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Evaluation of scale-up parameters of bioethanol production from *Escherichia coli* KO11

[*Escherichia coli* KO11 suşundan biyoetanol üretimi için ölçek büyütme parametrelerinin değerlendirilmesi]

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ABSTRACT

Objective: In recent years, increased attention has been devoted to the conversion of biomass into fuel ethanol, as one of the cleanest liquid fuel alternatives to fossil fuels. However, industrial production of bioethanol is related with successful scaling-up studies.

Methods: In this study, the experimental designs of scale-up procedures based on constant mixing time, impeller tip speed and oxygen mass transfer coefficient were performed in 8 L stirred tank reactor and were compared in terms of product yield and productivity with those obtained from 2 L stirred tank reactor using quince pomace as a substrate for bioethanol production by *Escherichia coli* KO11.

Results: Scale-up based on constant mixing time yielded a maximum ethanol concentration of 23.42 g/L which corresponded to 0.4 g ethanol/ g reduced sugar in 8 L stirred tank reactor. Moreover, shear stress increased only 1.1 fold which resulted in low cell damage and high cell viability.

Conclusion: Constant mixing time was identified as the most important key parameter especially for scaling-up of viscous fermentation broths of bioethanol production due to the significance of the homogeneity.

Key Words: Bioethanol production, *Escherichia coli* KO11, mixing time, quince pomace, scale-up

Conflict of Interest: The authors have no conflict of interest.

ÖZET

Amaç: Son yıllarda fosil yakıtlara alternatiflerden biri olarak biyokütlenin dönüşümüyle etanol eldesi çok önem kazanmıştır. Endüstriyel ölçekte biyoetanol üretimi, başarılı bir ölçek büyütmeye bağlıdır.

Metod: Bu çalışmada, 8 L karıştırmalı tank reaktörde sabit karışma süresi, sabit bıçak ucu hızı ve sabit oksijen kütle transfer katsayısına göre ölçek büyütme prosedürleri gerçekleştirilmiştir. Sonuçlar, *Escherichia coli* KO11 suşundan ayva posası ile 2 L reaktörde elde edilen sonuçlarla ürün verimi ve verimliliği bakımından karşılaştırılmıştır.

Bulgular: 8 L karıştırmalı tank reaktörde maksimum etanol konsantrasyonu (23.42 g/L), 0.4 g etanol/g indirgen şeker verimiyle sabit karışma süresine göre yapılan ölçek büyütmede elde edilmiştir. Ayrıca, kayma gerilimi sadece 1.1 kat artmıştır ve bu durum düşük hücre hasarı ve yüksek hücre canlılığıyla sonuçlanmıştır.

Sonuç: Sabit karışma süresi, biyoetanol üretiminde homojenitenin önemi açısından özellikle viskoz fermentasyon sıvıları için en önemli parameter olarak tanımlanmıştır.

Anahtar Kelimeler: Biyoetanol üretimi, *Escherichia coli* KO11, karışma süresi, ayva posası, ölçek büyütme.

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Introduction

During the last two decades, the conversion of agro-industrial wastes to ethanol has been intensively studied using genetically engineered *Escherichia coli* KO11. Recombinant *E. coli* KO11 has high ethanol yields, a high phenotypic stability and is capable of efficiently producing ethanol from all sugar constituents which make it a promising biocatalyst for large scale ethanol production [1].

