Periodica Polytechnica
Chemical Engineering

60(4), pp. 298-312, 2016
DOI: 10.3311/PPch.8286

RESEARCH ARTICLE

Production of β-galactosidase in a Batch Bioreactor Using Whey through Microbial Route – Characterization of Isolate and Reactor Model

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Received 29 May 2015; accepted after revision 16 September 2015

Abstract

Whey was used as a source of isolation of bacterial strains, symbolized as, IB1, IB2 and IB3, capable of synthesizing β-galactosidase. The microbe labeled as IB1 was tested to be most tolerant against pH and temperature shocks, as well as, heavy metals. Subsequently, IB1 was identified as Bacillus safensis (JUCHE 1) by genetic information. In a later exercise, it was used for β-galactosidase production using whey through fermentative route. The initial concentration of substrate, i.e., lactose in microbial growth medium was varied ranging from of 5-50 g/L. It was found that the classical Monod kinetics and substrate inhibited Monod kinetics are able to describe the microbial growth kinetics at low (5-20 g/L), and high (>20 g/L) concentration ranges of lactose in growth medium respectively. Kinetics of β-galactosidase production followed the Monod incorporated modified Luedeking-Piret model and the Monod incorporated Luedeking-Piret model with substrate inhibition in the low, and high ranges of lactose concentration in growth medium respectively.

Keywords

Isolation of Bacillus safensis (JUCHE 1), β-galactosidase production, Whey, Kinetic model

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1 Introduction

Whey, the major waste product of dairy industry, is a potential contaminant with a BOD value between 30,000 ppm to 50,000 ppm and COD value between 60,000 ppm to 80,000 ppm. Therefore, its direct disposal into water courses is strictly forbidden [1, 2]. Although membrane based processes are being utilized to produce useful whey proteins, like, α-lactalbumin, β-lactoglobulin, bovin serum albumin etc from whey, there are sufficient scopes to develop processes to produce valuable products, like, β-galactosidase, galacto-oligosaccharide (GOS), ethanol, tagatose, etc. from whey [2-4]. The enzyme β-galactosidase is the key biomolecule used for the treatment of population suffering by hypolactasia, reduction of crystallization of lactose, increase the solubility of milk product, etc. It is an exoglycosidase which hydrolyzes the β-glycosidic bond formed between galactose and its organic moiety. It also participates in transgalactosylation reaction, producing prebiotic GOS [5]. Although β-galactosidase may be produced using different microorganisms, bacterial sources are recommended for β-galactosidase production because it provides high yield of enzyme and they are considered as 'Generally Recognized as Safe' microorganism [6-7]. It is expected that the bacterial strains isolated from dairy effluent are more suitable for the production of β-galactosidase using whey. This is the reason why few research studies have recently been reported on isolation of β-galactosidase producing bacteria from dairy effluent [8-10]. However, there are scopes to isolate other bacterial strains from dairy waste and their subsequent application in large reactor to produce β-galactosidase. As the kinetics of growth of the microorganism, substrate utilization and β-galactosidase production play key role for predicting the reactor performance and scale up, therefore, systematic and judicious studies to evaluate fermentation kinetics are very much prerequisite. Unfortunately, few research studies have been reported on kinetics of β-galactosidase production through microbial route [11-15].

The aim of this present investigation is fermentative production of β-galactosidase by microbial route considering whey as a fermentation medium. To archive this goal, a great effort has been

placed to isolate most robust strain (potential to sustain in hostile condition) which is suitable for fermentative β-galactosidase production. During the screening of the most potent strains among all isolated ones, priority has been given to the strain which exhibits at high thermal and pH shocks, as well as heavy metal resistance. Under the present study, different unstructured models for growth kinetics of the bacteria, substrate utilization and β-galactosidase production have been attempted. The most appropriate kinetic models for microbial growth, substrate utilization and β-galactosidase production have been selected on the basis of comparison of experimental data of small reactors (Erlenmeyer flasks) with predicted ones. Furthermore, selected kinetic equations for each component (concentration of biomass, lactose and β-galactosidase) have been applied for lab-scale 5 L batch bioreactor to understand the actual system dynamics, as well as validity of selected kinetic model.

2 Materials and Methods

2.1 Materials

Prior to every experiment, freshly prepared casein whey was collected from local sweet meat processing industries, situated in-and-around Kolkata, India. Highly purified whey proteins, ortho-Nitrophenyl-β-galactoside (ONPG), de-hydrated tris buffer, sodium phosphate buffer and citrate buffer were procured from Sigma Aldrich, USA. All inorganic salts, Folin-Ciocalteau reagent and acetonitrile were procured from Merck, Mumbai, India. Hydrochloric acid and sodium hydroxide were procured from Ranbaxy, India. All other chemicals were procured from HIMEDIA, India.

2.2 Equipment

A B.O.D incubator with rotary shaker, a UV laminar flow hood, a hot air oven, a water bath (Bhattacharya & Co., Kolkata, India), a microfiltration unit along with cellulose acetate membrane of 47 mm diameter and 0.1 μm pore size, a sonicator (Sartorius AG, Göttingen, Germany), a magnetic stirrer, a refrigerated centrifuge (lower limit of temperature is -10°C) (Remi Instruments Ltd., Mumbai, India), an autoclave (G.B. Enterprise, Kolkata, India) and a 5 L Jar fermenter (working volume 2 L) (Eyla, Japan) were used. The deionized water used in all the experiments was obtained from Arium 611DI ultrapure water system (Sartorius AG, Göttingen, Germany).

2.3 Analytical instruments

A digital pH meter, a digital balance machine (Sartorius AG, Göttingen, Germany), high performance liquid chromatography (HPLC), equipped with RI detector and Spheri 5 amino column (5 μm, 4.6 mm×220 mm) (Perkin Elmer, Series 200) and a VARIAN UV-Visible spectrophotometer (Cary50 Bio) were used.

