Effective ethanol production from whey powder through immobilized *E. coli* expressing *Vitreoscilla* hemoglobin

Taner Sar, Benjamin C. Stark & Meltem Yesilcimen Akbas

To cite this article: Taner Sar, Benjamin C. Stark & Meltem Yesilcimen Akbas (2017) Effective ethanol production from whey powder through immobilized *E. coli* expressing *Vitreoscilla* hemoglobin, Bioengineered, 8:2, 171-181, DOI: 10.1080/21655979.2016.1218581

To link to this article: https://doi.org/10.1080/21655979.2016.1218581

© 2017 Taylor & Francis

Published online: 31 Aug 2016.

Submit your article to this journal

Article views: 1111

View related articles

View Crossmark data

Citing articles: 14 View citing articles
Effective ethanol production from whey powder through immobilized *E. coli* expressing *Vitreoscilla* hemoglobin

Taner Sar, Benjamin C. Stark, and Meltem Yesilcimen Akbas

ABSTRACT

Ethanol production from whey powder was investigated by using free as well as alginate immobilized *E. coli* and *E. coli* expressing *Vitreoscilla* hemoglobin (VHb) in both shake flask and fermenter cultures. Media with varying levels of whey (lactose contents of 3%, 5%, 8% or 15%) and yeast extract (0.3% or 0.5%) were evaluated with fermentation times of 48–96 h. Immobilization and VHb expression resulted in higher ethanol production with all media; the increases ranged from 2% to 89% for immobilization and from 2% to 182% for VHb expression. It was determined that growth medium containing 8% lactose with 0.5% yeast extract yielded the highest ethanol production for free or immobilized strains, with or without VHb expression, in both shake flask and fermenter cultures. Immobilization with alginate was found to be a promising process for ethanol production by VHb-expressing ethanologenic *E. coli*.

KEYWORDS

alginate; ethanol; entrapment method; fermentation; *Vitreoscilla* hemoglobin; whey

Introduction

Bioethanol is the most widely used biofuel for transportation because it is nontoxic and renewable. Its production from corn starch is not terribly efficient regarding the energy economy of the process, and also has drawbacks regarding the competition it creates with farm acreage used to produce food. Because of this a good deal of effort has gone into production of bioethanol from both farm and food processing wastes. Cheese whey is an abundant by-product of the dairy industry. Whey produced in large amounts is considered a highly polluting waste due to its high organic content. The global production of whey is about 180 to 190 × 10^6 tons per year with 9 L of whey produced for each kg of cheese obtained. In the past 50 years half of the world wide whey production has been disposed as waste to the environment while about 50% has been converted to valuable food products.

Cheese whey contains 5–6% lactose, 1% protein and 0.06% fat, and therefore could be used as a renewable and inexpensive raw material for microbial fermentations to produce fuels as well as food additives. Ethanol production from cheese whey has been investigated by many groups. In some countries (New Zealand, the United States, and Denmark) bioethanol is produced from whey.

Due to the low lactose content of whey, however, its fermentation yields only about 2–3% (v/v) ethanol; this makes this process unfeasible at an industrial level, because of the costs of distillation. Cheese whey powder is a dried and concentrated form of cheese whey which can be produced at a cost of between 20 to 40 cents per kg. The use of high lactose fermentation medium made with cheese whey powder could result in higher ethanol concentrations and thus lower distillation costs.

Bioethanol is produced naturally by some lactose fermenting microorganisms such as *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Candida pseudotropicalis*. *Kluyveromyces marxianus* cannot ferment lactose rapidly or with high ethanol concentrations. *S. cerevisiae* has many advantages compared to bacteria due to its efficient ethanol production and tolerance to wide ranges of pH and ethanol concentrations.

Wild type *S. cerevisiae*, however, cannot ferment lactose to ethanol. However, the fermentation of enzymatically hydrolysed lactose can be used for lactose
fermentation with \textit{S. cerevisiae}, although this process is expensive and may be complicated by catabolite repression.\textsuperscript{26,27}

Enhancing ethanol production and tolerance of microorganisms are alternative research areas for economical ethanol production. (reviewed by Lin and Tanaka\textsuperscript{28,29-31}) Included among these is immobilization of cells. Generally immobilization techniques are simple and low cost.\textsuperscript{32} Sodium alginate, calcium alginate beads or agar-agar cubes are the most utilized media for immobilization of microorganisms. (reviewed by Tesfaw and Assefa\textsuperscript{33,34}).

Immobilization provides enhanced ethanol productivity in many ways. These include reduction of contamination;\textsuperscript{35-37} easy separation of cell mass from the liquid medium;\textsuperscript{36} enhanced stability of ethanol production;\textsuperscript{38} reduced production costs;\textsuperscript{39,40} decreased fermentation times;\textsuperscript{34} providing cells for several cycles of fermentation;\textsuperscript{41} and providing good barriers against inhibitors.\textsuperscript{38} Disadvantages of immobilization may include gel degradation, significant substrate and product mass transfer limitation, and gel particle disruption due to CO\textsubscript{2} production and leakage of microorganisms.\textsuperscript{42}

Immobilization of the major ethanol producers \textit{S. cerevisiae} and \textit{Z. mobilis} for ethanol production has been investigated using various substrates including mahula flowers,\textsuperscript{43} molasses,\textsuperscript{44} starchy sweet potato,\textsuperscript{45} and cassava.\textsuperscript{46}

\textit{Escherichia coli} FBR5 is a strain that ferments various sugars and has been engineered to produce ethanol as its only fermentation product.\textsuperscript{17-49} Further engineering of strain FBR5 to express the hemoglobin (VHb) from the bacterium \textit{Vitreoscilla} (strain TS3) has been shown to improve its growth and ethanol production capabilities.\textsuperscript{50-52} This is one application of a much more general use of engineering with VHb to enhance microbial growth and productivity.\textsuperscript{53,54,50-52}

Strains FBR5 and TS3 have been compared regarding ethanol production from a variety of substrates, including pure sugars or corn stover,\textsuperscript{55,56} potato processing waste,\textsuperscript{3} sugar beet molasses, whey, and whey powder;\textsuperscript{4} and mixed waste containing corn lignocellulose and potato starch.\textsuperscript{5} The objectives of the work reported here are to extend the work on ethanol production by strain TS3 using whey powder as a substrate and to assess whether immobilization of TS3 cells provides additional advantages regarding ethanol production.

