Biodegradation kinetics and modeling of whey lactose by bacterial hemoglobin VHb-expressing *Escherichia coli* strain

Muayad M. Abboud [a], Isam H. Aljundi [b,*], Khaled M. Khleifat [c], Saif Dmour [c]

[a] *Pharmacology Department, Mutah University, Al-Karak 61710, Jordan*
[b] *Chemical Engineering Department, Mutah University, Al-Karak 61710, Jordan*
[c] *Biology Department, Mutah University, Al-Karak 61710, Jordan*

ABSTRACT

The batch fermentation of cheese whey lactose was achieved using *Escherichia coli*:pUC8:16 recombinant strain that was transformed with *Vitreoscilla* hemoglobin gene(*vgb*). In this process, 70% of the initial whey lactose was biodegraded during 24 h of incubation time. Biodegradation was accompanied with a turnover of glucose intermediate and a production of lactic acid. Total lactic acid produced by this recombinant strain was 57.8 mmol/L compared with a reference lactic acid producing strain, *Lactobacillus acidophilus*, that yielded only 55.3 mmol/L of lactic acid from the same initial whey lactose concentration. The engineering of *vgb* gene transformation in *E. coli* strain has led to increase in bacterial biomass and boosted lactic acid production, relative to other strains that lack the vgb gene like *E. coli*:pUC9 or *E. coli* wild type or *Enterobacter aerogenes*. Contrary to Monod's, Haldane's model gave a good fit to the growth kinetics data. Kinetic constants of the Haldane equation were $\mu_m = 0.5573\,h^{-1}$, $K_s = 4.8812\,g/L$, $K_I = 53.897\,g/L$. Biomass growth was well described by the logistic equation while Luedeking–Piret equation defined the product formation kinetics. Substrate consumption was explained by production rate and maintenance requirements. In simulation studies including the Haldane model, an evident agreement was observed between measured and calculated biomass, product, and substrate concentrations.

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1. Introduction

Whey is a watery by-product of the cheese manufacturing process which is composed mainly of 5–6% lactose and 0.8–1% protein [1,2]. It is produced world wide in large quantity reaching over 10^8 tons per year [3]. Cheese whey is considered environmentally high strength wastewater pollutant due to its high biological oxygen demand (BOD) and chemical oxygen demand (COD) contents which approaches values of 50 and 80 g/L, respectively [4].

The disaccharide lactose is the major carbohydrate of milk and dairy products. It can be considered as a renewable and thus biotechnologically important carbon source which accumulates primarily as a by-product from cheese manufacture or whey processing industries [5]. Only 56% of the whole whey solids are currently processed into animal or human food products [6]. Several problems are encountered in whey disposal, including uneconomical transport due to its high water content and the difficulty of prolonged storage because of whey susceptibility to spoilage by bacteria and fungi. In addition, drying the whey requires

a large capital investment and energy consumption which are not economically profitable [7]. Therefore, the currently adopted biotechnology of whey disposal is based on recycling of the main nutrient components: lactose and proteins.

There has been increased interest in lactic acid production from whey lactose, because of its utilization as raw material for the production of polylactic acid polymer that has special industrial applications in manufacturing medical and biodegradable plastics [8]. Lactic acid can be produced either by chemical synthesis or fermentative processes [9,10] but the fermentative means has advantages over chemical synthesis, due to a better efficiency and desirable optically pure lactic acid being generated [11]. Usually the complete fermentation of whey lactose requires the supplementation of whey with an additional nitrogen source such yeast extract or whey protein hydrolyzate in order to insure rapid production of lactic acid [12–14].

Various bacterial and fungi microorganisms are capable of producing Lactic acid from whey lactose disposal [15–18], however few efforts have been made to improve the production of lactic acid through metabolic engineering techniques. One such attempt was conducted involving the expression of L-lactate dehydrogenase and D-lactate dehydrogenase genes in *L. helveticus* for the production of pure D(−)- and L(+)-lactic acids [19]. In another attempt, a

* Corresponding author. Tel.: +962 3 2372380; fax: +962 3 2375540.
E-mail address: aljundi@mutah.edu.jo (I.H. Aljundi).

recombinant *Escherichia coli* was constructed for the conversion of hexose and pentose sugars in to L(+)-lactic acid, which was also metabolically engineered for carbon catabolite repression [20].