2.4 Methods

2.4.1 Isolation and screening of bacterial strains

Bacterial strains capable of producing β-galactosidase were isolated from casein whey by conventional serial dilution method and repetitive streaking on modified deMan Rogosa and Sharpe (MMRS) agar medium (proteose peptone, 10.0 g/L; beef extract, 10.0 g/L; yeast extract, 5.0 g/L; polysorbate-80, 1.0 g/L; ammonium citrate, 2.0 g/L; sodium acetate, 5.0 g/L; magnesium sulphate, 0.1 g/L; manganese sulphate, 0.05 g/L; di-potassium phosphate, 2.0 g/L; bactriological agar, 18 g/L; lactose, 20.0 g/L). Final pH of the MMRS medium was adjusted 6.5±0.2 (at 25°C) by either 0.1 N sodium hydroxide or 0.1 N hydrochloric acid. Conventionally, deMan Rogosa and Sharpe (MRS) agar medium, where glucose is sole carbohydrate source, is used for isolation of lactic acid bacteria. As the ultimate goal of proposed investigation was isolation of most suitable baceria, potential for β-galactosidase synthesis, as well as lactose utilization, a short modification of the growth medium was adopted. In MRS medium glucose was replaced by lactose, known as MMRS. In serial dilution method, dilution level was maintained ranging from 10^{-1}-10^{-9}. Comparative batch studies on all isolated strains were conducted with respect to their withstanding capacity against thermal and pH shocks, as well as heavy metal resistance. The temperature and pH of each bacterial culture were varied in the range of 10-70°C and 4.5-12 respectively. The responses of each bacterial growth against temperature and pH were observed. Heavy metals, namely, chromium (Cr^{6+}), cadmium (Cd^{2+}), lead (Pb^{2+}), arsenic (As^{3+}), mercury (Hg^{2+}) and copper (Cu^{2+}) were individually added to the growth medium of each isolates. The concentration of each metal ion was varied in the range of 1.0-200.0 ppm. The MICs (minimum inhibitory concentration) of metallic ions for all cultures were determined by identifying the maximum concentration of heavy metals corresponding to extinction of cell growth. Based on the comparative performance of each bacterial isolate in response to variation of temperature, pH, as well as resistivity towards metallic ions, the most suitable bacterium was selected. The most suitable bacterium was characterized by standard biochemical assays [16]. Subsequently, the strain was identified by MTCC (Microbial Type Culture Collection & Gene Bank, of Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, India) through 16s rDNA analysis.

2.4.2 Growth medium preparation

2.4.2.1 Model whey preparation

Different sets of experiments were conducted using model whey in small bioreactors (Erlenmeyer flasks). The composition (per liter basis) of individual proteins, inorganic salts, vitamins in model whey medium is described in Table 1.

Table 1 Composition of model whey medium.

Ingredient	Concentration (g)
β-lactoglobulin	3.5
α-lactalbumin	1.4
Bovin serum albumin	0.4
IgG	0.2
IgA	0.3
IgM	0.1
Lactoperoxidase	0.06
Lactoferrin	0.05
Vitamin A	13
Thiamin	0.65
Pyridoxin	1.04
Riboflavin	3.25
Pantothenic acid	7.8
d-biotin	0.039
Cyanocobalamin	0.0065
Folic acid	5.2
Potassium di-hydrogen phosphate	0.94
di-potassium hydrogen phosphate	0.94
di-sodium hydrogen phosphate	0.234
Sodium di-hydrogen phosphate	0.234
calcium chloride	0.16
Ferrous sulphate	0.04
Cobalt sulphate	0.05
Copper sulphate	0.03
Nickel sulphate	0.04
Sodium acetate	5
Ammonium citrate	2
Manganese sulfate	0.05
Magnesium sulfate	0.05
Zinc sulphate	0.04

Initial lactose concentration in model whey medium was varied ranging from 5-200 g/L. Initial pH of the growth medium was adjusted by either 0.1 N sodium hydroxide or 0.1 N hydrochloric acid. Sterilization of all inorganic salts of the growth medium were done in an autoclave at 121°C for 15 min. Contradictorily, solution of lactose, vitamins and amino acids were sterilized using the microfiltration unit equipped with cellulose acetate membrane because of its sensitivity towards high temperature. After proper sterilization of all components, they were mixed together appropriately in sterile condition.

2.4.2.2 Real whey preparation

Experiments were also conducted in 5 L batch bioreactor (fermenter) with real casein whey. For experimental purpose, lactose concentrations in real whey were varied ranging from 5-50 g/L. Initially, the concentration of lactose in real whey was near about 40-42 g/L. Therefore, to maintain the lactose concentration 5-40 g/L, 0.05 M sodium phosphate buffer was used. Also to make the lactose concentration more than 40 g/L in real whey, appropriate amount of dehydrated lactose was dissolved in fermention medium. Sterilization of whey was performed by microfiltration unit equipped with cellulose acetate membrane.

2.4.3 Pre-culture

Adaptations of the strain to a medium containing high concentration of lactose (50 g/L) were performed by three times repetitive sub-culturing. The pre-culture process was conducted in an incubator at 37°C using 250 mL Erlenmeyer flasks for 1 day, based on sufficient growth (2.94×10^9 cfu/mL). The cell from the last adaptation experiment was stored for using the experiments conducted in the Erlenmeyer flask, as well as lab-scale batch fermenter.

2.4.4 Cell concentration determination

The concentration of cell was determined by dry cell weight method and CFU counting on agar plate [17].