Effective scale-up is essential for successful bioprocessing. The design of microbial processes depends on the product, microbial strain, growth conditions, bioconversion/biotransformation conditions and bioreactor geometry. Hence, for a given product, an adequate and comprehensive approach has to be established that includes the detailed characterization of the process parameters which directly linked to the product yield. A particular scale-up strategy is carried out by maintaining a specific set of parameters constant throughout the scale-up process, in order to ensure success of the production [2]. However, this is quite complex since there are several parameters influencing transport phenomena and dynamics within a bioreactor. Moreover, these parameters are directly related to mass transfer, mixing, power input, bulk rheology and shear induced by agitation or aeration, substrate and products concentration, nutrients and microconditions in the reactor [3]. Among several scale-up parameters, impeller tip speed is the most common parameter. As a rule of thumb it is known that microbial damage may occur at tip speeds above 3.2 m/s and this number depends on many factors such as broth rheology [4]. If the scale-up is carried out using constant tip speed, the volumetric power consumption is often lowered, which can adversely affect the bioconversion. In many processes, oxygen behaves as a limiting substrate thus constant oxygen mass transfer coefficient can be used as scale up parameter. The mass transfer of oxygen into the bulk is influenced by several variables, such as physical properties of the fluid, operational conditions and geometry of the reactor. The oxygen transfer rate can be kept constant by altering stirring speed which concomitantly alters the power input. The use of constant oxygen mass transfer coefficient as scaling criterion is widely applied in conventional scales from laboratory to large scales if there is a strong link between cell growth and dissolved oxygen tension profiles [5]. However if the fermentation broth is viscous, constant mixing time is used through scaling. Mixing time is defined as the time required for the reactor composition to achieve a specified level of homogeneity following addition of a tracer pulse at a single point in the vessel [6]. Mixing time contains some information on flow and mixing within the reactor and can be useful for the scale-up of growth-regulated products.

In this study bioethanol production using quince pomace and scaling up was investigated. Cemeroglu et al. [7] reported that the quince pomace was directly used as a substrate without any chemical pretreatment (such as acid or base hydrolysis) due to the availability of the sugars (mostly glucose and fructose) in the pomace for microorganisms. There are several studies on the utilization of the fruit pomaces such as apple pomace without pretreatment process for the bioconversion of value-added bioproducts [8-10]. In this study quince pomace as an agro-industrial biomass was used for bioethanol production under microaerated conditions, eliminating the pretreatment step. In our previous study, we reported that microaerated conditions enhanced bioethanol production via promoting sugar consumption [11]. Considering the increasing demand on ethanol utilization worldwide, a suitable scale-up technology with a suitable scale-up parameter for bioethanol production from *E. coli* KO11 needs to be identified. To this end, the two main objectives of the present study were: (i) to evaluate the use of constant impeller tip speed, mixing time and oxygen mass transfer coefficient as scale-up methodologies under laboratory conditions for the scale-up process from 2 L reactor to 8 L stirred-tank reactor, considering whether an increase in the bioethanol yield can be achieved, and (ii) to validate the kinetic parameters for better describing the behavior of *E. coli* KO11 during bioethanol fermentation from quince pomace.

Materials and Methods

Growth conditions

Recombinant *E. coli* KO11 (pLOI 1910) strain was provided by courtesy of Professor L.O. Ingram from University of Florida. Stock cultures were stored in 40% glycerol at –80°C. Seed cultures of KO11 were maintained on modified Luria-Bertani (LB) agar containing 5 g of NaCl, 5 g of yeast extract, 10 g of tryptone, 20 g of glucose, 15 g of agar, and 600 mg of chloramphenicol per liter and kept at 4°C.

For inoculation, 3 colonies were transferred into 250 mL flasks containing 50 mL LB medium supplemented with 60 g/L glucose. Seed cultures were incubated under static conditions for 16 hours at 30°C. Cells were harvested by centrifugation (5000 g, 5 min) and washed with the fermentation medium.

Substrate

Quince pomace was used as a substrate for ethanol production instead of glucose. Quinces were pressed and dried to constant weight at 70°C in pasteur oven (Memmert GmbH & Co. KG D-91126, Germany) to remove bound-water. Dried pomace was grinded to 0.1 mm in size.

The total carbon (C) and nitrogen (N) content of dried quince pomace were determined using the Barbano and Walkley-Black methods, respectively [12,13]. Total C and N content were detected as 34.5% and 0.23% of the total dry biomass, respectively. Quince pomace was also reported to compose of 28.8% glucose, 55.7% fructose and 10.1% sucrose based on dry mass [7].