The vgb gene encoding VHb hemoglobin protein of *Vitreoscilla*, has been cloned and expressed in a wide range of bacterial strain [21]. The main physiological function of this protein is confined to its efficient supply of oxygen to terminal oxidases under limited aerobic conditions. Therefore, its expression inside various heterologous hosts often yields an enhancement of cell density, oxidative metabolism, engineered product formation, and bioremediation [22–25].

The present work evaluates the efficiency of expressing VHb protein in *E. coli* strain, as a novel approach to enhance whey lactose degradation and lactic acid production.

2. Materials and methods

2.1. Microorganisms and culture conditions

E. coli, the principle bacterial strain used in present work was characterized and transformed with the plasmid pUC8:16 as described elsewhere [26]. The resulted recombinant strain from this transformation either was lacking the vgb gene (*E. coli*:pUC9) or bearing the vgb gene (*E. coli*:pUC8:16 or VHb strain) on the transformed plasmid. *Enterobacter aerogenes* and *E. coli* (WT) were maintained on Luria Broth (LB) media while, the transformed strains *E. coli*: pUC8:16 (VHb) and *E. coli*:pUC9 were maintained on LB broth containing 50 μg/mL ampicilline.

Another strain used in this study was *Lactobacillus acidophilus* ATCC 4962 which was obtained from the Faculty of Agriculture, Mutah University, Jordan. This strain was maintained on Man Rogosa and Sharpe (MRS) medium (Difco, USA) containing per one litter of distilled water: peptone 10 g, meat extract 8 g, yeast extract 4 g, glucose 20 g, dipotassium hydrogen phosphate 2 g, Sodium acetate trihydrate 5 g, triammonium citrate 2 g, magnesium sulphate hepatahydrate 0.2 g and manganous sulphate tetrahydrate 0.05 g.

2.2. Biodegradation study

To carry out the whey fermentation, 1×10^8 cells/mL inoculum of previously maintained bacterial culture on LB agar medium was transferred to a flask containing 50 mL of minimal salt medium. This medium contained per one litter of distilled water: 1.36 g

KH_2PO_4, 0.5 g $(NH_4)SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCL_2 \cdot 2H_2O$, 0.005 g $FeSO_4$, 0.0025 g $MnSO_4 \cdot 7H_2O$, 0.0025 g $NaMO_4 \cdot 2H_2O$, 2.43 g Na_2HPO_4. After sterilization, the pH was adjusted to 7.3 then supplemented with whey lactose as sole carbon source. The culture was incubated for 24–28 h at 37 °C and agitation rate of 150 rpm. The bacterial growth was determined by measuring the optical density at 600 nm.

At different time intervals of the whey fermentation, the broth was centrifuged at 7000 rpm for 20 min, and the supernatant was used for the estimation of residual lactose remaining, glucose intermediate and pH or lactic acid production.

The whey was obtained locally from cheese processing factory and subjected to heating at 121 °C for 15 min to denature proteins. The precipitate was removed by centrifugation while the supernatant was sterilized and adjusted to pH 7.3. The initial whey was designated as the whey filtrate which is subjected to serial dilutions with minimal salt medium before added to the culture.

2.3. Analytical techniques

To determine the whey lactose concentration, the proteins in broth supernatant were precipitated by 12% trichloroacetic acid (TCA) and discarded after centrifugation. The lactose in collected supernatant was determined by treatment with picric acid under alkaline condition to give pink color which is measured at 520 nm and compared with a standard solution of lactose [27]. The glucose concentration was measured using the glucose oxidase reaction as described by Barham and Trider [28].