2.4.5 Estimation of carbohydrate concentration of abiotic phase

The supernatant obtained after centrifugation of 20 mL culture broth was analyzed using the HPLC to determine the concentrations of lactose in the abiotic phase. Centrifugation was done at 10,000 rpm and 4°C for 15 min. The temperature of the HPLC column was maintained at 15°C. Acetonitrile 75% (v/v) was used as the mobile phase at a flow rate 1.67×10^{-8} m^3/s [18].

2.4.6 Estimation of concentration of β-galactosidase

The pellet of microbial biomass, obtained after centrifugation (10000 rpm and 4°C for 15 min) of 20 mL harvested culture was washed twice with 20 mL distilled water and was finally re-suspended in 5 mL, 0.05 M sodium phosphate buffer (pH 7.0). The cell pellet was sonicated at 16 kHz with a probe of 9.5 mm outer diameter using a constant power 400 W. For sonication purpose optimum sonication time was considered as 300 s. Inter stage cooling for 10 s was maintained after sonication period of 30 s [8, 9]. The sonicated cellular mass was centrifuged and the supernatant was assayed for β-galactosidase activity according to the Miller's method considering ONPG as a substrate [19]. In the present investigation the unit of activity of the enzyme in unit bacterial cell fluid is defined as the 'μkatal' (μmol/s) [20]. Concentration of β-galactosidase is described by activity of enzyme per unit volume of bacterial cell fluid.

2.4.7 β-galactosidase production in bench-top reactor

Experiments were also carried out in the bench-top 5 L bioreactor (working volume 2 L) under batch operation using the selected microorganism. Real casein whey obtained from a local dairy industry was used as the growth medium. The temperature, stirrer speed and pH were maintained at their optimum values (Temperature 37°C, stirrer speed 170 rpm, pH 7). Inoculation was done with the adapted culture and the initial inoculum size was maintained at 4.0% (v/v). Samples were withdrawn at every 2 hr intervals and the experiments were conducted until the cells entered into the stationary phase of growth. The samples were withdrawn from the bioreactor through a capillary needle and evacuated into sample tubes. The sample tubes were immediately placed in a refrigerator at 4°C for inactivation of cellular activities. The samples were analyzed for the determination of concentration of biomass, abiotic lactose and β-galactosidase.

2.4.8 Estimation of kinetic parameters

Different sets of batch experiments using the model whey medium were conducted in the 50 mL Erlenmeyer flasks to determine the kinetic parameters of model equations for microbial growth, lactose utilization and β-galactosidase production (Table 2). Initial lactose concentration in the microbial growth medium was varied in the range of 5-200 g/L. Inoculation was done with the adapted culture and the inoculum size was 4.0% (v/v). The optimum values of operating parameters, such as, rate of agitation = 170 r.p.m., incubation temperature = 37°C and initial pH = 7 were maintained for experiment purpose [21, 22].

2.4.8.1 Estimation of maintenance coefficients

The maintenance coefficient during the lag phase of microbial growth was determined by calculating the ratio of substrate consumption rate to the corresponding biomass concentration [23]. Therefore,

$$m_{lac} = \frac{1}{[c_x]}\left|\frac{d[c_{lac}]}{dt}\right| \quad (1)$$

2.4.8.2 Estimation of yield coefficients

The yield coefficient of biomass, $Y_{X/lac}$ and synthesized β-galactosidase, $Y_{\beta\text{-}gal/lac}$ with respect to substrate were determined. The yield coefficient, $Y_{X/lac}$ and $Y_{\beta\text{-}gal/lac}$ were determined using the mass of biomass grown, β-galactosidase formed, and lactose utilized over a period of 2 hr during the exponential growth [23]. Yield coefficients ($Y_{X/lac}$ and $Y_{\beta\text{-}gal/lac}$) may be defined as follows,

$$Y_{X/lac} = \frac{\Delta c_x}{|\Delta c_{lac}|} \quad (2)$$

$$Y_{\beta-gal/lac} = \frac{\Delta c_{\beta-gal}}{|\Delta c_{lac}|} \quad (3)$$

2.4.8.3 Estimation of specific substrate utilization rate

The experimental data of biomass and rate of decrease of abiotic lactose concentration in exponential phase of microbial growth have been used to determine the specific substrate utilization rate, q_{lac}, which is as follows [24].

$$q_{lac} = \frac{1}{[c_x]}.\left|\frac{d[c_{lac}]}{dt}\right| \quad (4)$$

2.4.8.4 Estimation of specific β-galactosidase production rate

The experimental data of biomass and rate of increase of concentration of β-galactosidase in exponential phase of microbial growth have been used to determine the specific β-galactosidase production rate, $q_{\beta\text{-}gal}$, which is as follows [23].

$$q_{\beta-gal} = \frac{1}{[c_x]}.\left|\frac{d[c_{\beta-gal}]}{dt}\right| \quad (5)$$

2.4.8.5 Determination of growth kinetic parameters

The values of maximum specific growth rate, $\mu_{max_{lac}}$ and the Monod constant, $K_{s_{lac}}$ were determined through regression analysis using the experimental data of specific growth rate, μ, and corresponding initial substrate concentration in microbial growth medium [23]. From the experimental results it was observed that microbial growth rate was inhibited by high lactose concentration (>20 g/L) and there was no microbial growth at lactose concentration 180 g/L in microbial growth medium. Therefore, critical concentration of lactose, [c_{lac}*] in microbial growth medium was considered to be 180 g/L [25-27]. The kinetic parameter of the logistic model, k, was evaluated through regression analysis for each batch type of experiment using the experimental values of initial specific growth rate, μ, and corresponding biomass concentration [11, 23, 33].

