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Emulsifier properties of the mannoprotein extract from yeast isolated from sugar palm wine

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ABSTRACT: *Saccharomyces cerevisiae* KA01 was isolated from palm wine obtained from a local brewer in Songkhla province, Thailand. Mannoproteins with emulsification properties were extracted from the cell walls of *S. cerevisiae* KA01 cultivated in YM medium by autoclaving in a pH 7.0 citrate buffer for 60 min with a yield of 0.32 g/g wet cells. The mannoprotein extract obtained was evaluated for its chemical and physical stability to establish its potential use as a natural emulsifier in processed foods. The mannoprotein extract exhibited emulsion with the vegetable oils tested and showed emulsion activity of 65% towards palm oil as oil-in-water with a critical emulsifier concentration of 20 g/l. The mannoprotein extract had similar emulsifying properties to the commonly used food emulsifiers gum arabic and lecithin. Palm oil-in-water emulsions were stabilized over a broad range of conditions from pH 5 to 8 with up to 3% (w/v) sodium chloride and up to 0.1% (w/v) $CaCl_2$ and $MgCl_2$ in the aqueous phase. Temperature did not affect the emulsion activity of the mannoprotein extract. Preliminary trials showed that mannoproteins from *S. cerevisiae* KA01 had potential for use in salad dressing.

KEYWORDS: bioemulsifier, emulsion, *Saccharomyces cerevisiae*, salad dressing

INTRODUCTION

Surfactants and emulsifiers are amphipathic molecules. They have both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases of different polarity and hydrogen bonding such as oil/water or air/water interfaces[1]. As a result, surfactants and emulsifiers reduce the forces of repulsion between different phases at interfaces or surfaces and allow the two phases to mix easily. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits making surfactants and emulsifiers integral to many industrial, agricultural, and food processes. Mannoprotein has been shown to be an effective bioemulsifier. The presence of hydrophilic mannose polymers covalently attached to the protein backbone provides the mannoprotein with the amphiphilic structure common to surface active agents and effective emulsifiers[2].

Emulsifiers derived from natural sources, although more expensive to produce, may have certain advantages over the chemically synthesized emulsifying agents. Due to the increasing consumer demand for natural products, bioemulsifiers may eventually become cost-effective for various applications. Some natural, plant-derived, food emulsifiers such as lecithin and gum arabic are already in the market. However, these emulsifiers have limited functionality in many food products[3]. The production of food emulsifiers by microbial cultivation would remove some of the constraints associated with the properties and supply of natural, plant-derived emulsifiers.

In the production of wine, natural fruit juice fermentation is carried out by a succession of different yeast populations. The early stages of the alcoholic fermentation are characterized by the activity of mostly apiculate, non-*Saccharomyces* yeasts from the *Kloeckera* and *Hanseniaspora* genera[4–7]. The growth of these yeasts is generally limited to the first two or three days of fermentation, after which they die off, giving way to the more tolerant strains of *Saccharomyces cerevisiae*[5,8–10]. Its tolerance to high concentrations of ethanol is the principal feature that allows this yeast to survive in this specific environment[11].

Mannoprotein extracted from *S. cerevisiae* is an effective bioemulsifier[2,12]. The emulsions produced are thick and viscous. Since *S. cerevisiae* is edible (it is used in food and beverage products), the emulsifier would be expected to be nontoxic and could have applications in the food and cosmetics industries. This product also satisfies the current consumer demand for natural and environmentally safe products.

Mannoproteins are freely soluble in water and can be extracted from the cell wall of *S. cerevisiae* in high yields[2,13,14]. Thus strains of *S. cerevisiae* produced by low-cost biotechnology methods using water-soluble substrates, as well as brewing industries, have become important sources from which bioemulsifiers are extracted[12,15]. These sources offer the advantages of low cost and a high volume of yeast biomass, which translates into high bioemulsifier yields than from synthetic sources.