Lactic acid concentration was estimated by a kit method (Ranox laboratories Ltd., UK), which depends on the oxidation of L-Lactate by lactate oxidase to produce pyruvate and H_2O_2. The hydrogen peroxide produced by this reaction is then used in an enzymatic reaction to generate a purple colored dye that is measured at 550 nm. All experiments were repeated 2–3 times and the data were presented as means ± absolute error. *P* value of <0.05 was considered to be statistically significant.

3. Modeling

In order to describe and predict the kinetic behavior of cell growth, product formation and substrate consumption, unstructured mathematical models were constructed for these variables.

3.1. Cell growth

The relationship of specific growth rate to substrate concentration often assumes the form of saturation kinetics where it is assumed that a single chemical specie, *S*, is growth rate limiting. These kinetics are similar to the Michaelis-Menten kinetics for enzyme reactions. When applied to cellular systems, these kinetics can be described by the widely used Monod equation:

$$\mu = \frac{\mu_m S}{K_s + S} \tag{1}$$

where μ is the specific growth rate, μ_m is the maximum specific growth rate when $S \gg K_s$ and all other essential concentrations are not limiting. The constant K_s is known as the saturation constant.

At high concentrations of substrate or product and in the presence of inhibitory substances in the medium, growth becomes inhibited, and growth rate depends on inhibitor concentration. One of the most widely used inhibition expression is the non-competitive substrate inhibition:

$$\mu = \frac{\mu_m}{(1 + (K_s/S))(1 + (S/K_I))} \tag{2}$$

where K_I is the substrate inhibition constant. This equation will reduce to Haldane equation when $K_I \gg K_s$:

$$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_I}} \tag{3}$$

In order to describe the growth kinetics with both exponential and stationary phases, keeping in mind that there is a limit to the maximum attainable cell mass concentration, the logistic equation is used:

$$\frac{dX}{dt} = \mu X \left(1 - \frac{X}{X_m}\right) \tag{4}$$

where X is the cell mass concentration, t is the incubation time, and X_m is the stationary population size above which bacteria do not grow.

3.2. Product formation

The product formation was described by Luedeking–Piret expression [29]:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \tag{5}$$

where P is the product concentration, α is a growth-associated constant for product formation, and β is a non-growth-associated constant for product formation. In this expression, the rate of product formation depends on the instantaneous biomass concentration as well as the growth rate.

3.3. Substrate consumption

In fermentation, part of the substrate is converted to product and another part is used by cells to form new bacterial protoplasm as well as to perform normal metabolic activities irrespective of growth. Therefore, a maintenance coefficient, m, was introduced in the substrate utilization kinetics:

$$\frac{dS}{dt} = -\frac{1}{Y_{PS}} \frac{dP}{dt} - mX \tag{6}$$

where S is the substrate concentration, and Y_{PS} is the product yield coefficient (mass of lactic acid produced per mass of lactose utilized).

To predict the kinetics of the fermentation process, Eqs. (3)–(6) has to be solved simultaneously with respect to time. This set of coupled ordinary differential equations along with their initial conditions was solved using MATLAB 6.5 software (MathWorks Inc., USA).