Table 1. Equations of scale-up processes for bioethanol production

Mathematical equations			
$Re_i = \frac{\rho N_i D_i^2}{\eta}$	(Eq. 8)	$\gamma = k N_i$	(Eq. 11)
$N_P = \frac{P_o}{\rho N_i^3 D_i^5}$	(Eq. 9)	$\eta = \frac{\tau}{\gamma}$	(Eq. 12)
$P_g = k \left(\frac{P_o^2 N_i D_i^3}{Q^{0.56}}\right)^{0.45}$	(Eq. 10)	$\lambda = \left(\frac{\upsilon^3}{N_P N_i^3 D_i^2}\right)^{0.25}$	(Eq. 13)

Reactor conditions

Batch fermentations were carried out in 5 L (Sartorius A plus stat.) and 10 L (Sartorius B plus stat.) bioreactors under semi-aerobic conditions with working volumes of 2 and 8 L, respectively. Quince pomace was added to the reactor as carbon source to obtain a C/N ratio of 14.33 g/g (which calculated as 73 g/L for fermentors), to simulate the elemental composition of LB medium supplemented with glucose of 60 g/L. Quince pomace and LB (without glucose) were autoclaved separately and mixed aseptically prior to fermentation. The fermentation was carried out at pH 6 under 35°C with an aeration of 0.035 vvm for the first 8 hours, as aeration was found to promote ethanol production previously [11]. The initial cell density was adjusted to the concentration of 0.33 g cell/L for both scales of fermentors. 2 M KOH solution was automatically added to prevent the broth pH from declining below 6.0. Solids were separated using centrifugation (5000 g, 5 min, 5°C) and supernatant was used for ethanol and reduced sugar determinations. All experiments were carried out in duplicate.

Settings and calculations of scale-up parameters

The adjustments of constant mixing time, constant impeller tip speed and constant oxygen mass transfer coefficient in the culture broth were carried out by varying the agitation rate which was set to 300 rpm in 2 L control reactor.

Mixing time

Mixing time was experimentally determined using the pH-response technique [14]. The agitation rate of 330 rpm was used in 8 L reactor through scaling-up of constant mixing time.

Impeller tip speed

Impeller tip speed (υ_{tip}) was calculated as a function of tip speed and reactor diameter by Eq. 1 [15]. The agitation rate was set to 225 rpm in 8 L reactor in the scale up studies based on the constant υ_{tip}.

$$\upsilon_{tip} = \pi D_i N_i \quad (1)$$

Oxygen mass transfer coefficient

Oxygen mass transfer coefficient was measured using the unsteady state method described elsewhere [16]. The agitation rate was 370 rpm in 8 L reactor based on constant k_La and the oxygen transfer rate (OTR) which was determined as 5 mmol O_2/L/h, was calculated by Eq. 2 [17].

$$OTR = dO_2/dt = k_L a(C^* - C_L) - q_{O_2} X \quad (2)$$

Mathematical equations of scale-up process for bioethanol production are presented in Table 1. Calculated process parameters are given in Table 2.

Analytical measurements and calculations

Biomass was determined and validated by counting colony forming units, measuring absorbance and dry cell mass. Absorbance was measured at 600 nm (A_{600}) using Unicam-Helios-α spectrophotometer, and the cell concentration was converted to g dry cell mass per liter (DC*m*/L) using the conversion factor of 0.33 g-DC*m*/L/A_{600}, for *E. coli* KO11 [18].

Considering the cell death was negligible, maximum specific growth rate (μ_{max}) was calculated (Eq. 3) [15].

$$\mu = \frac{\ln X_2 - \ln X_1}{\Delta t} \quad (3)$$

where X_2 is the final cell concentration, X_1 is the initial cell concentration and Δt is the time required for the increase in concentration from X_1 to X_2.

Fermentation broth viscosity was measured twice at the beginning and at the end of the fermentation period by a rotational viscometer (Brookfield model DV-E, USA) with LVtype spring torque using LV1 (61) spindle and determined by Poiseuille equation (Eg. 4). Average viscosity of 1.36×10^{-6} m^2/s was used in the equations.

$$\frac{dV_L}{dt} = \frac{\pi D_C}{8\,\eta}\frac{dP}{L} \quad (4)$$

The density of the fermentation broth was measured by 25 mL pycnometer (Isolab, Germany) at the beginning and at the end of the fermentation and an average density value was used in the study (1033 kg/m).

Total soluble reducing sugar content of quince pomace was determined using dinitrosalicylic acid (DNS) method where the absorbance was measured at 540 nm [19].

Ethanol concentrations were measured using a Gas Chromatograph (6890N Agilent Technologies Network GC System) equipped with a flame ionization detector and a DB-FFAP 30 m×0.32 mm×0.25 mm capillary column (J&W Scientific) [20].