The objective of this study was to study the emulsification properties of mannoproteins extracted from yeast isolated from palm wine produced in a local brewer in Songkhla province, Thailand. The potential use of mannoproteins in salad dressing has been also studied.

MATERIALS AND METHODS

Chemicals

Commercial vegetable oils (soybean oil, palm oil, corn oil, olive oil, sunflower oil, rice bran oil, and sesame oil) were purchased from a supermarket. Gum arabic was purchased from Nacalai Tesgue, Kyoto. Lecithin was obtained from Fluka. All other chemicals used were of analytical grade.

Yeast strain, medium, and growth conditions

Palm wine at the final stage of fermentation was collected from a local brewer in Singhanakorn District, Songkhla. Direct isolation was performed by serially diluting samples in 0.85% sterile normal saline to 10^{-1}–10^{-7} dilution. Aliquots were spread-plated on the YM agar containing malt extract powder (Himedia, Thailand), yeast extract (Lab-scan, Thailand), and bacteriological peptone (Hi-media, Thailand) and incubated at room temperature for 48 h. From each plate 5–10 morphologically distinct yeast colonies were randomly selected and streaked on YM agar. This procedure was repeated in order to purify the isolates. The isolated yeast was maintained on YM slants at 4 °C and transferred at 1 month intervals. The isolated yeast strain was identified based on 26S rDNA sequence analysis. The 26S rDNA fragment of isolated yeast strain was amplified using universal oligonucleotide primers F63-Forward 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and LR-Reverse 5′-GGTCCGTGTTTCAAGACGG-3′. Sequences of the amplified fragments were analysed with an ABI PRISM 3100 DNA sequencer (PE Amplified Biosystems, Foster City, CA) and a BioDye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The BLAST program was used to analyse the sequence homology.

S. cerevisiae KA01 was grown in 200 ml of YM medium in 500 ml flasks. The flasks were incubated at room temperature for 12 h (early stationary phase) and shaken at 150 rpm.

Extraction of a yeast extract enriched in mannoproteins

Mannoproteins were extracted from cells of *S. cerevisiae* KA01 by the method of Torabizadeh et al[12]. Yeast cells (20 g) were suspended in 100 ml 0.1 M potassium citrate and 0.02 M potassium metabisulphite buffer (pH 7) and autoclaved (121 °C) for various periods (15–120 min). The resulting suspensions were centrifuged at 6000g for 10 min at 4 °C. The supernatant was retained and mixed with five volumes of chilled ethanol and incubated overnight at 4 °C for complete precipitation. The precipitates were recovered by centrifugation at 6000g for 10 min at 4 °C, and then they were washed twice with chilled ethanol. The precipitates were freeze-dried for 48 h. Mannoproteins were dissolved in distilled water and dialysed (8 kDa molecular weight cut-off) against distilled water overnight. The whole mannoprotein extract was then freeze-dried and used throughout this study. Total protein was determined by the dye-binding assay[16] using bovine serum albumin as the standard. Total sugar was determined by phenol-sulphuric assay[17] using mannose as the standard.

Analytical methods

An average of molecular weight of the mannoprotein extract was estimated on the basis of the calibration curve obtained by HPLC with an Ultrahydrogel linear 1 column (7.8 i.d. × 300 mm, Waters, Madison, USA) using a 0.05 M sodium bicarbonate buffer (pH 11, Lab Scan, Bangkok) as the mobile phase (column temperature 25 °C, flow rate 0.6 ml/min). The column was standardized with pullulans (Polymer Laboratories, Shropshire, UK) of diverse molecular mass and monitored by RI and a photodiode array detector (Water 410, Madison). Emulsification activity was measured according to a modified version of the method of Cameron et al[2]. Vegetable oil (1 ml) was added to 1 ml of mannoproteins suspension and vortexed at high speed for 3 min. The emulsion activity was determined after 1 h (Emulsification activity, %EA) whereas the emulsion stability was determined after 24 h (emulsification index, $\%E_{24}$). The %EA and $\%E_{24}$ were calculated by dividing the measured height of emulsion layer by the mixture's total height. All experiments were done in triplicate and results were

reported as the average from the determinations in triplicate.

