis* EMCC 120 produced the highest yield (910 mg /l) followed by *Saccharomyces cervisiae* EMCC 69 (850 mg l^{-1}). The highest emulsification activity (91.5%) was recorded for *Candida utilis* EMCC 120 followed by *Pichia anomala* EMCC 122 (91%), whereas the highest emulsification activity (91.5%) was recorded for *Candida utilis* EMCC 120 (Table 1).

All of bioemulsifiers produced from yeast strains tested showing emulsifying activity, expressed as % butter oil phase emulsified, varied from 54% for commercial strain of baker's yeast (2) to 91.5% for that *Candida utilis* EMCC 120 (Table 1).

Data in Table (1) clearly indicated that some yeast strains, such as *C. utilis* EMCC 69, produced high yield with superior activity of bioemulsifier. For this reason and due to long history of safe human consumption, these strain was chosen for further studies.

Data in Table (2) and Fig (9) clearly indicate that *Candida utilis* EMCC 120 grown on modified Czapek 's yeast broth at 25 °C for72 h using 1-L bioreactor as a batch culture produced the highest bioemulsifier production (2.82 g/l) after 60 h of

fermentation at 25 °C. These results are in agreement with those reported by Shephered *et al* (1995).

Data in Table (3) clearly indicated that carbohydrate and protein contents of the purified ultrafiltrated extractr were, respectively, 76% and 23.75 % for *C. utilis* EMCC 120 The presence of proteinaceous material covalently bound to a polysaccharide-based bioemulsifier would lead to erroneous conclusions about the emulsification properties of that polysaccharide as proteins are known for their surfactancy (Patel and Fry, 1987).

Table 1. Extracellular bioemulsifier yield, activity and stability produced from yeast strains grown in Czapek's yeast broth in at 25°C for72 h using shaking flasks.

Yeast strain	Extracellular bioemulsifier (g/l)	Extracellular bioemulsifier yield (%)	Emulsification activity (%)
Glycerol monoleate (control)	-	-	90
Candida utilis EMCC 120	0.910	3.61	91.5
Candida kefyr EMCC 68	0.400	0.36	61
Creptococcus marinus EMCC 121	0.345	1.51	42
Candida utilis EMCC 41	0.275	2. 52	60
Candida tropicalis EMCC 2	0.198	1.97	87
Pichia anomala EMCC 122	0.475	2.90	91
Saccharomyces cervisiae EMCC 69	0.850	3.86	90
Saccharomyces cervisiae EMCC 71	0.755	5.51	89
commerical strain of baker's yeast (1)	0.770	4.09	90
commerical strain of baker's yeast (2)	0350	2.05	54
Candida bombicola EMCC 91	0.250	2.03	76

Table 2. Extracellular bioemulsifier Production by C. utilis EMCC 120 grown on modified Czapek ,s yeast broth at 25 oC for72 h using 1-L bioreactor as a batch culture.

Fermentation period (h)	Extracellular bioemulsifier (g/l)
0	0
6	1.2
12	2
18	2.4
24	2.55
30	2.5
36	1.6
42	2.15
48	2.25
54	2.45
60	2.82
66	2.62
72	1.9

Table 3. Characteristics of extracellular bioemulsifier from C. utilis EMCC 120 grown on modified Czapek ,s yeast broth at 25 oC for72 h using 14-L bioreactor as a batch culture.

Property / Composition	C. utilis EMCC 120
Emulsification activity (butter oil phase emulsified) (%)	92.0
Carbohydrate content (%)	76.0
Kjeldahl nitrogen (%)	3.80
Protein content %	23.75
Bioemulsifier (g/l)	3.20
Bioemulsifier yield (%)	10.66

The highest bioemulsifier production of extracellular bioemulsfier produced by *C. utilis* EMCC 120 increased gradually during fermentation period and reached the maximum value of (3.2 g/l) after 60 h in 14-L bioreactor of fermentation, then decreased gradually until the end of fermentation period (Fig. 1). These results are in agreement with those reported by Shephered *et. al.,* (1995).

The purified emulsifiers (polysaccharide and protein) were tested for its stabilization under a range of chemical and physical conditions which might be encountered in various applications. To facilitate the detection of the possible detrimental effects of pH, sodium chloride or sucrose on emulsification stability, emulsions were made with 0.2% (w/v) purified emulsifier. The pH of the aqueous phase had little effect on the amount of the kerosene phase emulsified between pH 2 and pH 11. In the presence of 0.5 to 5% (w/v) sodium chloride in the aqueous phase, stable emulsions were formed. Similar results were noticed on the sucrose aqueous solutions (Tables 3). These results are in accordance with those reported by Cameron *et. al.,* (1988) and Roushdy (1997).

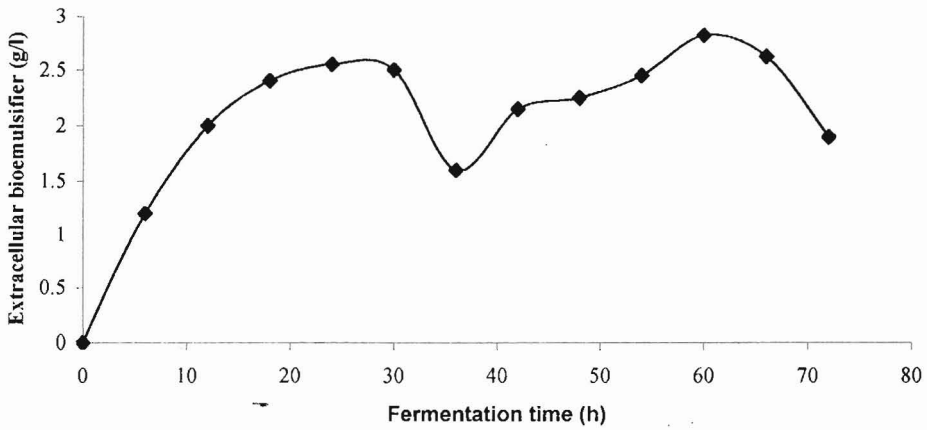

Fig. 1. Extracellular bioemulsifier Production by *C. utilis* EMCC 120 grown on modified Czapek 's yeast broth at 30 °C for 60 h using 1-L bioreactor as a batch culture.