Ethanol yield ($Y_{P/S}$) was defined as the amount of ethanol produced per the amount of sugar consumed during fermentation (Eq. 5). Total ethanol yield against theoretical yield and volumetric productivity were calculated by the Eq. 6 and 7, respectively.

$$Y_{P/S} = \frac{dE}{dS} \quad (5)$$

$$Total\ Ethanol\ Yield = \frac{dE}{dS} x \frac{1}{0{,}51} x\ 100 \quad (6)$$

Table 2. Summary of the rheological behavior of fermentation broth and hydrodynamic process parameters for stirred-tank bioreactors

	2 L control reactor	8 L reactor		
		Scale-up criteria		
		Constant t_m	Constant υ_{tip}	Constant k_La
N_i (rpm)	300	330	225	370
t_m (s)	17	17	22.27	16.49
υ_{tip} (m/s)	0.94	1.38	0.94	1.55
k_La (1/s)	1.2 x 10^{-2}	0.62 x 10^{-2}	0.46 x 10^{-2}	1.2 x 10^{-2}
Re_i	1.32 x 10^4	2.58 x 10^4	1.76 x 10^4	2.89 x 10^4
P_o (W)	0.52	2.93	0.93	4.13
P_g (W)	0.50	2.58	0.77	3.69
P_o/V_L (W/m^3)	260	360	120	520
P_g/V_L (W/m^3)	250	322.5	96.25	461.25
P_g/P_o	0.96	0.88	0.83	0.89
γ (1/s)	3000	3300	2250	3700
τ (N/m)	4.23	4.65	3.17	5.22
λ (m)	3.23 x 10^{-5}	2.60 x 10^{-5}	3.47 x 10^{-5}	2.39 x 10^{-5}

Table 3. Kinetic parameters for scale up studies of bioethanol production by *E. coli* KO11

	2 L control reactor*	8 L reactor		
		Scale-up criteria		
		Constant t_m	Constant υ_{tip}	Constant k_La
$Y_{P/S}$ (g/g)	0.36±0.01	0.40±0.05	0.36±0.04	0.32±0.09
μ_{max} (1/h)	0.23±0.02	0.23±0.04	0.22±0.03	0.26±0.12
ϑ_p (g/L/h)	0.41±0.11	0.49±0.04	0.38±0.01	0.40±0.03
Total yield (%)	70.10±0.05	78.67±0.03	69.68±0.01	62.83±0.02

t_m=17 s, υ_{tip}=0.94 m/s, k_La= 0.012 s^{-1} in 2 L control reactor.

$$Q_P = \frac{dE}{dt} \quad (7)$$

Statistical analyses

Statistical analyses of the collected data were performed by one-way analysis of variance (ANOVA). A probability value of $p<0.05$ was considered to denote a statistically significant difference of two batches. Data are presented as mean values±SEM (standard error of the mean).

Results and Discussion

Scaling up based on constant t_m

In this study, three common scale-up parameters (mixing time; t_m, impeller tip speed; υ_{tip} and oxygen mass transfer coefficient; k_La) of 8 L stirred tank reactor were compared with 2 L control reactor to obtain maximum ethanol yield.

As seen in Figure 1a, the constant t_m experiment yielded a maximum bioethanol production of 23.42 g/L in 48 hours. The increase in bioethanol concentration gradually decreased after 48 hours so the suitable process duration was chosen as 48 hours for the maximization of the volumetric productivity. Moreover, the ethanol concentration ($p<0.05$) obtained in 8 L stirred tank reactor was 19% higher than in 2 L stirred tank reactor at 48 hours based on constant t_m of scale-up. Similar to the ethanol production, the reducing sugar concentration decreased from 60 g/L to 4.52 g/L in 48 hours for 8 L stirred tank reactor and sugar consumption was 0.22 fold higher compared to 2 L stirred tank reactor based on constant t_m of scale up (Figure 1a and Figure 1d). It was reported that using scale-up based on constant t_m, bioethanol concentration of 70 g/L and 65 g/L, was obtained from sucrose and sugar beet juice in 18 hours, respectively using *Saccharomyces cerevisiae* IR2 immobilized on loofa sponge in 8 L [21]. In another study, 23 g/L ethanol was obtained from *L. japonica* hydrolysates by *E. coli* KO11 in 1 L reactor [22].