Materials and methods

Strains and media

Ethanologenic \textit{E. coli} strain TS\textsuperscript{55} is a derivative of pLOI297-bearing strain FBR5,\textsuperscript{49} which was used as a VHb-negative control. LB plates supplemented with 8% xylose were used for maintenance of the strains. 100 \textmu g/mL ampicillin was added for strain FBR5, and 100 \textmu g/mL ampicillin and 50 \textmu g/mL streptomycin were added for TS\textsuperscript{55}.

Cheese whey powder (CWP) was supplied by Bahcivan Gida (Kirkareli, Turkey); it contained, on a dry weight basis, 75% total sugar, 14% protein, 2.3% fats, 3% nitrogen, and 1.6% total phosphorous. 128 g of whey powder was suspended in 400 mL of distilled water and sterilized in an autoclave at 121°C for 15 min. After autoclaving the mixture was cooled down and centrifuged at 15,000 g for 10 min. The supernatant was removed and saved as “whey powder solution” (adapted from Ozmihci and Kargi).\textsuperscript{17-20} Yeast extract was obtained from Merck (Darmstadt, Germany), dissolved in distilled water at a concentration of 30 or 50 g/L, and autoclaved.

Whey powder media were prepared by adding whey powder solution and yeast extract solution to 100 mL of CaCl\textsubscript{2} (50 g/L),\textsuperscript{57} and an appropriate amount of sterile distilled water to give a 1 liter final volume. Seven media with various concentrations of whey and yeast extract were prepared: WPM1 (3% lactose and 0.3% yeast extract); WPM2 (3% lactose and 0.5% yeast extract); WPM3 (5% lactose and 0.3% yeast extract); WPM4 (5% lactose and 0.5% yeast extract); WPM5 (8% lactose and 0.3% yeast extract); WPM6 (8% lactose and 0.5% yeast extract); and WPM7 (15% lactose and 0.5% yeast extract). Phosphate buffer was omitted, as it causes destruction of alginate gel beads.

Immobilization method

The calcium alginate gel-entrapping method was used in this study. Sodium alginate powder (Sigma Aldrich, United Kingdom) was sterilized under UV light in a sterile chamber for 30 min to avoid degradation of the alginate by high temperature during autoclaving,\textsuperscript{58} and diluted with sterilized distilled water to give a 6% (w/v) alginate solution. The bacterial cell suspension was prepared by overnight growth in the same medium to be used in the subsequent experiment,
followed by centrifugation at 4000 × g for 15 min at 4°C and resuspension of the pellet with 0.9% NaCl (to give 10^7 cfu/mL). 6% (w/v) sodium alginate solution was mixed with each cell suspension in a 1:1 volume ratio. The mixture was then placed in a syringe (3P 21G 0.80 × 38 mm) and dripped into 250 mL of sterile 0.1 M CaCl₂, which was stirred continuously at room temperature for 30 min.

The CaCl₂ solution was replaced with 0.05 M sterile CaCl₂ and beads were allowed to harden for 1 h. The mean diameter of the resulting Ca-alginate gel beads was approximately 3.0 mm. The beads were washed with 0.9% NaCl to remove excess calcium ions and non adhered cells. The beads were stored at 4°C in 0.2% (w/v) glucose and 0.2% yeast extract solution for 24 h before use. They were rinsed with sterile water before being used for the fermentation experiments.

In order to determine viable counts in beads, beads were immersed in 1 mL phosphate buffer (1 M, pH 7.0) and disrupted by vigorous mixing on a shaker for about 1 min. Following serial dilutions, viable cell counts were determined on nutrient agar plates incubated at 37°C for 24 h. For determination of leakage of immobilized cells from beads, 100 μL samples were taken from the 0.05 M CaCl₂ used for bead formation after 30 min at 25°C and from the 0.2% (w/v) glucose and 0.2% yeast extract solution after 12 hour of storage at 4°C or 37°C. Serial dilutions were performed on these samples with sterile 0.9% NaCl followed by determination of viable cell counts by plating on nutrient agar plates incubated at 37°C for 24 h.

**Shake flask cultures**

Precultures were obtained by inoculating a single colony in 5 mL of whey powder medium (WPM) containing appropriate antibiotics and incubating at 37°C at 180 rpm overnight. For free cells, shake flask cultures were started by inoculation of approximately 1 mL of preculture (OD₆₀₀nm = 0.8) into 400 mL of the same WPM used for the preculture in 500 mL flasks. Inoculation volumes were determined by optical density to equalize the biomass of all inocula across all cultures with the same initial OD₆₀₀nm of 0.02. For immobilized cells, shake flask cultures were started with about 40 beads containing immobilized cells (containing a total of 1 mL of bacterial preculture of OD₆₀₀nm = 0.8) added to 400 mL of medium in 500 mL flasks; again the same medium was used for both preculture and the experimental culture.

Each culture was capped with rubber stopper pierced with a 22-gauge needle for CO₂ exhaust and incubated at 37°C and 180 rpm for 96 h. At 24 h intervals, about 15 mL of fermentation medium was removed for ethanol, sugar level and VHb measurements. The samples were centrifuged at 4000 × g for 10 min at 4°C; the pellets were used for VHb measurements and the supernatants were transferred to sterile microfuge tubes and stored at −20°C for eventual ethanol and residual sugar measurements.

**Fermenter cultures**

The free and immobilized FBR5 and TS3 strains were also tested for their ethanol production capacity in a 2 L fermenter (Biostat B plus Sartorius, Göttingen, Germany). Medium was WPM6 (8% lactose and 0.5% yeast extract), chosen because it yielded the highest ethanol levels in shake flask cultures for both free and immobilized cells. In addition, WMP7 (15% lactose and 0.5% yeast extract) was also tested. The medium volume was 1600 mL. Approximately 4 mL of precultures (OD₆₀₀nm = 0.8) for free cells or 160 beads (containing a total of 4 mL of of preculture (OD₆₀₀nm = 0.8 )) for immobilized cells were used as inocula. In each experiment the same medium was used for both preculture and the experimental culture. The fermentor system was equipped with a dissolved oxygen (DO) probe and pH electrode. The aeration system was an air inlet through a ring sparger with air-flow meter and filter. 10% antifoam A solution (Sigma Chemical Company, St. Louis, MO) was added.