4. Results

4.1. Whey lactose biodegradation

The fermentation of whey lactose as sole carbon source was assessed in a bacterial batch culture containing minimal salt medium. The biochemical profile of this fermentation process involves biodegradation of whey lactose to produce two monosaccharides, glucose and galactose, that subsequently degraded further to pyruvate and then to lactate. A typical pattern of whey lactose fermentation by the recombinant bacterial strain *E. coli* VHb is depicted in Fig. 1. A time dependent degradation of whey lactose was observed which lasted for 28 h incubation period. Concomitantly the rate of glucose formation as an intermediate of biodegradation was increased, reaching a peak value at 12 h of incubation time followed by a lagging phase of decay which was extended to the stationary phase of bacterial growth. Also the culture pH was reduced from a starting value of 7.3 to a final low value
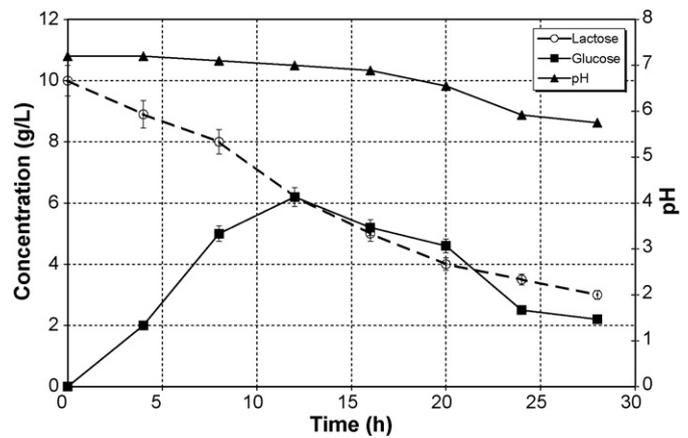

Fig. 1. Fermentation of whey lactose by *E. coli* VHb in batch culture.

of 5.7. By using highly specific lactic acid oxidase assay, this increase in acidity was later found to be due to the gradual formation of lactic acid in the bacterial culture.

The supplementation of *E. coli* VHb culture with 4% yeast extract improved the bacterial growth as well as enhancing the process of whey lactose biodegradation (data not shown).

4.2. Effect of initial whey concentration on the biodegradation rate

To determine the rate of whey lactose fermentation (expressed as gram of lactose disappeared in bacterial culture per 1 h incubation time), the effect of initial whey concentration on this process was examined. A characteristic biphasic shape of whey lactose fermentation was observed, showing an early elevation in the rate of fermentation followed by a late decrease as the initial whey lactose concentration was increased in the culture medium (data not shown). The maximum rate of whey lactose degradation apparently occurred at an initial whey concentration of 20%; beyond which the rate of lactose degradation started to fall down reaching a minimum level at the undiluted (100%) initial whey concentration. This unexpected finding of biphasic whey fermentation stimulated further investigation to find out if the initial whey lactose has osmotic influence on bacterial cell growth. Fig. 2 shows the effect of free lactose on the growth of *E. coli* VHb, using free lactose instead of the whey lactose. A maximum rate of bacterial growth was obtained at 10 g/L free lactose, then growth started to drop down as the lactose concentration increased until it stopped completely at 75 g/L. Sim-
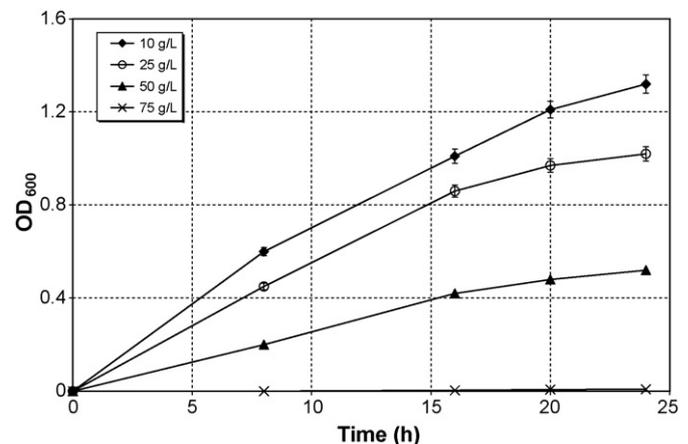

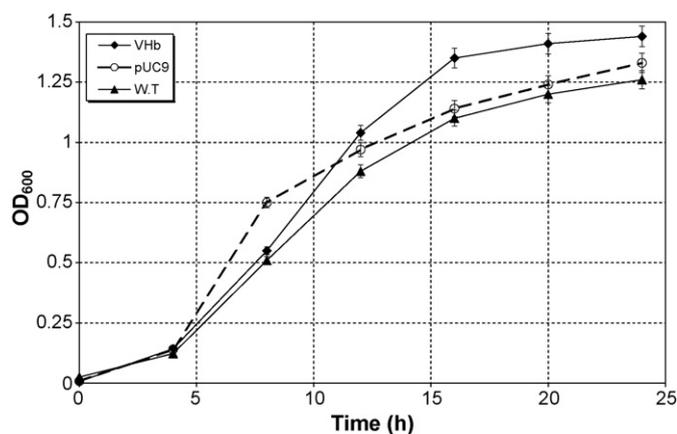**Fig. 2.** Effect of free lactose concentrations on *E. coli* VHb growth.