2.4.8.6 Determination of production kinetics of β-galactosidase

Constants of the Luedeking-Piret equation and the modified Luedeking-Piret equation, i.e. growth associated enzyme synthesis, $\alpha_{\beta\text{-}gal}$, and non growth associate enzyme synthesis, $\beta_{\beta\text{-}gal}$ were determined through regression analysis by different values of specific β-galactosidase production rate, $q_{\beta\text{-}gal}$, and specific growth rate, μ [11, 23]. Regression analysis by different initial values of $\left(\frac{1}{[c_{\beta-gal}]}.\frac{d[c_{\beta-gal}]}{dt}\right)$ and corresponding values of β-galactosidase concentration were attempted to estimate the kinetic constants of the Mercier equation [11, 28].

Table 2 Values of estimated kinetic parameters of model equation for biomass concentration, substrate utilization and β-galactosidase production, and the corresponding correlation coefficient (R^2).

Biomass concentration	Substrate utilization	β-galactosidase production
Classical Monod model (Without substrate and product inhibition) [23] $\mu = \frac{\mu_{max_{lac}}.[c_{lac}]}{K_{s_{lac}}+[c_{lac}]}$ μ_{max} = 0.75 hr^{-1}, $K_{s_{lac}}$ = 12 g/L, R^2 = 0.98 (for $[c_{lac}]_0$ = 5-200 g/L)	Classical Monod substrate utilization model [24] $\frac{d[c_{lac}]}{dt} = -\frac{1}{Y_{X/_{lac}}}.\frac{\mu_{max_{lac}}.[c_{lac}].[c_x]}{K_{s_{lac}}+[c_{lac}]}$ $Y_{X/lac}$ = 0.14, R^2 = 0.99 (for $[c_{lac}]_0$ = 5-20 g/L)	Monod incorporated modified Luedeking-Piret model $\frac{d[c_{\beta-gal}]}{dt} = \alpha_{\beta-gal}.\frac{\mu_{max_{lac}}.[c_{lac}].[c_x]}{K_{s_{lac}}+[c_{lac}]}$ $\alpha_{\beta\text{-}gal}$= 12.76 (µkatal/L bacterial cell fluid). (L/g biomass), R^2 = 0.99 (for $[c_{lac}]_0$ = 5-20 g/L)
Substrate inhibited Monod model [25-27] $\mu = \frac{\mu_{max_{lac}}.[c_{lac}]}{K_{s_{lac}}+[c_{lac}]}.\left(1-\frac{[c_{lac}]}{[c_{lac}^*]}\right)^m$ $\mu_{max_{lac}}$ = 0.75 hr^{-1}, $K_{s_{lac}}$ = 12 g/L, R^2 = 0.98; m = 2.7, $[c_{lac}^*]$= 180 g/L, R^2 = 0.98 (for $[c_{lac}]_0$ > 20 g/L)	Substrate inhibited Monod substrate utilization model $\frac{d[c_{lac}]}{dt} = -\frac{1}{Y_{X/_{lac}}}.\frac{\mu_{max_{lac}}.[c_{lac}].[c_x]}{K_{s_{lac}}+[c_{lac}]}.\left(1-\frac{[c_{lac}]}{[c_{lac}^*]}\right)^m$ $Y_{X/lac}$ = 0.14; R^2 = 0.99 (for $[c_{lac}]_0$ >20 g/L)	Monod incorporated Luedeking-Piret substrate inhibited model $\frac{d[c_{\beta-gal}]}{dt} = \alpha_{\beta-gal}.\frac{\mu_{max_{lac}}.[c_{lac}].[c_x]}{K_{s_{lac}}+[c_{lac}]}.\left(1-\frac{[c_{lac}]}{[c_{lac}^*]}\right)^m + \beta_{\beta-gal}.[c_x]$ $\alpha_{\beta\text{-}gal}$= 4.27 (µkatal/L bacterial cell fluid). (L/g biomass), $\beta_{\beta\text{-}gal}$= 1.82 (µkatal/ L bacterial cell fluid).(L/(g biomass. hr)), R^2 = 0.98 (for $[c_{lac}]_0$ >20 g/L)
Logistic growth Model [11, 23, 33] $\mu = k.\left(1-\frac{[c_x]}{[c_x]_\infty}\right)$ k = 0.261 hr^{-1}, $[c_x]_\infty$= 0.43 g/L, R^2 = 0.98 (for $[c_{lac}]_0$= 5 g/L); k = 0.362 hr^{-1}, $[c_x]_\infty$= 1.3 g/L, R^2 = 0.99 (for $[c_{lac}]_0$ = 10 g/L)	Logistic substrate utilization model [33] $\frac{d[c_{lac}]}{dt} = -\frac{1}{Y_{X/_{lac}}}.k.\left(1-\frac{[c_x]}{[c_x]_\infty}\right).[c_x]$ $Y_{X/lac}$= 0.14; R^2 = 0.99 (for $[c_{lac}]_0$ = 5-10 g/L)	Logistic incorporated modified Luedeking-Piret model [11] $\frac{d[c_{\beta-gal}]}{dt} = \alpha_{\beta-gal}.k.\left(1-\frac{[c_x]}{[c_x]_\infty}\right).[c_x]$ $\alpha_{\beta\text{-}gal}$= 12.76 (µkatal/L bacterial cell fluid). (L/g biomass), R^2 = 0.99 (for $[c_{lac}]_0$= 5-10 g/L)
	Logistic incorporated modified Luedeking-Piret substrate utilization model $\frac{d[c_{lac}]}{dt} = -\left(\frac{1}{Y_{X/_{lac}}}+\frac{\alpha_{\beta-gal}}{Y_{\beta-gal/_{lac}}}\right).\frac{d[c_x]}{dt} - m_{lac}.[c_x]$ $Y_{X/lac}$ = 0.14, R^2 = 0.99; $Y_{\beta\text{-}gal}$= 1.55 (µkatal/L bacterial cell fluid)/ (g lactose /L microbial growth medium), R^2 = 0.98; $\alpha_{\beta\text{-}gal}$= 12.76 (µkatal/L bacterial cell fluid). (L/g biomass), m_{lac} = 0.0021 hr^{-1}, R^2 = 0.99 (for $[c_{lac}]_0$= 5-10 g/L)	Logistic incorporated Luedeking-Piret model [11] $\frac{d[c_{\beta-gal}]}{dt} = \alpha_{\beta-gal}.k.\left(1-\frac{[c_x]}{[c_x]_\infty}\right).[c_x] + \beta_{\beta-gal}.[c_x]$ $\alpha_{\beta\text{-}gal}$= 4.27 (µkatal/L bacterial cell fluid). (L/g biomass), $\beta_{\beta\text{-}gal}$= 1.82 (µkatal/ L bacterial cell fluid).(L/(g biomass. hr)), R^2 = 0.98 (for $[c_{lac}]_0$ = 5-10 g/L)
	Logistic incorporated Luedeking-Piret substrate utilization model [11] $\frac{d[c_{lac}]}{dt} = -\left(\frac{1}{Y_{X/_{lac}}}+\frac{\alpha_{\beta-gal}}{Y_{\beta-gal/_{lac}}}\right).\frac{d[c_x]}{dt} - \frac{\beta_{\beta-gal}.[c_x]}{Y_{\beta-gal/_{lac}}} - m_{lac}.[c_x]$ $Y_{X/lac}$ = 0.14, R^2 = 0.99; $Y_{\beta\text{-}gal}$= 1.55 (µkatal/L bacterial cell fluid)/ (g lactose /L microbial growth medium), R^2 = 0.98; $\alpha_{\beta\text{-}gal}$= 4.27 (µkatal/L bacterial cell fluid). (L/g biomass), $\beta_{\beta\text{-}gal}$= 1.82 (µkatal/ L bacterial cell fluid).(L/(g biomass. hr)), R^2 = 0.98; m_{lac} = 0.0021 hr^{-1}, R^2 = 0.99 (for $[c_{lac}]_0$ = 5-10 g/L)	Mercier model [11, 28] $\frac{d[c_{\beta-gal}]}{dt} = [c_{\beta-gal}]_r.[c_{\beta-gal}].\left(1-\frac{[c_{\beta-gal}]}{[c_{\beta-gal}]_{max}}\right)$ $[c_{\beta\text{-}gal}]$= 0.364 hr^{-1}, $[c_{\beta\text{-}gal}]_{max}$= 5.01 (µkatal/L bacterial cell fluid) R^2 = 0.9 (for $[c_{lac}]_0$ = 5 g/L); $[c_{\beta\text{-}gal}]$= 0.402 hr^{-1},$[c_{\beta\text{-}gal}]_{max}$= 16.31 (µkatal/L bacterial cell fluid) R^2 = 0.88 (for $[c_{lac}]_0$= 10 g/L); $[c_{\beta\text{-}gal}]$= 0.475 hr^{-1},$[c_{\beta\text{-}gal}]_{max}$= 26.38 (µkatal/L bacterial cell fluid) R^2 = 0.86 (for $[c_{lac}]_0$ = 15 g/L); $[c_{\beta\text{-}gal}]$= 0.518 hr^{-1}, $[c_{\beta\text{-}gal}]_{max}$= 35.08 (µkatal/L bacterial cell fluid) R^2 = 0.86 (for $[c_{lac}]_0$= 20 g/L)
	Logistic incorporated Mercier substrate utilization model [11] $\frac{d[c_{lac}]}{dt} = -\left(\frac{1}{Y_{X/_{lac}}}.\frac{d[c_x]}{dt} + \frac{1}{Y_{\beta-gal/_{lac}}}.\frac{d[c_{\beta-gal}]}{dt}\right)$ $Y_{X/lac}$ = 0.14, R^2 = 0.99; $Y_{\beta\text{-}gal}$= 1.55 (µkatal/L bacterial cell fluid)/ (g lactose /L microbial growth medium), R^2 = 0.98 (for $[c_{lac}]_0$ = 5-10 g/L)	