Determination of the type of emulsion formed

A filter paper wetting test and a dilution test[18] were used to determine the type of emulsion formed. For the filter paper wetting test, a droplet of emulsion was dropped onto filter paper. A water-in-oil (w/o) emulsion droplet of emulsion remains as a droplet on the filter paper. If the emulsion is oil-in-water (o/w) it disperses rapidly on the filter paper. For the dilution test, a droplet of emulsion was dropped into water and oil. If the emulsion is w/o, a droplet of emulsion disperses in the oil but remains as a droplet in the water. If the emulsion is o/w, a droplet of emulsion disperses in water turning it cloudy but remains as a droplet in oil.

Stability of the fraction enriched in mannoproteins

The freeze-dried mannoprotein extract (20 g/l) was prepared in distilled water. To investigate the effects of pH, salts concentration (NaCl, $CaCl_2$, and $MgCl_2$), and temperature on emulsification activity of mannoproteins, the mannoprotein extract solution was adjusted with 1 N HCl or NaOH to obtain a range of pH between 3 and 12. NaCl was added to the sample to obtain the final concentrations of 0–3% (w/v). $CaCl_2$ and $MgCl_2$ were also added to the samples to obtain the final concentrations of 0–0.1% (w/v). For the study of thermal stability, the mannoprotein extract solution was incubated at 63 °C for 30 min, at 100 °C for 15 min, and at 121 °C for 15 min, and then cooled to 30 °C. The remaining emulsification activity was then determined. Commercial bioemulsifiers (lecithin and gum arabic) at the same concentrations were also subjected to the stability study.

Determination of droplet size distribution

The droplet size distributions for the fresh emulsions were measured approximately 1 h after preparation by a laser diffraction method[19]. Distilled water was used as the dispersant to determine the emulsion lipid globule size distribution. The software used a refractive index (RI) of dispersant (water) of 1.33 to calculate the dispersion index (or span) from span $= [d(90) - d(10)]/d(50)$. The $d(10)$, $d(50)$, and $d(90)$ values are size values corresponding to the cumulative distribution at 10%, 50%, and 90%, respectively. Thus the $d(10)$ represents a size value below which 10% of the cumulative distribution is present. Drops of emulsion were introduced into the sample presentation unit until the concentration reached the optimum one, as indicated by the instrument.

Preparation of salad dressing

The salad dressing formulation consisted of soybean oil 24.4% (w/w), vinegar 54.2% (w/w), sugar 14.1% (w/w), salt 4.3% (w/w), pepper 1.6% (w/w), and mustard 1.4% (w/w) and with or without bioemulsifiers (mannoproteins, gum arabic, or lecithin, 0.2–0.6%, w/w). The dried ingredients were mixed, vinegar was added when the dried ingredients were dispersed, and the soybean oil was gradually incorporated into the mixture.

Statistical analysis

Data were subjected to ANOVA. A comparison of means was carried out by Duncan's multiple-range test. Statistical analysis was performed using SPSS 10.0.

RESULTS AND DISCUSSION

Isolation of yeast

Generally Thai traditional wine (rice wine, palm wine) is produced with indigenous microorganisms and fermented in earthenware. The back-slopping technique to produce wine is also used. In the present study palm wine in the last stage of fermentation before subjection to the distillation process was used as the source of microorganisms. We found that only one yeast strain dominated in the palm wine at the last fermentation stage. The isolated yeast strain produced an off-white, smooth, raised, and glistening colony. Under the microscope isolated yeast had an oval shape with buds and 2–4 ascospores when cultivated in ascospore-inducing medium (data not shown). From 26S rDNA sequence analysis, the isolated yeast was identified as *S. cerevisiae*. The rDNA sequence was deposited in DDBJ/EMBL/GenBank (Accession No. AB510531).