Fig. 3. Growth comparison of three *E. coli* strains at 20% whey lactose concentration.

Fig. 5. Time profiles of lactic acid production by different bacterial strains.

ilar osmotic effects were found with other free sugars like glucose, galactose and fructose which influenced range of different bacterial strains like lactic acid producing, halophilic and waste water inhabitants (data not shown).

The above data implied that 10 g/L whey lactose (corresponding to 20% initial whey) is the optimum concentration for getting the appropriate rate of whey lactose fermentation. By using this fixed whey concentration as sole carbon source together with a supplementation of 4% yeast extract, the bacterial growth (OD at 600 nm) was compared between the three *E. coli* strains. During incubation time, all tested bacterial strains gave a typical growth curve with variations of growth rate in the order VHb-expressing *E. coli* strain > VHb-lacking pUC9 strain > wild type *E. coli* strain (Fig. 3).

In keeping with these growth conditions, the pattern of lactose fermentation showed a slow rate at the lag phase, followed by rapid rise during exponential phase and a terminal decline in the stationary phase (Fig. 4). The highest proportion of whey lactose degradation (about 70%) was expressed by *E. coli* VHb strain, compared with nearly 55 and 60% degradation carried out by the VHb-lacking strains *E. coli* pUC9 and *E. coli* wild type, respectively.

4.3. Bioproduction of lactic acid

In addition to the previous effects on lactose degradation, the increase in initial whey concentration was found to interfere with the accurate determination of lactic acid production during the fermentation process. This interference was due to the detection of various basal lactic acid levels in the whey preparation sam-
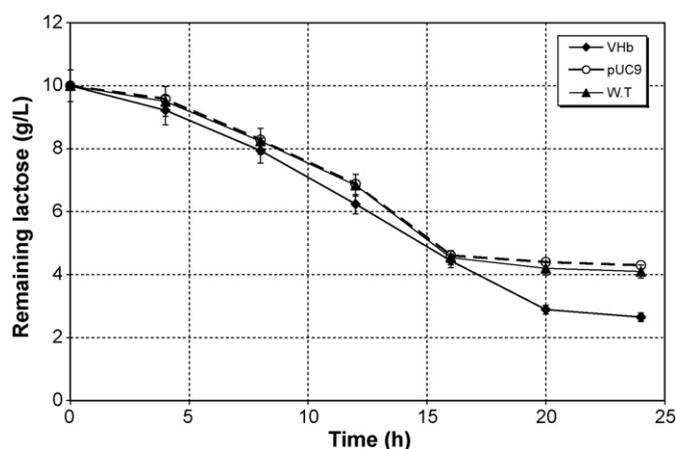
ples, even before these samples were added to the fermentation medium.

Therefore, to obtain the net lactic acid actually produced from whey lactose fermentation the basal level of lactic acid was subtracted from the total lactic acid measurement at the end of the fermentation process. The least negative effect of basal lactic acid was observed when the initial whey concentration being used at proportions <20%.

4.4. Correlation of lactic acid production with bacterial growth

Fig. 5 shows that the formation of lactic acid was almost linear up to 24 h of incubation time in a culture supplied with fixed concentration of 20% whey. Similar to whey lactose fermentation, the production of lactic acid exhibited initial slow down during the lag phase with subsequent rapid increase in the exponential phase and later decline at the stationary phase. The VHb strain expressed higher capacity for lactic acid production from whey lactose than either *Enterobacter*, pUC9 harboring *E. coli* and wild type *E. coli* strains.