The fermentors were run at 180 rpm (using internal stirring bars) at a constant temperature of 37°C and a starting pH of 7.0. Well-mixed samples were taken using a syringe for measurements of cell optical densities, ethanol concentrations, residual sugar levels and VHb measurements at 48, 72 and 96 h. Aeration was maintained by continous air sparging at a rate of 1 vvm (1 L/min) through a fine porous stainless steel air filter mounted 2 cm above the vessel bottom. Ethanol yield (Yₛₑ) (g/g) is defined as ethanol produced (g/L) divided by lactose consumed (g/L). The theoretical yield of ethanol is 0.538 g for each g of lactose consumed.
**Analytical measurements**

OD values were determined using a spectrophotometer with cuvette path length of 1.0 cm (BioRad, SmartSpec 300). For the OD measurements OD$_{600nm}$ readings were kept below 0.6 by dilution, if necessary, with the corresponding WPM. Measurements of ethanol, residual sugar, and lactose contents were determined using an HPLC (Shimadzu 10A, Shimadzu, Colombia, MD) equipped with an autosampler (SIL-20AC), an exchange column and a refractive index detector (RID-10A). Concentrations of lactose and residual sugar were measured by injecting 20 µL samples into a NH$_2$ column (Interstil NH$_2$ column, 5 µm, 4.6 x 250 mm, GL Sciences Inc., Shinjuku, Tokyo, Japan) operated at 25°C and 0.5 mL/min with an acetonitrile (60%) and ultra distilled water (40%) mixture used as mobile phase. The metabolites were quantified using standard curves correlating peak areas with concentrations of standard solutions. VHb levels were calculated by dithionite treated-minus untreated difference spectra ($\Delta$A$_{435-405}$ = 34 M$^{-1}$ cm$^{-1}$) of cell lysates as previously described$^{61}$ and normalized to g wet weight of cells.

**Data analysis**

Averages and sample standard deviations were determined using Microsoft Excel.

**Results**

**Shake flasks**

**VHb levels**

Levels of VHb (strain TS3) were essentially the same for free versus immobilized cells across all media. Absolute VHb levels were almost all between 6 and 64 nmol/g (wet weight) of cells (Table 1). This very similar to the levels measured previously for medium made with whey powder at a lactose concentration of 5.5%.$^4$

**Effect of immobilization and VHb expression on ethanol production**

Immobilization or VHb expression by themselves or in combination had either a small positive effect or no effect on ethanol production for any media containing 3% lactose or 15% lactose or medium containing 5% lactose and 0.3% yeast extract (Fig. 1). There was a

| Time (hours) | Medium | Efficiency (%) | Lactose Consumption (%) | VHb (nmol/g) |
|--------------|--------|----------------|-------------------------|--------------|
|               |        | FBR5 (f) | FBR5 (i) | TS3 (f) | TS3 (i) | FBR5 (f) | FBR5 (i) | TS3 (f) | TS3 (i) | TS3 (f) | TS3 (i) |
| 48           | WPM1   | 91 (4)   | 89 (0)   | 91 (1) | 95 (3) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 16 (0) |
|              | WPM2   | 89 (4)   | 93 (0)   | 87 (1) | 99 (9) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 29 (4) |
|              | WPM3   | 54 (2)   | 80 (5)   | 80 (0) | 93 (7) | 44 (1)  | 45 (0)  | 64 (0)  | 60 (0)  | 60 (0)  | 6 (0)  |
|              | WPM4   | 54 (7)   | 85 (1)   | 78 (1) | 76 (3) | 55 (6)  | 65 (6)  | 72 (1)  | 82 (2)  | 82 (2)  | 11 (7) |
|              | WPM5   | 58 (1)   | 63 (3)   | 97 (4) | 78 (1) | 37 (5)  | 41 (2)  | 34 (1)  | 35 (1)  | 35 (1)  | 27 (1) |
|              | WPM6   | 56 (6)   | 73 (3)   | 87 (1) | 84 (1) | 58 (4)  | 48 (1)  | 55 (0)  | 53 (0)  | 53 (0)  | 63 (4) |
|              | WPM7   | 24 (4)   | 28 (3)   | 28 (2) | 30 (2) | 43 (2)  | 42 (3)  | 51 (3)  | 43 (4)  | 27 (8)  | 26 (3) |
| 72           | WPM1   | 89 (3)   | 96 (1)   | 83 (7) | 87 (1) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 11 (7) |
|              | WPM2   | 95 (0)   | 97 (1)   | 97 (3) | 97 (3) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 16 (4) |
|              | WPM3   | 69 (5)   | 80 (1)   | 87 (3) | 97 (2) | 57 (2)  | 87 (1)  | 75 (7)  | 77 (1)  | 77 (1)  | 6 (0)  |
|              | WPM4   | 82 (3)   | 86 (2)   | 95 (8) | 89 (7) | 60 (5)  | 88 (2)  | 74 (3)  | 91 (1)  | 91 (1)  | 7 (1)  |
|              | WPM5   | 48 (1)   | 64 (3)   | 78 (1) | 97 (3) | 80 (2)  | 67 (1)  | 58 (2)  | 54 (1)  | 54 (1)  | 21 (1) |
|              | WPM6   | 61 (1)   | 65 (3)   | 86 (1) | 99 (3) | 81 (2)  | 80 (0)  | 59 (2)  | 56 (1)  | 56 (1)  | 31 (4) |
|              | WPM7   | 32 (3)   | 30 (2)   | 30 (3) | 32 (4) | 48 (2)  | 51 (4)  | 57 (4)  | 56 (2)  | 56 (2)  | 23 (3) |
| 96           | WPM1   | 98 (5)   | 98 (1)   | 99 (2) | 99 (2) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 12 (1) |
|              | WPM2   | 93 (1)   | 95 (0)   | 97 (0) | 97 (2) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 13 (4) |
|              | WPM3   | 84 (2)   | 89 (1)   | 87 (4) | 93 (2) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 11 (4) |
|              | WPM4   | 87 (2)   | 93 (3)   | 95 (2) | 99 (3) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 100 (0) | 10 (2) |
|              | WPM5   | 56 (8)   | 74 (2)   | 71 (2) | 84 (1) | 88 (6)  | 73 (1)  | 80 (5)  | 74 (1)  | 74 (1)  | 16 (2) |
|              | WPM6   | 69 (2)   | 71 (5)   | 76 (5) | 84 (2) | 84 (5)  | 82 (2)  | 82 (5)  | 77 (1)  | 77 (1)  | 21 (2) |
|              | WPM7   | 26 (5)   | 24 (1)   | 32 (3) | 33 (2) | 67 (3)  | 73 (7)  | 58 (4)  | 58 (2)  | 58 (2)  | 18 (2) |
Figure 1. Ethanol production (a, v/v; b, w/v % per unit of biomass (OD$_{600nm}$)) of free and immobilized FBR5 and TS3 (color coding indicated in inset) in 500 mL flasks (containing 400 mL of medium) at low aeration in all 7 media. Values are averages of 3 individual experiments. Error bars indicate sample standard deviations, which were determined individually for each average. A-D, growth in various media (indicated in each panel).
slight enhancement for medium containing 5% lactose and 0.5% yeast extract, but the greatest positive effects of VHb expression and immobilization occurred for the 2 media containing 8% lactose. Thus, at least for shake flask growth, both immobilization and VHb expression are advantageous for ethanol production, but only for media of intermediate richness; in addition, in this range of media the VHb and immobilization effects are additive.