In all cases non-linear regression analysis was performed by MATLAB 7.0.

All the experiments were performed in triplicate.

3 Theoretical analysis

The mathematical model of any fermentation process is the relationship among substrate consumption, biomass growth and product formation. To describe the microbial growth, substrate utilization and β-galactosidase production by proposed consortium, the present research group developed a non-segregated unstructured mathematical model based on the following assumptions.

3.1 Assumptions

1. No biomass is produced in the lag phase of microbial growth.
2. Lactose is the only growth limiting substrate.

The differential mass balance equations for concentration of different components, namely, biomass, lactose, and β-galactosidase for the large scale reactor may be written as follows;

3.2 Biomass generation

$$\frac{d[c_x]}{dt} = 0 \qquad \text{for } 0 \le t \le t_{lag} \tag{6}$$

$$\frac{d[c_x]}{dt} = \mu \cdot [c_x] \qquad \text{for } t > t_{lag} \tag{7}$$

3.3 Lactose utilization

$$\frac{d[c_{lac}]}{dt} = -m_{lac} \cdot [c_x] \qquad \text{for } 0 \le t \le t_{lag} \tag{8}$$

$$\frac{d[c_{lac}]}{dt} = -\frac{1}{Y_{X/lac}} \frac{d[c_x]}{dt} \qquad \text{for } t > t_{lag} \tag{9}$$