Extraction of mannoproteins

The yield of the bioemulsifier increased with increasing extraction time (Table 1). The critical emulsifier concentration for all extraction times was 20 g/l. The effectiveness of the bioemulsifier is evident from its low critical emulsifier concentration[15,20]. Nevertheless, autoclaving for 15 min gave the lowest bioemulsifier yield and emulsification activity value. In addition, it also resulted in a lower stability of the emulsion towards palm oil after being left to stand at room temperature for many days (data not shown). Although autoclaving for 30 min brought the critical emulsifier concentration to 20 g/l, it gave a less stable emulsion

Table 1 Effect of extraction time on the mannoprotein extract yield, and emulsification index (%E_{24}).

Time* (min)	Mannoproteins yield (g/5 g wet cell yeast)	Emulsification index (%E_{24})
15	1.466 ± 0.023^{d}	49 ± 4^{b}
30	1.588 ± 0.019^{c}	63.4 ± 0.8^{a}
60	1.63 ± 0.06^{bc}	64.7 ± 0.7^{a}
90	1.71 ± 0.04^{a}	64.3 ± 0.0^{a}
120	1.682 ± 0.009^{ab}	64.3 ± 0.0^{a}

* Holding time in autoclave.
Values are given as mean ± SD from triplicate determinations.
Different letters in the same column indicate significant differences ($p < 0.05$).

Table 2 Vegetable oil emulsification by the mannoprotein extract, gum arabic, and lecithin (%E_{24}).

Oil type	Mannoproteins	Gum arabic	Lecithin
Olive	64.7 ± 0.7^{ABa}	65.5 ± 0.0^{Aa}	54.3 ± 2.7^{Cb}
Soybean	57.0 ± 1.7^{Cb}	61.4 ± 1.2^{Ba}	59.5 ± 2.1^{Bab}
Palm	65.5 ± 1.2^{Aa}	0.0 ± 0.0^{Cb}	64.2 ± 2.1^{Aa}
Rice bran	0.0 ± 0.0^{Db}	58.1 ± 3.6^{Ba}	61.4 ± 2.6^{ABa}
Sesame	57.6 ± 0.9^{Ca}	0.0 ± 0.0^{Cb}	0.0 ± 0.0^{Db}
Corn	57.1 ± 0.0^{Cb}	61.6 ± 2.6^{Ba}	0.0 ± 0.0^{Dc}
Sunflower	62.8 ± 2.5^{Ba}	60.0 ± 1.2^{Ba}	55.5 ± 1.6^{Cb}

Values are given as mean ± SD from triplicate determinations.
Different superscript capital letters in the same column indicate significant differences ($p < 0.05$).
Different superscript lower-case letters in the same row indicate significant differences ($p < 0.05$).

as big droplets were observed. Emulsifier yield, emulsification index, and critical emulsifier concentration of emulsifiers from extraction time at 60, 90, and 120 min showed no significant difference ($p < 0.05$). Accordingly, autoclaving for 60 min was the best condition for emulsifier extraction from *S. cerevisiae* KA01 in terms of cost and energy consumption. In the present study the extraction time (60 min) was shorter than the optimum autoclaving time (120 min) for bioemulsifier extraction from *S. cerevisiae* used in other studies[2, 12, 15].

The apparent molecular weight of mannoproteins, when compared with that of pullulan standards, was 76 kDa (data not shown). The composition of the mannoproteins was 58% carbohydrate and 42% protein.