As seen in Figure 2a, the levels of log (CFU/mL) and the dry cell mass were 2.1% and 9.6% higher in 8 L stirred

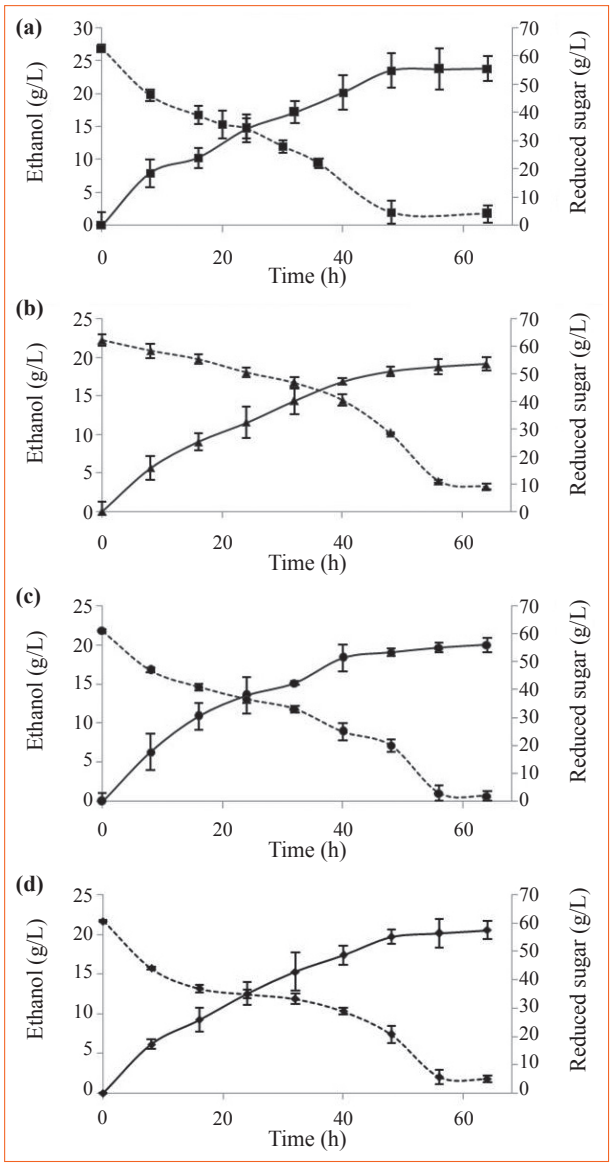


Figure 1. Ethanol (solid) and reduced sugar (dashed) concentrations of scale-up experiments based on **(a)** constant t_m (■), **(b)** constant v_{tip} (▲), **(c)** constant k_La (●) in 8 L stirred tank reactor compared to **(d)** 2 L control reactor (♦).