A comparison between the conditions of *E. coli* VHb growth and lactic acid production indicated that both processes had similar optimum pH, temperature and agitation rate (Table 1). In addition, these two processes exhibited parallel type of inhibitions after the addition of extra carbon or nitrogen sources to the bacterial media that contained whey preparations.

4.5. Comparison of lactic acid production by E. coli VHb and Lactobacillus acidophilus

Having established the appropriate conditions for whey lactose fermentation, the efficiency of lactic acid production was compared between *E. coli* VHb strain and *Lactobacilli acidophilus* as a reference lactic acid manufacturing strain. The selected optimum fermentation conditions for *E. coli* VHb strain were 20% whey, temperature 37 °C, pH of 6.5, and an agitation rate of 250 rpm. For *Lactobacil-*

Fig. 4. Time profile of whey lactose biodegradation by three *E. coli* strains.

Table 1
Correlation of growth conditions and lactic acid production for *E. coli* VHb.

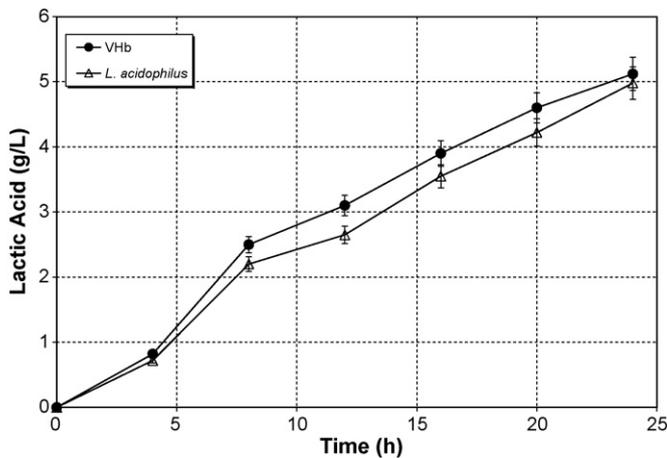| Conditions of lactose fermentation with 20% whey	*E. coli* VHb growth	Lactic acid production
Optimum pH	6.5	6.5
Optimum temperature (°C)	37	37
Optimum agitation rate (rpm)	250	250
Additional carbon sources	Inhibition	Inhibition
Additional nitrogen sources	Inhibition	Inhibition

Fig. 6. Comparison of lactic acid production between *E. coli* VHb and *Lactobacillus acidophilus* strains.

Fig. 7. Simulations of whey lactose biodegradation, growth, and lactic acid production by *E. coli* VHb strain.

lus acidophilus the optimum conditions included the same whey concentration, a temperature of 42 °C, pH of 4.8, and an agitation rate of 150 rpm. Fig. 6 indicates a slight increase in rate of lactic acid production by *E. coli* VHb culture which yielded 5.2 g/L of lactic acid relative to the culture containing the *Lactobacillus acidophilus* strain that generated 4.98 g/L lactic acid after 24 h of incubation time.

Due to the maximal amount of lactic acid produced and high rate of lactose degradation, the VHb-expressing strain was selected for mathematical modeling interpretation as a representative bacterial strain model.

4.6. Kinetics and mathematical modeling

To determine the kinetic parameters of the rate equations, batch fermentation experiments was performed at seven different lactose concentrations. The specific growth rate in the exponential phase was calculated as

$$\mu = \frac{\ln(OD_2/OD_1)}{t_2 - t_1} \tag{7}$$

where OD_1 is the cell's optical density at time t_1 (start of the exponential phase), OD_2 is the optical density at time t_2 (end of the exponential phase). When the specific growth rate was plotted against the initial whey concentration, the experimental data revealed that lactose substrate at certain concentration has an inhibitory effect and that Monod kinetic model was not applicable to this fermentation system. Haldane equation, on the other hand, gave an excellent representation of the experimental data with a correlation coefficient of 0.994.