3.4 β-galactosidase synthesis

$$\frac{d[c_{\beta-gal}]}{dt} = 0 \qquad \text{for } 0 \le t \le t_{lag} \tag{10}$$

$$\frac{d[c_{\beta-gal}]}{dt} = q_{\beta-gal} \cdot [c_x] \qquad \text{for } t > t_{lag} \tag{11}$$

The initial time, i.e., t = 0;

$$[c_x] = [c_{x_0}],\ [c_{lac}] = [c_{lac_0}],\ [c_{\beta-gal}] = [c_{\beta-gal_0}]$$

3.5 Simulation and statistical analysis

The above unstructured model describes the dynamic behavior of the system. The model involves set of ordinary differential equations of the following form,

$$\frac{d\bar{C}}{dt} = f(\bar{C}, \bar{P}) \tag{12}$$

where, $\bar{C}$ is a vector of concentrations of different components under the consideration and $\bar{P}$ is a vector of model parameters. The parameters of the most suitable growth, substrate utilization and β-galactosidase production kinetics have been used during simulation using the proposed model. The mass balance equations of different components of the system have been solved using the 4th order Runge-Kutta method by MATLAB 7.0. Validity of the model has also been established by comparing the predicted data with the experimental ones using real casein whey as a growth medium. The accuracy of the proposed model has also been assessed by the evaluation of M_{SE} values [29, 30].

4 Results and Discussion

4.1 Selection of suitable microbe and subsequent characterization

Following the procedure mentioned in Section 2.4.1, three bacteria, namely, IB1, IB2 and IB3 were isolated. The potential for β-galactosidase production, tolerance of temperature, pH and resistance against heavy metals have been assessed for three isolated bacteria, IB1, IB2 and IB3. These are reported in Table 3.

Table 3 Tolerance limit of temperature, pH, β-galactosidase production and MIC values of heavy metals for isolated bacteria.

	IB1	*IB2*	*IB3*
β-galactosidase activity (μkatal)	42.27	48.69	40.25
Temperature tolerance (°C)	4-45	20-40	25-40
pH tolerance	4.5-12	5.5-7.5	5.5-7.2
MIC (ppm)			
Cr^{+6}	100	<1.0	<1.0
Pb^{+2}	1500	<1.0	<1.0
Cd^{+2}	50	<1.0	<1.0
As^{+5}	1500	<1.0	<1.0
Hg^{+2}	1.0	<1.0	<1.0
Cu^{+2}	1500	5.0	1.0

Although it is observed that the β-galactosidase activity is similar for all types of isolated bacteria, IB1 is the more tolerant to temperature and pH shocks and has the maximum resistance to different heavy metal ions. Therefore, IB1 has been selected for further studies to produce β-galactosidase in a large bioreactor.

Subsequently, morphological, biochemical and genetic analysis of IB1 were performed. Colonies of the IB1 bacterium are round, undulated, dull white, no luminescent and has irregular margin on nutrient agar plate. They are rod shaped and gram positive. Cells are 0.5-0.7 μm in diameter and 1.2-1.4 μm in length during log phase. It has been observed that IB1 is not motile, i.e., no cilia, flagella, pili, or fimbriae are present. Also, IB1 forms spore during adverse conditions. The results of biochemical assays of IB1 are reported in Table 4.

Table 4 Biochemical characteristics of isolated IB1.

Biochemical characteristics	Result
Catalase	+
Oxidase	+
Nitrate reduction	-
Growth in N_2 free Asby's medium	+
Indole	-
Methyl red	-
Voges proskauer	-
Citrate	+
Sulfate reduction	-
Amylase	+
Casein hydrolysis	+
Urease	-
Cellulase	+
Alkaline phosphatase	+
Acid phosphatase	-
DNase	-
RNase	-
de-amination of tryptophan, lysine, ornithin, L-phenyl alanine	-
de-carboxylation of tryptophan, lysine, ornithin, L-phenyl alanine	+

N.B. +: Positive result, -: Negative result

According to the literature review, the present isolated bacterium IB1 is very much similar with *Bacillus safensis* (13 sub sp.), as well as, *Bacillus pumilus* (13 sub sp.). It has been reported that the cell size and cell morphology of *Bacillus safensis* and *Bacillus pumilus* are totally similar with isolated bacterium IB1. Moreover, all of them are aerobic. Similar with *Bacillus safensis* and *Bacillus pumilus,* present isolated bacterium IB1 is also spore forming. All of *Bacillus safensis* can grow upto 10% (w/v) NaCl concentration and able to grow at the temperature range 10–50°C, which is very similar with present isolated bacterium IB1. On agar plate their colonies are round, undulate, dull white, non-luminescent and have irregular margins. It has been reported that all of *Bacillus safensis* are able to synthesis oxidase, catalase, β-galactosidase, β-glucosidase, alkaline phosphatase. Contradictorily, they are not able to produce H_2S, urease, DNase and indole. They have negative results on de-amination of amino acids (tryptophan, phenylalanine, arginine, lysine and ornithine). All of *Bacillus safensis* are not able to reduce nitrate. Casein hydrolysis varies among strains. Similar with IB1, all *Bacillus pumilus* are able to synthesis lipase and they are able to casein hydrolysis [31]. Some other pioneering researcher isolated *Bacillus safensis* from saline desert area in Gujarat, India. They reported that *Bacillus safensis* is able to synthesis protease, lipase, chitinase, and pectinase [32]. Utilization of triple sugar by IB1 is described in Table 5.

Table 5 Triple sugar utilization of IB1.

Time	Butt	Slant
24 hr	+	++
48 hr	++	++

N.B. ++: Rapid acid production, +: Slow acid production

The results are reported in Table 5, signify that isolated bacterium IB1 has a high affinity to grow in presence of oxygen (slant turns into yellow within 24 hr due to faster acid production), although it can grow in anaerobic condition (Butt turns into orange yellow from initial red within 24 hr due to slower acid production). The growth of isolated bacterium IB1 is slow under anaerobic condition than in presence of oxygen. Therefore, the present isolated bacterium considered to be a facultative anaerobe. Fermentation of different types carbohydrates by IB1 for different time periods are reported in Table 6.