The highest level of ethanol achieved for free cells was 3.4% (v/v) by strain TS3 in medium containing 8% lactose and 0.5% yeast extract, and, for immobilized cells, 3.5% (v/v) with strain TS3 in the same medium. The time course of ethanol accumulation was generally similar for most media and all 4 combinations of free/imobilized and VHb+/VHb− cultures, except for the 2 media containing 5% lactose, in which it was much slower for the VHb− free cells, and the 2 media containing 3% lactose, in which ethanol accumulation was essentially maximum at 24 h. For all other media ethanol levels increased steadily during the course of culturing, with maxima at 96 h.

**Ethanol production per biomass**

The trends for this parameter followed those of ethanol production on a v/v basis fairly closely, at least qualitatively (Fig. 1). The outlier here was for the medium containing 15% lactose in which the ethanol produced per biomass was enhanced substantially by both VHb expression and immobilization, even though there was little effect of either on the actual ethanol accumulation.

**Lactose consumption**

Both free and immobilized cells consumed the lactose in 3% lactose containing media completely within 48 h, and in 5% lactose containing media within 96 h. For 8% lactose containing media 54%–81% and 73%–88% of the lactose was consumed within 72 h and 96 h, respectively. The lowest lactose consumption was observed for both free and immobilized cells (ranging between 58%–73% at 96 h) when 15% lactose containing medium was used (Table 1). Generally lactose consumption was lower in 5% and 8% lactose containing media for all immobilized strains than for the corresponding free cells at 72 and 96 h.

**Ethanol production efficiency**

Immobilization of cells resulted in an inconsistent trend of increased efficiency of conversion of lactose into ethanol. This weak trend encompassed both TS3 and FBR5 and a variety of media (Table 1). A more consistent increase in conversion efficiency was associated with VHb expression (TS3 compared to FBR5) for both free and immobilized cells across all media.

**Fermenter cultures**

**VHb levels**

Similar to what was seen in shake flasks, levels of VHb (strain TS3) were essentially the same for free vs. immobilized cells across both media. Absolute VHb levels were also similar to those seen in shake flasks (Table 2).

**Effect of immobilization and VHb expression on ethanol production**

Only two media were tested for fermenter growth, WPM6 (8% lactose and 0.5% yeast extract) and WPM7 (15% lactose and 0.5% yeast extract) (Fig. 2). Similar to what was seen in shake flasks, growth in WPM6 resulted in increases in ethanol accumulation

---

Table 2. Theoretical ethanol production (efficiency, %), lactose consumption (%), and VHb (nmol/g) of free and immobilized FBR5 and TS3 in a 2 L fermenter at low aeration in WPM6 and WMP7 media. Values are averages of three individual experiments. Standard deviations (in parentheses) indicate sample standard deviations, which were determined individually for each average.

| Time (hours) | Medium | Efficiency (%) | Lactose Consumption (%) | VHb (nmol/g) |
|--------------|--------|----------------|-------------------------|--------------|
|              |        | FBR5 (f) | FBR5 (i) | TS3 (f) | TS3 (i) | FBR5 (f) | FBR5 (i) | TS3 (f) | TS3 (i) | TS3 (f) | TS3 (i) |
| 48           | WPM6   | 50 (4)   | 63 (3)   | 65 (3)   | 72 (1)   | 32 (5) | 36 (6) | 57 (4) | 89 (2) | 46 (13) | 45 (12) |
|              | WPM7   | 20 (5)   | 20 (4)   | 33 (3)   | 35 (3)   | 51 (3) | 41 (3) | 42 (5) | 40 (4) | 26 (2)  | 26 (5)  |
| 72           | WPM6   | 65 (5)   | 97 (2)   | 67 (4)   | 78 (1)   | 58 (3) | 58 (3) | 70 (6) | 91 (3) | 32 (15) | 23 (10) |
|              | WPM7   | 26 (4)   | 28 (4)   | 33 (3)   | 37 (3)   | 59 (2) | 45 (3) | 50 (4) | 52 (3) | 32 (1)  | 28 (1)  |
| 96           | WPM6   | 80 (4)   | 82 (2)   | 74 (4)   | 74 (1)   | 61 (4) | 74 (2) | 83 (3) | 97 (2) | 25 (5)  | 20 (8)  |
|              | WPM7   | 22 (2)   | 22 (2)   | 32 (3)   | 35 (2)   | 75 (6) | 63 (5) | 57 (4) | 61 (4) | 26 (2)  | 26 (2)  |
correlated with both VHb expression and immobilization, which were additive. Also similar to shake flask results, ethanol accumulation was much lower for WPM7 than for WPM6 medium. Thus the maximum ethanol level (3.9% (v/v)) occurred for immobilized strain TS3 grown in WPM6 medium. Unlike the shake flask results with WPM7 medium, however, the expression of VHb and immobilization were both correlated with increased ethanol accumulation.