The estimation of Haldane's parameters requires the use of a non-linear regression technique. The model parameters were fitted to the experimental data using CurveExpert 1.34 software [30]. The obtained parameters from the Haldane equation are: 0.5573 h^{-1}, 4.8812, and 53.897 g/L for maximum specific growth rate, the saturation constant, and the substrate inhibition constant, respectively.

The fermentation process was modeled by solving the simultaneous set of equations, and the parameters obtained by fitting the model to the experimental data are: 0.5573, 4.8812, 53.897, 3.425, 0.001, 1.532, and 0.001 for μ_m, K_S, K_I, α, β, X_m, and m, respectively. The value for product yield on substrate, Y_{PS}, was taken as 0.74 in the model development. This value was calculated using the experimental data of the substrate and the product at the beginning and the end of the fermentation process. Fig. 7 shows the model prediction of the cell concentration, substrate utilization, and the product formation against time.
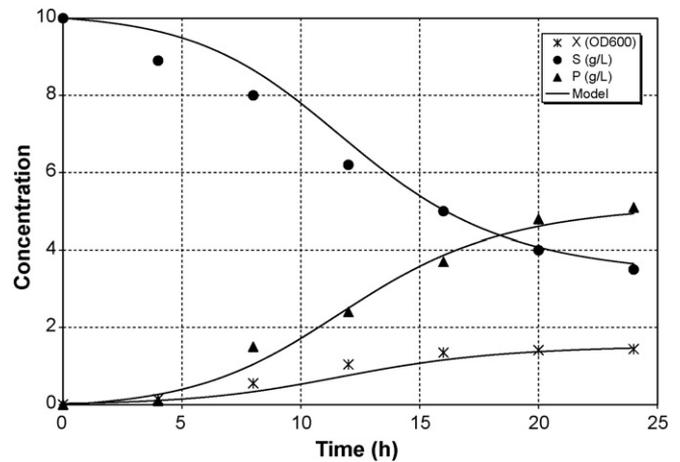
The experimental data shows that the production of lactic acid increased with fermentation time. The highest lactic acid concentration was attained at the corresponding maximum cell concentration. Lactic acid was produced during all growth phases; however, lower rate of acid production was noticed in the stationary phase. This suggests that the acid production is strongly associated with growth. The simulated behavior was in a very good agreement with the experimental data. The cell growth appeared to be fast, resulting in a rapid increase in biomass production. A stationary phase was predicted by the model when the optical density was 1.532. The model prediction of the three growth phases was in a good agreement with the experimental data. The model gave a good prediction of the whey lactose degradation as well. The general behavior of slow decrease during the lag period followed by a rapid decrease during the exponential growth, and finally slow decrease during the stationary phase is excellently simulated.

5. Discussion

The whole process of whey lactose fermentation was demonstrated in *E. coli VHb* culture by detecting whey lactose disappearance during the incubation period which was associated with a corresponding turnover in the intermediate glucose as well as lowering in pH of the medium due to lactic acid generation. The observed lagging in glucose degradation at the end of incubation period suggested an incomplete fermentation process, which could have been reflected on the short outcome of 70% instead of completely degraded whey lactose. The reported inhibition of β-galactosidase by accumulated glucose and galactose in the culture medium [25,31] may have important role in reducing this efficiency of whey lactose hydrolysis.

In present work, the main objective initially addressed was to establish a batch biotechnical system that can biodegrade large proportion of whey lactose as well as facilitating the production of maximum amount of lactic acid. Such attempt was hindered by two challenging problems. The first difficulty involved the encounter of osmotic effect associated with the increase consumption of whey lactose concentration. Such phenomenon was also observed with free lactose as well as other sugars which involved range of bacterial strains being tested including the halophilic, acidophilic lactic acid producing, and waste water strains. A similar osmotic stress of high sugar concentration on bacterial cells was documented by other workers [3,32,33]. The second challenging problem was related to the natural presence of proportional lactic acid in whey prepara-