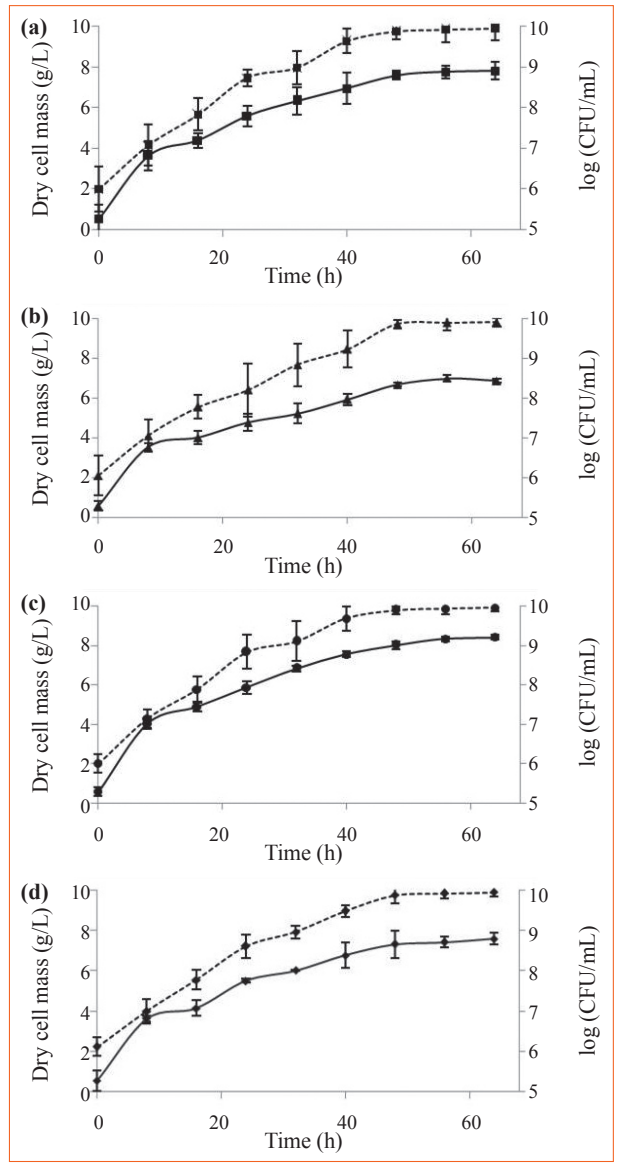


Figure 2. Dry cell mass (solid) and log (CFU/mL) (dashed) values of scale-up experiments based on **(a)** constant t_m (■), **(b)** constant v_{tip} (▲), **(c)** constant k_La (●) in 8 L stirred tank reactor compared to **(d)** 2 L control reactor (♦).

tank reactor than 2 L control reactor, respectively based on constant t_m experiment ($p<0.05$). This was due to the large surface area of the reactor, which led to more exposure to oxygen. The biomass concentration reached a maximum of 9.88 g/L around 48 h in scaling up based on constant t_m experiment (Figure 2a). These findings were compatible with the depletion of reduced sugar at the same point (Figure 1a).

As a rule of thumb, when the Kolmogorov eddy size becomes equivalent to the cell diameter or gets smaller, the movement of the flow lines can shear cells. In the scale-up study based on constant t_m, the size of Kolmogorov eddies was calculated to be 2.6×10^{-5} m which was considerably larger than an average *E. coli* cell size (Table 2). This could be attributed to the lower cell stress potential based on constant t_m experiment.

In this study, power number was taken as 5.20 at fully turbulent flow for Rushton turbine impeller design equipped with baffles [4,23]. The characteristic empirical constant (k) for a standard Rushton turbine impeller was taken as 10 [24]. Power consumption per unit volume (P_o/V_L) is considered as a measure of mixing intensity and mass transfer rate. In this study, the increases in impeller tip speed and Kolmogorov eddy size from 2 L control reactor to 8 L stirred tank were considered as negligible, because the P_o/V_L ratio in the constant t_m experiment was shown lower increase than the constant v_{tip} and constant k_La experiments (Table 2). This finding was in accordance with the study of [25].

Productivity is a significant parameter to determine the

cost-effectiveness value of an industrial production. As seen in Table 3, scale-up based on constant t_m results in the highest volumetric productivity of 0.49 g/L/h and the total ethanol yield of 78.67% with the highest sugar consumption rate (Figure 1a). It was shown that when the sugar consumption rate was increased, higher volumetric productivity of 0.42 g/L/h was observed for glucose supported LB medium with the total yield of 70% [26]. The ethanol production was achieved by *Saccharomyces cerevisiae* in 13 L semi-pilot scale production and the maximum ethanol concentration was 46 g/L for 10 days with the lower volumetric productivity of 0.015 g/L/h [27].