Stability of mannoproteins

Mannoproteins, gum arabic, and lecithin at the critical emulsifier concentration (20 g/l) were checked for specificity of vegetable oil emulsion. All the vegetable oils tested, except rice bran oil, served as substrates for emulsification by the mannoprotein extract (Table 2). The poor emulsification properties of some vegetable oils, such as rice bran oil, might be due to the inability of the bioemulsifier to stabilize the microscopic droplets. Maximum emulsifying activity was observed with palm oil; however, the emulsification index of isolated mannoproteins towards palm oil and olive oil were not significantly different ($p < 0.05$). All the tested oils comprise mainly of three fatty acids, oleic acid, linoleic acid, and palmitic acid, in varying proportions. Oleic and linoleic acids are unsaturated fatty acids, whereas palmitic acid is a saturated fatty acid[21]. Accordingly, palm oil displays a lower degree of unsaturation than olive oil which is rich in unsaturated oleic acid ($C_{18:1}$). Perhaps the mannoprotein extract had more specificity with palmitic acid, which is the main constituent of palm oil, than oleic and linoleic acid. This suggests that palm oil showed high emulsification specificity with mannoproteins. Study of specificity for substrates has indicated that mannoproteins are capable of forming stable emulsions with various vegetable oils, and mannoprotein can be used as an emulsifying agent for these compounds[2]. The capacity to form emulsions with vegetable oils suggests potential applications as an emulsifying agent in the food industry.

Mannoproteins exhibited higher emulsification activity than the commercial emulsifiers, gum arabic and lecithin. Gum arabic could not emulsify palm oil or sesame oil. Lecithin could not emulsify sesame oil or corn oil. The stability of the emulsion is affected by the composition of the oil dispersed phase[22].

The structure of mannoproteins, gum arabic, and lecithin was also considered. The structure of gum arabic is more similar to mannoproteins than lecithin. It is composed of a highly branched arrangement of the simple sugars galactose, arabinose, rhamnose, and glucuronic acids. It also contains a protein component (about 2%, w/w) covalently bound within its molecular arrangement[23]. Lecithin is composed of glycerol, two fatty acids, and phosphate and has a nitrogenous base. Accordingly, the emulsification activity of mannoproteins was much more similar to gum arabic than lecithin.

The mannoprotein extract was most active for pH 5–8 (Fig. 1). A slight decrease in emulsifying activity was observed for pH less than 5 due to the precipitation of mannoproteins. The emulsifying

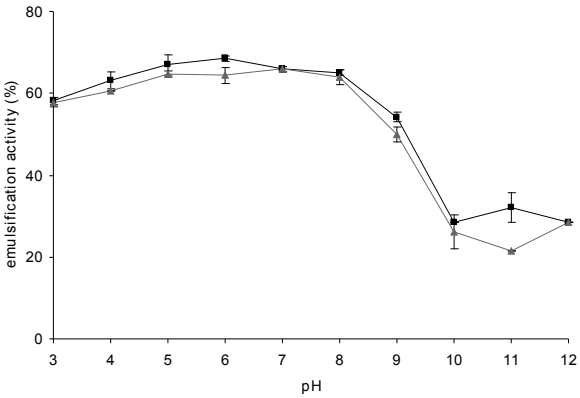

Fig. 1 Effect of pH on emulsification activity of mannoproteins. ■: emulsification activity (%EA), ▲: emulsification index (%E_{24}). Bars represent the standard deviation from three determinations.

activity decreased with increasing pH when the pH was above 9. Different emulsifiers are known to have different optimal pH levels. For example, the emulsifier from *Kluyveromyces marxianus* FII 510700 is stable at pH 3–11 [24] whereas the emulsifier from *Yarrowia lipolytica* is stable at pH 3–9 [25].

The stability of the bioemulsifier was tested over a wide temperature range. Studies of the effect of heat treatment demonstrated that temperatures which are usually used in food processing, such as pasteurization, cooking, and food thermal processing, have no appreciable effect on emulsifying activity (Table 3). Each temperature tested showed no influence on the emulsification activity of mannoproteins towards palm oil. This feature of mannoproteins may be attributed to certain chemical groups that protect it from hydrolytic degradation. This is a useful property for the many commercial applications that involve surface-active or emulsifying agents in formulations subjected to high temperature treatments [6]. The thermal stability of the bioemulsifier indicates the usefulness of the bioemulsifier in industries where heating to achieve sterility is of paramount importance.