tions that interfered with the accurate determination of lactic acid produced by bacterial fermentation. The amounts of this basal lactic acid were increased with the use of high whey proportions. These two limitations hampered the possibility of using whey at high proportions in the fermentation process. Therefore a mixture of 20% whey and 80% minimal salt medium made good compromise as an appropriate culture for *E. coli* VHb fermentation of whey lactose and production of lactic acid. Under these conditions the *E. coli* VHb produced 5.2 g/L lactic acid (equivalent to 57.8 mmol/L) from 10 g/L whey lactose which corresponds to an overall yield of 74%. Our data also revealed that a well known lactic acid producing bacteria; *Lactobacillus acidophilus*, produced less amounts (55.3 mmol/L) of lactic acid under analogous conditions. In another study, Pescuma et al. [34] observed that *Streptococcus thermophilus* CRL 804 consumed only 12% of the initial lactose to produce 45 mmol/L of lactic acid at 24 h. The possibility of generating products other than lactic acid in this fermentation process cannot be excluded. However, the high yield of lactic acid production would make the existence of such side products in present bacterial system very minor. Besides, the lactic acid formation is known to be favored over other side products when *E. coli* ferments whey lactose under acidic conditions [18].

The cloning of vgb gene in *E. coli*:pUC8:16 strain proved to be an efficient mechanism for enhancing bacterial growth and lactic acid production, when compared with other strains that lack the vgb gene such as *E. coli*:pUC9, *E. coli* wild type, or *Enterobacter aerogenes*. The efficiency of vgb gene recombination can be attributed to a selective enrichment of bacterial biomass [24,35] which may lead to enhancement of lactic acid productivity. The effects of vgb gene is believed to be mediated through an oxygen-trapping process, that may generate enough ATP needed for stimulation of β-galactosidase as well as other enzymes involved in whey lactose biodegradation [25,24,36].

In accordance with these findings our results indicated that all incubation conditions which supported an optimum production of *E. coli* VHb biomass such as pH; temperature and aeration, also were optimum for lactic acid formation. Moreover, when an extra carbon and nitrogen sources were used, a simultaneous reduction in both bacterial biomass as well as lactic acid production was observed. The inhibition effects of these additional supplementary sources are likely to be the outcome of catabolite repression on whey lactose utilization [37,38] though more detail studies are needed to elucidate the mechanism of this inhibition.

Kinetics of whey lactose degradation was studied using different microbial cultures in batch system. Haldane's kinetic model was found to describe quite accurately the specific growth rate profile of *E. coli* VHb strain at different whey lactose concentrations. The cell growth and lactose biodegradation corresponded well with each other throughout the studied concentration range indicating that the bacterial cells grow and biodegrade whey lactose. However, whey lactose exhibited inhibition on the growth and biodegradation above a lactose concentration of 10 g/L in the media. Biomass growth was well described by the logistic model while Luedeking–Piret equation defined the product formation kinetics. Substrate consumption was successfully explained by production rate and maintenance.

6. Conclusions

A recombinant strain of *E. coli* (VHb strain) which was genetically engineered by the transformation of a plasmid carrying the hemoglobin gene (*vgb*) from *Vitreoscilla* bacteria, was able to produce lactic acid from whey lactose in batch culture medium. Optimum conditions for whey lactose fermentation were established, though certain limitations encountered. It was proved that the VHb strain is more efficient in lactic acid production than the wild type or another recombinant *E. coli* strain carrying the same plasmid but lacking the *vgb* gene expression. In addition, the VHb strain was found slightly better in lactic acid manufacturing than a reference lactic acid producing strain; *Lactobacillus acidophilus*. The boost of lactic acid formation in VHb was attributed to hemoglobin induction of bacterial biomass. A good concurrence to the experimental data was observed when the unstructured model was used to simulate the bacterial growth, lactose consumption, and lactic acid production. Further improvement in whey lactose fermentation and lactic acid productivity could be achieved in the future by attempting an alternative continuous fermentation with recycling system that is characterized by increased dilution rate and reduced substrate concentration in the effluent.

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