**Ethanol production per biomass**

As was the case in shake flasks, the qualitative trends for this parameter followed somewhat those of ethanol production measured on a v/v basis (Fig. 2).

**Lactose consumption**

Lactose consumption levels were higher in fermenters than in shake flask cultures for strain TS3 when medium containing 8% lactose and 0.5% yeast extract was used. In the same medium in fermenters, immobilized cells consumed more lactose than free cells.

**Ethanol production efficiency**

Immobilization of cells had little effect on efficiency of conversion of lactose into ethanol for both media tested (Table 2). A more consistent increase in conversion efficiency was associated with VHb expression (TS3 compared to FBR5) for both free and immobilized cells, but only in WPM7 medium (15% lactose, 0.5% yeast extract).

**Discussion**

**Ethanol production as a function of medium richness**

Not surprisingly the levels of ethanol produced in shake flask cultures increased as the lactose (fermentable sugar) concentration of the medium increased from 3% to 8%, although the 5% and 8% lactose media were nearly identical regarding this parameter. As has been seen previously, however, increasing the lactose concentration to 15% resulted in substantially lower ethanol than the 5% and 8% media in both fermenter and shake flask cultures. These latter results are consistent with previous reports that lactose concentrations above 100–200 g/L inhibit fermentation of lactose in whey.62,17-20

The general similarity in the patterns of accumulation of ethanol on a v/v basis and when normalized to cell biomass indicates that both immobilization and VHb expression enhanced ethanol production by increasing the efficiency of ethanol production rather than by increasing biomass accumulation. Similar conclusions were drawn concerning VHb correlated increases in ethanol with sugar sources of corn stover hydrolysate,55 potato processing waste,3 or corn plus...
potato processing wastes. VHb enhancement of ethanol production in LB medium supplemented with whey or whey powder, however, was found previously to occur by increases in both biomass and the amount of ethanol produced per unit of biomass.

The consumption of lactose and efficiency of conversion of lactose to ethanol were very high for all strains at lower lactose concentrations, but decreased at the higher lactose concentrations (8% and 15% in WPM5, WMP6, and WMP7 media) and were particularly low at 15% lactose. The low values for 15% lactose containing medium are likely related to the fermentation inhibition mentioned above. For 8% lactose containing media, both lactose consumption and efficiency were fairly high, but there was still an excess of lactose over that which could be used given the maximum growth possible by 96 h. Thus, media containing intermediate lactose concentrations (perhaps between 5% and 8%) may be the most effective at industrial scales.

Free vs immobilized cells

The effectiveness of immobilization in our work is indicated by very low cell leakage from the alginate beads and no gel degradation observed even after 96 h of fermentation in either shake flask or fermenter cultures. This may have been due to the inclusion of 5 g/L CaCl₂, which has been shown to prevent beads from swelling. Although in general in both shake flask and fermenter cultures higher ethanol levels were achieved by immobilized strains than free strains, the advantage of immobilization was not uniform. The clearest advantages of immobilization (in both shake flasks and fermenters) occurred for the media (WPM5 and WMP6, 8% lactose) which also yielded the greatest ethanol concentrations at the end of growth (96 h). Also, in general, even when immobilization did not substantially enhance the maximum ethanol levels achieved, it did result in much faster ethanol accumulation, and so provided that additional benefit.

The advantage of immobilized cells over free cells for ethanol production has been reported previously. Possible reasons, which may have occurred in the experiments reported here as well, include reduction of lag phase, resistance to inhibitory products, or effective uptake of nutrients. In addition, the fatty acid composition of membranes from immobilized cells may enhance resistance to ethanol transport of ethanol to the growth medium. Other possibilities are protection against osmotic pressure or presence of storage molecules such as glucan and mannan to sustain metabolic activities for long time periods.

VHb⁺ vs VHb⁻ cells

Enhancement of production of a variety of metabolites, including ethanol, has been achieved through VHb expression (reviewed by Frey and Kallio). The probable mechanism for the positive VHb effects is the increasing of oxygen transport to the respiratory chain, thus increasing ATP production to aid growth and production of secondary metabolites.

As mentioned above VHb expression by strain TS3 in the work reported here was between 6 and 64 nmol/g (wet weight) of cells (considering both shake flask and fermenter cultures), and this is very similar to our previous reports (27 and 47 nmol/g) for this strain in medium using whey powder as the source of fermentable sugar. Growth of TS3 in other media under aeration conditions such as used in this work and using various wastes as sugar sources (corn stover hydrolysate, whey, potato and corn processing wastes) have resulted in VHb levels ranging from about 14 to over 300 nmol/g. Lower levels of VHb (in the range of those seen in this work) have been shown to be more advantageous than higher levels.

In general the positive effects on ethanol production of immobilization and VHb expression were additive, although the combined effects were most substantial in media that produced the greatest absolute ethanol levels. The greatest combined enhancement at 96 h was seen in WPM6 medium where immobilized strain TS3 produced 1.37 X as much ethanol (3.56% (v/v) versus 2.60% (v/v) as free FBR5 cells in shake flasks and 1.47 X as much (3.90% (v/v) vs. 2.66% (v/v)) in fermenters.

From an economic point of view, the highest possible ethanol concentrations should be obtained from fermentations in order to decrease distillation costs. For example, ethanol separation from 2% (v/v) ethanol containing media is not economically feasible. The highest ethanol production observed in the work reported here was substantially higher than that (3.9% (v/v)). This compares with 3.7% (v/v) ethanol produced by fermenter grown free Kluyveromyces marxianus DSMZ 7239 in 100 g/L lactose containing whey...
powder medium, and 5.2% (v/v) ethanol by the same strain in batch cultures in 75 g/L lactose containing whey powder medium.20 It was also shown that as much as 4.9% (v/v) ethanol could be achieved in shake flask cultures after 48 h with 133% theoretical yield by using LB supplemented with whey powder containing 5.5% lactose.4

In the work reported here the highest ethanol production was obtained by combining VHB expression with immobilization for fermenter growth in a medium with an intermediate concentration of lactose, where an enhancement of 47% compared to free cells not expressing VHB was realized. It will be useful in the future to see if the same advantages can be obtained even with media more minimal than that used here, that is, those that do not include the expense of the addition of yeast media. We would like to thank the Gebze Technical University, Turkey and the Illinois Institute of Technology, USA.