Experiments were performed to examine the influence of NaCl, $MgCl_2$, and $CaCl_2$ concentration on the emulsification activity. The emulsifying activity against vegetable oil remained practically unchanged for the concentrations of NaCl and $MgCl_2$ tested (Fig. 2). NaCl and $MgCl_2$ had no effect on the activity of mannoproteins. However, the emulsification activity of mannoproteins decreased when $CaCl_2$ was added. On increasing the concentration of $CaCl_2$ the emulsification activity (%EA) remained stable whereas the emulsification index (%E_{24}) decreased to

Table 3 The effect of temperature on the stability of the mannoprotein extract.

Temperature (°C)	Emulsification index (E_{24},%)
63	65.1 ± 0.7^{a}
100	65.1 ± 0.7^{a}
121	63.5 ± 1.3^{a}

Values are given as mean ± SD from triplicate determinations.
Different superscript letters in the same column indicate significant differences ($p < 0.05$).

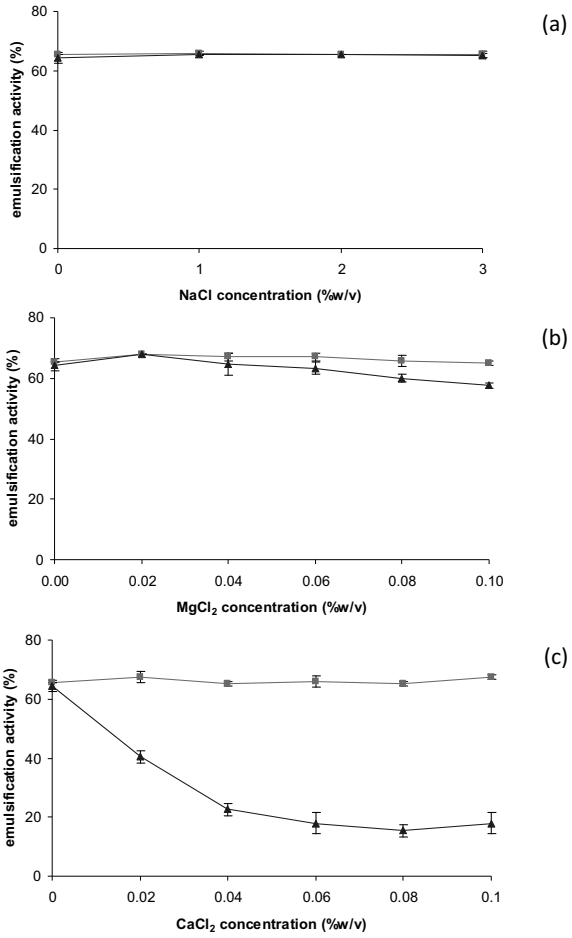

Fig. 2 Effect of salt concentrations on emulsification activity of mannoproteins (a) NaCl concentration (b) $MgCl_2$ concentration (c) $CaCl_2$ concentration. ■: emulsification activity (%EA), ▲: emulsification index (%E_{24}). Bars represent the standard deviation from three determinations.

20% at 0.1% (w/v) of $CaCl_2$. These were emulsions that were unstable at higher $CaCl_2$ concentration. The addition of more $CaCl_2$ might destabilize emulsions through reducing the electrostatic repulsion among

Table 4 Droplet mean diameter and dispersity index (span) of emulsions.

Samples	$d(10)$ (μm)	$d(50)$ (μm)	$d(90)$ (μm)	Span
Mannoprotein extract	40.49	123.39	241.58	1.63
Gum arabic	35.68	105.67	189.05	1.45
Lecithin	26.45	86.46	170.75	1.67

The experiment was done in triplicate and results were reported as the average from triplicate determinations.

droplets[26]. The stability of the bioemulsifier to salinity suggested that it is a good candidate for use in some industries that involve salt. This feature would be of great interest in food production that has a large amount of salted food. The mannoprotein extract from isolated yeast obtained from traditional liquor distillation was stable over a wide range of physical and chemical conditions. These findings revealed that the product obtained could be very useful in situations where extreme conditions of temperature, salinity, and pH 5–8 are present. The results agreed with previous studies that the mannoprotein extract from *S. cerevisiae* and *K. marxianus* FII 510700 were stable in a wide range of pHs, temperatures, and salts[12,24].