Scaling up based on constant v_{tip}

The maximum ethanol concentration was found to be 18.13 g/L in the constant v_{tip} experiment which was 29.39% lower than obtained in the constant t_m experiment in the 8 L stirred tank reactors (Figure 1a and Figure 1b). The amount of ethanol produced was considerably lower than all experiments based on constant v_{tip} of scale up and this result may be attributed to the usage of the lowest agitation rate, which influences the mass transfer adversely for viscous fermentation broths of bioethanol production. Since sugar content is directly associated to ethanol production, these findings were also supported by the lowest sugar consumption of only 53.3% at the end of the fermentation in the constant v_{tip} experiment (Figure 1b). During the production of ethanol from sugar beet juice by *Saccharomyces cerevisiae* IR2, it was concluded that the same or lower mixing rate used in 2 L bioreactor was not sufficient for 8 L bioreactor and consequently, the cells and the substrate were not uniformly distributed resulting in decreased ethanol productivity [21]. This can also be explained by the decrease of turbulent flow which was demonstrated by the decreases in P_o/V_L values (Table 2). It was also emphasized that when a scale up procedure resulted in a few increased Reynolds number, a very low P_o/V_L value is obtained, which is not sufficient for efficient mixing affecting product rate negatively [28]. Moreover, mixing time was 1.31 fold higher in 8 L stirred tank reactor than 2 L control reactor with the result of lower ethanol yield based on constant v_{tip} experiment (Table 2). In other words, the longest mixing time was obtained in the constant v_{tip} experiment and affected bioethanol yield unfavorably. These effects may be due to the phenomena that higher mixing time might influence the mass transfer adversely and causing probable death zones inside the reactor. It was reported that, the mixing of the fermentation broth and mixing time are important for the efficient operation in large scale of heterogeneous suspensions [29]. Longer mixing times can cause locally high sugar accumulation leaded to low dissolved oxygen levels [30,31]. In this study, the existence of death zones in 8 L reactor resulted lower biomass and product yields upon scale-up based on constant v_{tip} (Table 3).

The biomass concentration based on constant v_{tip} experiment was 15.15%, 21.21% and 10.6% higher than based on constant t_m, constant k_La, and 2 L control reactor experiments, respectively (Figure 2a, 2b, 2c and 2d). These decreased levels of biomass growths in 8 L stirred-tank bioreactor of constant v_{tip} experiment are attributed to the poor gas–liquid dispersion observed at the lower impeller speed with the higher Kolmogorov eddy size. It is worth mentioning that, as stirrer rate decreased, Kolmogorov eddy size increased throughout scaling-up for ethanol production by *E. coli* KO11 [18].

In this study, the minimum μ_{max} value of 0.22 (1/h) was obtained using scale-up strategy based on constant v_{tip}. Furthermore, ethanol yield was considerably lower when constant v_{tip} parameter was applied than all reactors, as a result of insufficient mixing when using quince pomace as a viscous substrate (Table 3). The reduced ethanol yield in the scale-up experiments based on constant v_{tip}, is also likely to be the result of operating at the reduced agitation rate, and the poor gas–liquid dispersion.

Scaling up based on constant k_La

In the scale-up studies based on constant k_La experiment, the ethanol concentration was 23.41% lower, whereas the biomass concentration was 9.59% higher than the scale-up studies based on constant t_m at 48 hours (Figure 1c and Figure 2c). It is important to underline that the maximum μ_{max} value of 0.26 (1/h) was obtained using scale-up strategies based on constant k_La (Table 3). It was stated that scale-up based on constant k_La is more appropriate for aerobic processes or biomass production as cells may tend to produce more biomass which leads to decrease in product yields [30,32]. Aerobic K99 antigen production was successfully scaled up from 5 to 200 L by keeping k_La constant using recombinant *E. coli* MC1061 with a product yield of 0.03 g/g [33]. The shear stress was only 1.23 fold higher in 8 L stirred tank reactor of constant k_La experiment, corresponding to a shear stress of 4.23 N/m^2 in 2 L control reactor (Table 2). It was shown that shear stress with the value of 12.5 N/m^2 had no significant decrease on cell lysis or cell viability by the wild type of *E. coli* [34]. Constant t_m was reported to be more applicable under microaerobic conditions compare to constant oxygen uptake rate for the scale-up of 2,3-butanediol fermentation by *Enterobacter aerogenes* in which homogeneity was important [35].

Since the k_La is a function of P_g, the Rushton turbine has a much larger mass transfer coefficient because it dissipates significantly more power than the other impellers studied at constant impeller speed [36]. The highest k_La value ($1.2x10^{-2}$ 1/s) was obtained in the constant k_La experiment at which also gave the best gas handling capacities shown by the highest P_g/V_L ratio (Table 2).

Conclusion

In this study, it was shown that the best approach is the application of constant t_m through scaling-up for viscous fermentation broths of bioethanol productions due to the

significance of the homogeneity. This work suggests that there is considerable potential from an economic perspective for using quince pomace waste as a substrate for bioethanol production since it is considered as waste for juice processing and cannot be used for further applications. The results obtained in this study will provide valuable guidelines for engineering of bioethanol producers.

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Conflict of Interest

There are no conflicts of interest among the authors.

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