Abbreviations
CWP cheese whey powder
VHB Vitreoscilla hemoglobin
WPM whey powder media

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

Acknowledgments
We would like to thank the Gebze Technical University, Turkey and the Illinois Institute of Technology, USA.

References
[1] Fairley P. Next generation biofuels. Nature 2011; 474:s2-5; PMID:21697838; http://dx.doi.org/10.1038/474S02a
[2] Peplow M. Cellulosic ethanol fights for life. Nature 2014; 507:152-53; PMID:24622185; http://dx.doi.org/10.1038/507152a
[3] Abanoz K, Stark BC, Akbas MY. Enhancement of ethanol production from potato-processing wastewater by engineering Escherichia coli using Vitreoscilla haemoglobin. Lett Appl Microbiol 2012; 55:436-43; PMID:22994421; http://dx.doi.org/10.1111/lam.12000
[4] Akbas MY, Sar T, Ozcelik B. Improved ethanol production from cheese whey, whey powder, and sugar beet molasses by "Vitreoscilla hemoglobin expressing" Escherichia coli. Biosci Biotecnol Biochem 2014; 78:687-94; PMID:25036968; http://dx.doi.org/10.1080/09168451.2014.896734
[5] Sumer F, Stark BC, Akbas MY. Efficient ethanol production from potato and corn processing industry waste using E. coli engineered to express Vitreoscilla haemoglobin. Environ Technol 2015; 36:2319-27; PMID:25766084; http://dx.doi.org/10.1080/09593330.2015.1026846
[6] Baldasso C, Barros TC, Tessaro IC. Concentration and purification of whey proteins by ultrafiltration. Desal 2011; 278:381-6; http://dx.doi.org/10.1016/j.desal.2011.05.055
[7] Kosikowski FV. Whey utilization and whey products. J Dairy Sci 1979; 62:1149-60; http://dx.doi.org/10.3168/jds. S0022-0302(79)83389-5
[8] Becerra M, Baroli B, Fadda AM, Mendez JB, Siso MIG. Lactose bioconversion by calcium alginate immobilization of Kluyveromyces lactis cells. Enzyme Microb Tech 2001; 29:506-12; http://dx.doi.org/10.1016/S0141-0220 (01)00409-4
[9] Siso MIG. The biotechnological utilization of cheese whey: A review. Bioresource Technol 1996; 57:1-11; http://dx.doi.org/10.1016/0960-8524(96)00036-3
[10] Ganzle MG, Haase G, Jelen P. Lactose: Crystallization, hydrolysis and value-added derivatives. Int. Dairy J 2008; 18:685-94; http://dx.doi.org/10.1016/j.idairyj.2008.03.003
[11] Dominguez L, Lima N, Teixeira JA. Alcohol production from cheese whey permeate using genetically modified flocculent yeast cells. Biotechnol Bioeng 2001; 72:507-14; PMID:11460240; http://dx.doi.org/10.1002/1097-0227(20010305)72:5%3c507::AID-BIT1014%3e3.0.CO;2-U
[12] Kourkoutas Y, Dimitrourou S, Kallilaki M, Marchant R, Nimig P, Banat IM, Koutinas AA. High-temperature alcoholic fermentation of whey using Klyuveroomyces marxianus IMB3 yeast immobilized on delignified cellulose material. Biore sour Technol 2002a; 82:177-81; http://dx.doi.org/10.1016/S0960-8524(01)00159-6
[13] Kourkoutas Y, Psarianos C, Koutinas AA, Kallilaki M, Banat IM, Marchant R. Continuous whey fermentation using kefir yeast immobilized on delignified cellulose material. J Agric Food Chem 2002b; 50:2543-47; http://dx.doi.org/10.1021/jf0113427
[14] Silveira WB, Passos FJv, Mantovani HC, Passos FML. Ethanol production from cheese whey permeate by Klyuveroomyces marxianus UFV-3: A flux analysis of oxido-reductive metabolism as a function of lactose concentration and oxygen levels. Enzyme Microb Technol 2005; 36:930-96; http://dx.doi.org/10.1016/j.enzmictec.2005.01.018
[15] Zafar S, Owais M. Ethanol production from crude whey by Klyuveroomyces marxianus. Biochem Eng J 2006; 27:295-8; http://dx.doi.org/10.1016/j.bej.2005.05.009
[16] Kargi F, Ozmihici S. Utilization of cheese whey powder (CWP) for ethanol fermentations: Effects of operating parameters. Enzyme Microb Tech 2006; 38:711-8; http://dx.doi.org/10.1016/j.enzmictec.2005.11.006
[17] Ozmihici S, Kargi F. Effects of feed sugar concentration on continuous ethanol fermentation of cheese whey powder solution (CWP). Enzyme Microb Technol 2007a; 41:876-80; http://dx.doi.org/10.1016/j.enzmictec.2007.07.015
[18] Ozmihici S, Kargi F. Ethanol fermentation of cheese whey powder solution by repeated fed-batch operation.
[19] Ozmihci S, Kargi F. Comparison of yeast strains for batch ethanol fermentation of cheese-whey powder (CWP) solution. Lett Appl Microbiol 2007c; 44:602-6; http://dx.doi.org/10.1016/j.lam.2006.10.005

[20] Ozmihci S, Kargi F. Kinetics of batch ethanol fermentation of cheese-whey powder (CWP) solution as function of substrate and yeast concentrations. Bioresource Technol 2007d; 98:2978-84; http://dx.doi.org/10.1016/j.biortech.2006.10.005

[21] Ozmihci S, Kargi F. Ethanol production from cheese whey powder solution in a packed column bioreactor at different hydraulic residence times. Biochem Eng J 2008; 42:180-5; http://dx.doi.org/10.1016/j.bej.2008.06.017

[22] Pesta G, Meyer-Pittroff R, Russ W. Utilization of whey. In. Utilization of by-products and treatment of waste in the food industry ed. Oreopoulou V, Russ W. Springer. 2007; 193-207.