Determination of droplet size distribution

A convenient way to evaluate the long-term stability of an emulsifier is to determine droplet size distribution[27] using parameters that indicate dispersion. The stability of an emulsion can be enhanced by reducing the droplet size. All the emulsions showed a monomodal distribution of droplets (Table 4). Emulsion from mannoproteins had 50% of the particles under 123 μm, whereas the corresponding $d(50)$ values for emulsions from gum arabic and lecithin were 106 μm and 86 μm, respectively. This revealed that the emulsion from lecithin had the smallest particle size. However, the span of lecithin emulsion was not the lowest. It showed that emulsion from lecithin had the broadest range of polydispersity. As a rule, large globules tend to coalesce faster than smaller ones[27]. Therefore obtaining an emulsion with a uniform smaller droplet size is essential for achieving a stable emulsion system.

Preparation of salad dressing

Finally, the mannoprotein extract was tested in a salad dressing by using four different formulations. The results revealed that salad dressing made by adding mannoproteins, gum arabic, and lecithin had sufficient emulsion stability after leaving it at 4 °C for 1 h.

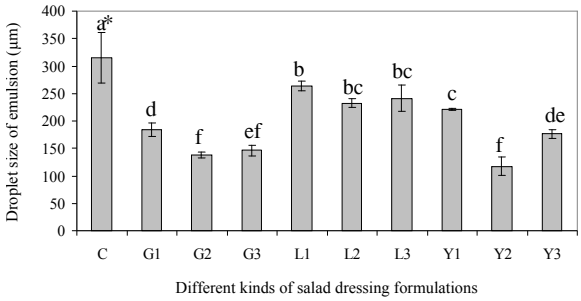

Fig. 3 Droplet sizes of emulsion of salad dressing formulations at different concentration of emulsifier. (C: control, G1: gum arabic 0.2%, G2: gum arabic 0.4%, G3: gum arabic 0.6%, L1: lecithin 0.2%, L2: lecithin 0.4%, L3: lecithin 0.6%, Y1: mannoproteins from *S. cerevisiae* cells 0.2%, Y2: mannoproteins from *S. cerevisiae* cells 0.4%, Y3: mannoproteins from *S. cerevisiae* cells 0.6%). Bars represent the standard deviation from three determinations. *Different letters indicate significant differences ($p < 0.05$).

In contrast, the control formulation with no added emulsifier showed rapid separation of oil. Every formulation showed a significantly higher emulsification activity than that of the control formulation ($p > 0.05$). The highest emulsification activities found were in the formulations with 0.6% (w/v) of mannoproteins added (%EA = 41%) and with 0.6% (w/v) gum arabic added (%EA = 39%).

The droplet sizes of emulsions with added emulsifier were smaller than that of the control (Fig. 3) and the difference was significant ($p < 0.05$). Droplet size distribution of every formulation was in the range of 120–315 μm. The control formulation showed the highest droplet size ($p < 0.05$) whereas adding 0.4% (w/v) of mannoproteins and 0.4% and 0.6% (w/v) of gum arabic gave the smallest droplet size ($p < 0.05$). Consequently, it should be possible to use mannoproteins in food products as a commercial emulsifier.

CONCLUSIONS

The mannoprotein extract from yeast, *Saccharomyces cerevisiae*, isolated from palm wine obtained from distillations of local brewers could provide a source of raw material for the mass production of mannoproteins emulsifier. This would eliminate the need to grow the yeast specifically for the production of the mannoprotein extract, and would help the environment by reducing waste discharge from local distillers. These findings reveal that the mannoprotein extract obtained could be very useful in situations where extreme conditions of temperature, salinity, and

pH 5–8 are present. The capacity to form emulsions with vegetable oils suggest potential applications as an emulsifying agent in the food industry such as in salad dressing. In addition, the production of mannoproteins would be economically favourable, since the process converts a low-value waste into a high-value product.

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