It has been observed that isolated bacterium cannot produce any gas during carbohydrate fermentation and sulfate reduction test is also found negative. Finally, isolated bacterium IB1 was identified with respect to genus and species by 16s rDNA analysis. The genus and species of IB1 were identified by MTCC (Microbial Type Culture Collection & Gene Bank, of Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, India) as *Bacillus safensis* (*JUCHE 1*).

4.2 Selection of microbial growth kinetics

In Table 2, values of the parameters of different kinetic equations for biomass growth are reported along with estimated correlation coefficients (R^2). The kinetic parameters of the Monod model, namely, maximum specific growth rate, μ_{max} and the saturation constant, K_s could be determined using the experimental data obtained using initial abiotic lactose concentration of 5-50 g/L. The parameter m of the substrate inhibited Monod model has been evaluated beyond the initial substrate concentration 20 g/L where inhibition is encountered. From Table 2, it is evident that good correlation coefficient (R^2) (more than 0.95) have been obtained for estimated parameters of the classical Monod model (without substrate and product inhibition)

Table 6 Fermentation characteristics of different carbohydrates by IB1.

Name of Carbohydrate	Incubation time		
	24 Hour	48 Hour	72 Hour
Maltose	-	+	+
Manitol	+	+	+
Sucrose	+	+	+
Fructose	+	+	+
Ribose	+	+	+
Galactose	-	+	+
Manose	+	+	+
Lactose	-	-	-
Arbinosol	-	-	-
D Xylose	-	+	+
D Dextrose	+	+	+
Adonitol	-	-	-
Rhamnose	-	-	-
L Arabinose	+	+	+
Sorbitol	-	-	-
Inositol	+	+	+
Cellobiose	+	+	+
Erytritol	-	-	-
D Arabinose	-	-	-
L Xylose	-	-	-
Sorbose	-	-	-
Innulin	-	-	-
Raffinose	-	-	-
Xylitol	-	-	-
D Fucose	-	-	-
L Fucose	-	-	-
D Arabitol	-	-	-
L Arabitol	-	-	-

N.B. +: Acid production, -: No acid production

and the substrate inhibited Monod model in the low (5-20 g/L), and high (> 20 g/L) concentration ranges of lactose in growth medium respectively. The values of kinetic constants of the logistic growth model could be evaluated up to 20 g/L initial lactose concentration in microbial growth medium. Beyond this concentration, the rate constant of logistic model, k could not be determined. Good correlation coefficient (more than 0.98) of rate constant of the logistic growth model, k for abiotic initial lactose concentration 5-10 g/L has been obtained. This is also in agreement with the observation of F. Kargi (2009) who showed that the logistic growth model is actually the first order form of the classical Monod model (without substrate and product inhibition) and is valid at low initial substrate concentration ($[c_s] << K_s$) in microbial growth medium [33].

In order to study the effect of initial substrate concentration on microbial growth, in Fig. 1 the simulated values of specific growth rate, μ, based on the different growth kinetic models as the ordinate and initial concentration of lactose in abiotic phase as the abscissa are plotted. In the same Figure, experimental data points are superimposed with simulated results.

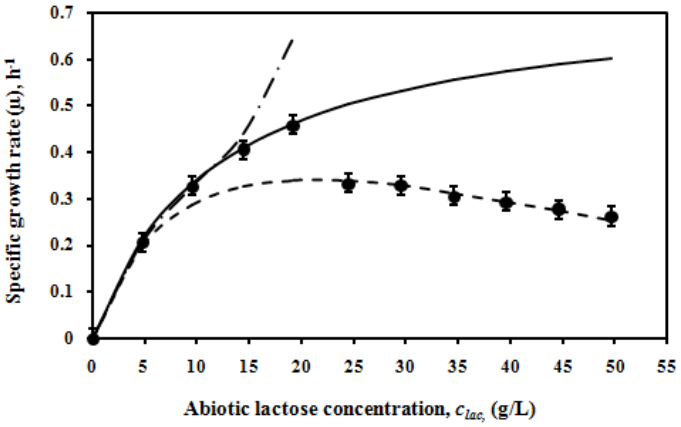

Fig. 1 Simulated and experimental values of specific growth rate, μ of consortium for different lactose concentration in microbial growth medium. Simulated; lines, Experimental; points. Classical Monod model (without substrate, and product inhibition) (——), Substrate inhibited Monod model (▪▪▪▪▪), Logistic growth model (— · -), and experimental (●).

The M_{SE} values were also calculated and reported in Table 2A (Supplementary section) to justify the model fit. It is notified that M_{SE} value is very low (10^{-4}-10^{-5}) for the classical Monod model (without substrate and product inhibition) at low initial substrate concentration (5-20 g/L) in microbial growth medium. Contradictorily, lower values of M_{SE} for the substrate inhibited Monod model (M_{SE} value in order to 10^{-4}-10^{-5}) are obtained in the regime of high initial lactose concentration (> 20 g/L). This may be due to the presence of steric hindrance of lactose specific enzyme, caused by overloading of lactose molecules at higher range of initial lactose concentration in microbial growth medium. At high concentration range of substrate, only a fraction of substrate may be bind with the active site of growth limiting enzyme, namely, hexokinase which lead to further metabolic activity, as well as cell growth, while the other active side of hexokinase may be overloaded by remaining fraction of substrate, produces inactive complex, leads to substrate inhibition (Fig. 2).

$$[E] + [S] \underset{}{\overset{k_s}{\rightleftharpoons}} [ES] \overset{k_p}{\rightleftharpoons} [E] + [P]$$
$$+$$
$$[S]$$
$$\Updownarrow K_i$$
$$[ES_2]$$

Fig. 2 Intracellular enzyme substrate reaction.