[23] Guimaraes PM, Teixeira JA, Domingues L. Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnol Adv 2010; 28:375-84; PMID:Can’t; http://dx.doi.org/10.1016/j.biotechnoladv.2010.02.002

[24] Lin Y, Zhang W, Li C, Sakakibara K, Tanaka S, Kong H. Factors affecting ethanol fermentation using Saccharomyces cerevisiae BY4742. Biomass Bioenergy 2012; 47:395-401; http://dx.doi.org/10.1016/j.biombioe.2012.09.019

[25] Prasertwasu S, Khumsupan D, Komolwanich T, Chaisuwann T, Luengnaruemitchai A, Kong H. Efficient process for ethanol production from Thai Mission grass (Pennisetum polystachion). Bioresource Technol 2014; 163:152-9; PMID:24811442; http://dx.doi.org/10.1016/j.biortech.2014.04.043

[26] Mheaia MA, Cheryan M. Ethanol from hydrolyzed whey permeate using Saccharomyces cerevisiae in a membrane recycle bioreactor. Bioprocess Eng 1990; 5:57-61; http://dx.doi.org/10.1016/j.biombioe.2012.09.019

[27] Gancedo JM. Yeast carbon catabolite repression. Microbiol Mol Biol Rev 1998; 62:334-6; PMID:9618445

[28] Lin Y, Tanaka S. Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 2006; 69:627-42; PMID:16331454; http://dx.doi.org/10.1007/s00253-005-0229-x

[29] Puligundla P, Smogrovicova D, Obulam VS, Ko S. Very high gravity (VHG) ethanolic brewing and fermentation: a research update. J Ind Microbiol Biotechnol 2011; 38:1133-44; PMID:21695540; http://dx.doi.org/10.1007/s10295-011-0999-3

[30] Yao W, Wu X, Zhu J, Sun B, Zhang YY, Miller C. Bacterial cellulose membrane – A new support carrier for yeast immobilization for ethanol fermentation. Process Biochem 2011; 46:2054-8; http://dx.doi.org/10.1016/j.procbio.2011.07.006

[31] Ylitervo P, Franzen CJ, Taherzadeh MJ. Ethanol production at elevated temperatures using encapsulation of yeast. J Biotechnol 2011; 156:22-9; PMID:21807041; http://dx.doi.org/10.1016/j.jbiotec.2011.07.018

[32] Zhou Z, Li G, Li Y. Immobilization of Saccharomyces cerevisiae alcohol dehydrogenase on hybrid alginate-chitosan beads. Int J Biol Macromol 2010; 3:21-6

[33] Tesfaw A, Assefa F. Current trends in bioethanol production by Saccharomyces cerevisiae: Substrate, inhibitor reduction, growth variables, coculture, and immobilization. International Scholarly Research Notices Article ID 532852, 11 pages. 2014.

[34] Karagoz P, Ozkan M. Ethanol production from wheat straw by Saccharomyces cerevisiae and Scheffersomyces stipitis co-culture in batch and continuous system. Bioresource Technol 2014; 158:286-93; PMID:24614063

[35] Razmovski R, Vucurovic V. Ethanol production from sugar beet molasses by S. cerevisiae entrapped in an alginate-maize stem ground tissue matrix. Enzyme Microb Tech 2011; 48:378-85; PMID:22112953

[36] Sembingri KC, Mulyani H, Fitria AI, Dahnum D, Sudiyani Y. Rice flour and white glutinous rice flour for use on immobilization of yeast cell in ethanol production. Energy Procedia 2014; 32:99-104

[37] Milessi TS, Antunes FA, Chandel AK, da Silva SS. Hemicellulosic ethanol production by immobilized cells of Scheffersomyces stipitis: Effect of cell concentration and stirring. Bioengineered 2015; 6:26-32; PMID:25488725

[38] Kirdponpattara S, Phisalaphong M. Bacterial cellulose-alginic acid composite sponge as a yeast cell carrier for ethanol production. Biochem Eng J 2013; 77:103-9

[39] Singh A, Sharma P, Saran AK, Singh N, Bishnoi NR. Comparative study on ethanol production from pre-treated sugarcane bagasse using immobilized Saccharomyces cerevisiae on various matrices. Renew Energ 2013; 50:488-93

[40] Pacheco AM, Gondim DR, Goncalves LR. Ethanol production by fermentation using immobilized cells of Saccharomyces cerevisiae in cashew apple bagasse. Appl Biochem Biotechnol 2010; 161:209-17; PMID:19798473

[41] Duarte JC, Rodrigues JAR, Moran PJS, Valença GP, Nunez JR. Effect of immobilized cells in calcium alginate beads in alcoholic fermentation. AMB express 2013; 3:2-8; PMID:23289832

[42] Phisalaphong S, Budiraharjo R, Bangkar P, Mongkolkaitsu J, Limtong S. Alginate-loofa as carrier matrix for ethanol production. J Biotech 2007; 104:214-7; PMID:17964486

[43] Behera S, Sar, K, Mohanty RC, Ray RC. Comparative study of bio-ethanol production from mahula (Madhuca latifolia L.) flowers by Saccharomyces cerevisiae cells immobilized in agar agar and Ca-alginic mats. Appl Energ 2010; 87:96-100

[44] Panesar PS, Chavan Y, Chopra HK, Kennedy JF. Production of microbial cellulose: Response surface methodology approach. Carbohydr Polym 2012; 87:930-4

[45] Yu B, Zhang F, Zheng Y, Wang P. Alcohol fermentation from the mash of dried sweet potato with its dregs using immobilised yeast. Process Biochem 1996; 31:1-6

[46] Nellaiah H, Gunasekaran P. Ethanol production from cassava starch hydrolysate by immobilized Zymomonas mobilis. Indian J Microbiol 1992; 32:435-42
[47] Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF. Genetic engineering of ethanol production in Escherichia coli. Appl Environ Microbiol 1987; 53:2420-5; PMID: 3322191

[48] Ingram LO, Conway T. Expression of different levels of ethanologenic enzymes from Zymomonas mobilis in recombinant strains of Escherichia coli. Appl Environ Microbiol 1988; 54:397-404; PMID:16347553