Table 4. Stability of emulsion with purified exteracellular bioemulsifier from *Candida utilis* EMCC 120 at different pH values, sodium chloride and sucrose concentrations.

pH value	Butter oil phase emulsified % in EXBE from *Candida utilis* EMCC 120	NaCl %	Butter oil phase emulsified % in EXBE from *Candida utilis* EMCC 120	Sucrose %	Butter oil phase emulsified % in EXBE from *Candida utilis* EMCC 120
2	91	0	91	*0*	91
4	90	1	90	0.5	91
6	90	2	90	1	91
8	89	3	90	2.5	91
10	89	4	88	5	91
11	88.5	5	87	7.5	91
				10	91
				15	91

EXBE: Exteracellular bioemulsifier.

Physical treatments known to reduce emulsion stability were tested on emulsion containing 0.2% purified emulsifier. Emulsification activity of only 91% after 3 cycles of freezing and thawing has been reported for *C. utilis* EMCC 120. Emulsification activity of only 15% after 2-3 cycles of freezing and thawing has been reported for *S. cerevisiae* bioemulsifier (Cameron *et al* 1988). However, they reported that addition of 30 mM tripotasium citrate, 5mM calcium chloride or 10 mM sodium chloride, sucrose prevented the breakage of emulsion with 0.2 % purified emulsifier during three cycles of freezing and thawing. Emulsions with *C. utilis* EMCC 120 bioemulsifier were not disrupted by pasteurization at 63°C for 30 min (Table 5).

The emulsifying agent showing superior emulsifying activity 91% for *C. utilis* EMCC 120 after storage at 4°C for 30 days (Table 5). In contrast, much loss in emulsion stability during storage at room temperature was observed (Table 5).

Table 5. Effect of pasteurization (63°C for 30 min), freezing and thawing, and storage at room temperature (25+1 °) and at 4°C on stability of emulsions with purified extracellular bioemulsifier from *Candida utilis* EMCC 120.

Treatment	Emulsification stability (%) (%Butter oil phase emulsified)	
	C. utilis EMCC 120	
	Before	After
Pasteurization	91	91
Freeze-thaw cycles: 1st 2nd 3rd	 91 90 89	 90 89 79
Storage at : 4°C for 30 days	91	91
25+1°C for 15 days	91	88

CONCLUSION

Since food or fodder yeasts such as *S. cerevisiae* and C. *utilis* are edible (El-Samragy *et al* 1988 and Roushdy 1997), it is expected that extracellular bioemulsifier, produced from such yeasts would be nontoxic.

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إنتاج مادة الاستحلاب الخارجية من الخمائر لإستخدامها فى منتجات الألبان

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تهدف هذه الدراسة إلى اختبار عدد كبير من الخمائر فى إنتاج مادة الاستحلاب الميكروبية من خارج جدار الخلية لإستخدامها إستخداما خاصا فى الأغذية. و لقد تم استخلاص مادة الاستحلاب هذه من كل نوع أو سلالة من الخمائر المختبرة و التى تضم تسعة أنواع من أجناس مختلفة بخلاف *C. utilis* و من أحد النواتج الأحدى عشرة المختبرة وجد أن ثمانية نواتج منها لها خواص استحلاب أفضل من خواص مواد الاستحلاب شائعة الاستخدام فى مجال الأغذية مثل الجليسرول مونو أوليت. و قد وجد أن السلالة (*C. utilis*) و التى تم اختبارها لاستكمال الدراسة المستقبلية قد أعطت مواد استحلاب خارجية ذات خواص استحلابية ممتازة ، بالاضافة إلى مناسبتها للاستخدام الغذائي.

وقد تم استخلاص مادة الاستحلاب الخارجية من (*C. utilis*) بعائد كبير و قد تم تنقية مادة الاستحلاب المستخلصة بالمعاملة الحرارية باستخدام الترشيح الفائق. و كان العائد الذى تم تنقيته من مادة الاستحلاب الخارجية للسلالات كالتالى:

٣،٢ جم/ لتر فى حالة ال *C. utilis* و ٨٥، جم/ لتر فى حالة الـ *S. cerevisiae* EMCC 69 و كان المحتوى من الكربوهيدرات ٧٦% و كان المحتوى من البروتين ٢٣،٧٥ % فى حالة ال *C. utilis* .

و قد حدث ثباتا لطور زيت الزبد فى الماء بالمستحلبات على مدى واسع من الاشتراطات فوجد أنه كان ثابتا خلال درجة pH ٢-١١ و كذلك مع تركيز ٥% كلوريد صوديوم حتى تركيز ١٥ % من السكروز فى الطور المائى. و فى وجود تركيزات منخفضة من مادة الاستحلاب الخارجية كانت المستحلبات ثابتة خلال ثلاث دورات من التجميد و التسييح.

الخلاصة:

نتيجة لاستخدام سلالات من الخمائر التى تستخدم عادة فى الأغذية مثل سلالة الـ *S. cerevisiae* EMCC 69 وسلالة الـ *C. utilis* فمن المتوقع ان مادة الاستحلاب الخارجية المنتجة امنة الاستخدام وغير سامة.