[49] Dien BS, Nichols NN, O'Bryan PJ, Bothast RJ. Development of new ethanologenic Escherichia coli strains for fermentation of lignocellulosic biomass. Appl Biochem Biotechnol 2000; 84:861-186; PMID:10849788; http://dx.doi.org/10.1002/(SICI)1097-0290(19960720)51:2%3c157::AID-ABAB84-861-9:181

[50] Stark BC, Dikshit KL, Pagilla KR. Recent advances in understanding the structure, function, and biotechnological usefulness of the hemoglobin from the bacterium Vitreoscilla. Biotechnol Lett 2011; 33:1705-14; PMID:21603987; http://dx.doi.org/10.1007/s10529-011-0621-9

[51] Stark BC, Dikshit KL, Pagilla KR. The biochemistry of Vitreoscilla hemoglobin. Comput Struct Biotechnol J 2012; 3:1-8; http://dx.doi.org/10.5936/csbj.201210002

[52] Stark BC, Pagilla KR, Dikshit KL. Recent applications of Vitreoscilla hemoglobin technology in bioprocess synthesis and bioremediation. Appl Microbiol Biotechnol 2015; 99:1627-1636; PMID:25575886; http://dx.doi.org/10.1007/s00253-014-6350-y

[53] Frey AD, Kallio PT. Bacterial hemoglobin and flavohemoglobin: versatile proteins and their impact on microbiology and biotechnology. FEMS Microbiol Rev 2003; 27:525-45; PMID:14550944; http://dx.doi.org/10.1007/s00253-014-6350-y

[54] Zhang L, Li Y, Wang Z, Xia Y, Chen W, Tang K. Recent applications of the hemoglobin from the bacterium Vitreoscilla. Biotechnol Lett 2011; 33:1705-14; PMID:21603987; http://dx.doi.org/10.1007/s10529-011-0621-9

[55] Zhang L, Li Y, Wang Z, Xia Y, Chen W, Tang K. Recent applications of the hemoglobin from the bacterium Vitreoscilla. Biotechnol Lett 2011; 33:1705-14; PMID:21603987; http://dx.doi.org/10.1007/s10529-011-0621-9

[56] Stark BC, Dikshit KL, Pagilla KR. Recent advances in understanding the structure, function, and biotechnological usefulness of the hemoglobin from the bacterium Vitreoscilla. Biotechnol Lett 2011; 33:1705-14; PMID:21603987; http://dx.doi.org/10.1007/s10529-011-0621-9

[57] Stark BC, Dikshit KL, Pagilla KR. Recent advances in understanding the structure, function, and biotechnological usefulness of the hemoglobin from the bacterium Vitreoscilla. Biotechnol Lett 2011; 33:1705-14; PMID:21603987; http://dx.doi.org/10.1007/s10529-011-0621-9

[58] Chen M-J, Chen K-N, Chiu HY, Lin C-W. Method for preparing alginate capsules. In: United States, Patent Application Publication. 2005.

[59] Ghorbani F, Younesi H, Sari AE, Najaﬁpour G. Cane molasses fermentation for continuous ethanol production in an immobilized cells reactor by Saccharomyces cerevisiae. Renew Energ 2011; 36:503-9; http://dx.doi.org/10.1016/j.renene.2010.07.016

[60] Chibata I, Tosa T, Sati T. Immobilized aspartase-containing microbial cells: preparation and enzymatic properties. Appl Microbiol 1974; 27:878-85; PMID:4208512

[61] Liu CY, Webster DA. Spectral characteristics and interconversions of the reduced oxidized and oxygenated forms of purified cytochrome o. J Biol Chem 1974; 240:4261-6

[62] Cheong SH, Park HK, Kim BS, Chang HN. Microencapsulation of yeast cells in the calcium alginate membrane. Biotechnol Tech 1993; 7:879-84; http://dx.doi.org/10.1007/BF00156366

[63] Yu J, Yue G, Zhong J, Zhang X, Tan T. Immobilization of Saccharomyces cerevisiae to modified bagasse for ethanol production. Renew Energ 2010; 35, 1130-4; http://dx.doi.org/10.1016/j.renene.2009.11.045

[64] Wendhausen R. Estudo sobre utilização de crisotila como suporte de células de Saccharomyces cerevisiae para uso em processo contínuo de fermentação alcoólica e bioreduções. Brazil: University of Campinas – UNICAMP (Dissertation), 1998.

[65] Shen H-Y, Moonjai N, Verstrepen K, Delvaux F. Impact of attachment immobilization on yeast physiology and fermentation performance. J Am Soc Brew Chem 2003; 61:79-87

[66] Jirk V, Masak J, Čejkova A. The potential of functional changes in attached biomass. Adv Environ Res 2003; 7:635-9, http://dx.doi.org/10.1016/S1093-0191(02)00045-X

[67] Yu J, Zhang X, Tan T. An novel immobilization method of Saccharomyces cerevisiae to sorghum bagasse for ethanol production. J Biotechnol 2007; 129:415-20; PMID:17383041; http://dx.doi.org/10.1016/j.jbiotec.2007.01.039

[68] Jirk V. Whole cell immobilization as a means of enhancing ethanol tolerance. J Ind Microbiol Biotechnol 1999; 22:147-51; http://dx.doi.org/10.1016/S0168-6445(03)00056-1

[69] Peinado RA, Moreno JJ, Villalba JM, González-Reyes JA, Ortega JM, Mauricio JC. Yeast biocapsules: A new immobilization method and their applications. Enzyme Microb Tech 2006; 40:79-84; http://dx.doi.org/10.1016/j.enzmicrotec.2005.10.040

[70] Doran PM, Bailey JE. Effects of immobilization on growth, fermentation properties, and macromolecular composition of Saccharomyces cerevisiae attached to gelatin. Biotechnol Bioeng 1986; 22:147-51; http://dx.doi.org/10.1016/S0168-6445(03)00056-1

[71] Peinado RA, Moreno JJ, Villalba JM, González-Reyes JA, Ortega JM, Mauricio JC. Yeast biocapsules: A new immobilization method and their applications. Enzyme Microb Tech 2006; 40:79-84; http://dx.doi.org/10.1016/j.enzmicrotec.2005.10.040