Intracellular lactose is hydrolyzed by β-galactosidase to form glucose and galactose. At high concentration intracellular glucose or galactose may also inhibit the formation of glucose-6p, responsible for biomass formation [34, 35]. At initial substrate concentration 5-10 g/L $\left(\left[c_{S_{lac}}\right] << K_{S_{lac}}\right)$ in microbial growth medium both the logistic growth model and the classical Monod model (without substrate and product inhibition) can explain the microbial growth adequately (M_{SE} value in order to 10^{-4}). While the kinetic parameter of semi-empirical logistic equation, k depends upon the values of initial substrate concentration $\left[c_{s_o}\right]$, the parameters, μ_{max} and K_s of mechanistic Monod kinetics are unique in nature. The range of validity of the classical Monod model (without substrate and product inhibition) is also wider (5-20 g/L) than that of the logistic growth model. Therefore, the classical Monod kinetics (without substrate and product inhibition) and substrate inhibited Monod kinetics are respectively selected to predict the growth of *Bacillus safensis (JUCHE 1)* in the low (5-20 g/L) and high (> 20 g/L) substrate concentration regimes.

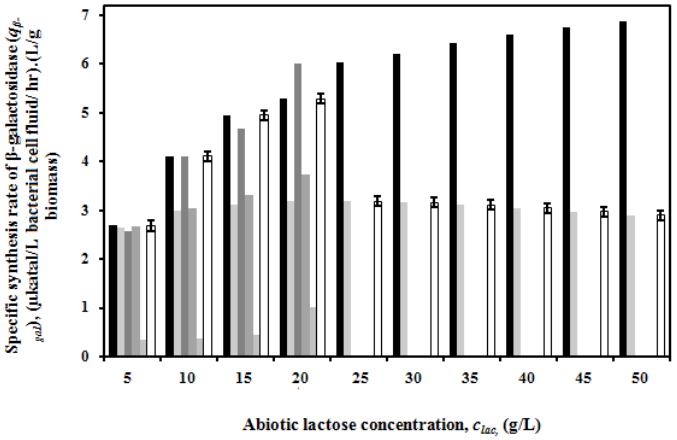

Fig. 3 Simulated and experimental values of specific β-galactosidase production rate, $q_{\beta\text{-gal}}$ for different lactose concentration in microbial growth medium. Simulated values of $q_{\beta\text{-gal}}$ based on the Monod incorporated modified Luedeking-Piret model (■), the Monod incorporated Luedeking-Piret model (□), the logistic incorporated modified Luedeking-Piret model (■), the logistic incorporated Luedeking-Piret substrate inhibited model (■), the Mercier model (■), and experimental (□).

4.3 Selection of β-galactosidase production kinetics

In Table 2, the kinetic parameters of the proposed model equations for β-galactosidase production are reported along with estimated correlation coefficient (R^2). The kinetic parameters of the Mercier model could not be determined above 20 g/L initial substrate concentration in microbial growth medium. High value of growth associated constant, $\alpha_{\beta\text{-}gal}$ of the Monod incorporated modified Luedeking-Piret model in the lower initial substrate concentration range (5-20 g/L) in microbial growth medium indicates that production of intracellular β-galactosidase is strongly growth associated in this regime. In contrast, the positive values of $\alpha_{\beta\text{-}gal}$ and $\beta_{\beta\text{-}gal}$ at high initial concentration of lactose (>20 g/L) in microbial growth medium signify that synthesis of intracellular β-galactosidase is both associated with growth, and non growth in this regime. Lower value of $\alpha_{\beta\text{-}gal}$ in this regime (>20 g/L) may signify the substrate inhibition on production of intracellular β-galactosidase. As β-galactosidase is an intracellular enzyme, its biosynthesis is strongly dependent upon biomass growth, as well as, lactose concentration in growth medium. Lactose is a natural inducer of biosynthesis of β-galactosidase from *lac operon* [36]. The inhibited growth of *Bacillus safensis (JUCHE 1)* at high substrate concentration results the decrease of β-galactosidase production. From Table 2, it is manifested that good correlation coefficient (R^2) (more than 0.95) have been obtained for estimated parameters of the Monod incorporated modified Luedeking-Piret model in the lower initial substrate concentration (5-20 g/L) and the Monod incorporated Luedeking-Piret model with substrate inhibition in the high concentration (> 20 g/L) ranges of lactose in growth medium respectively. To describe the Model fit, a bar plot is constructed in Fig. 3 with the experimental and the simulated values of specific β-galactosidase production rate, $q_{\beta\text{-}gal}$, against concentration of lactose in abiotic phase.

Moreover to indicate the strength of fitness of the β-galactosidase production kinetics, M_{SE} values, calculated by comparing with experimental and theoretical results of specific β-galactosidase production rate, $q_{\beta\text{-}gal}$ have been evaluated for different initial lactose concentration in microbial growth medium (Table 2B, Supplementary section). Low M_{SE} values for the Monod incorporated modified Luedeking-Piret model (M_{SE} value in order to 10^{-4}) and the Monod incorporated Luedeking-Piret model with substrate inhibition (M_{SE} value in order to 10^{-4}) indicate their validity at lower (5-20 g/L) and higher (> 20 g/L) concentration regime of initial lactose concentration in microbial growth medium respectively. Similar to the observations obtained in case of biomass formation, the logistic incorporated modified Luedeking-Piret model is able to describe the intracellular β-galactosidase production by proposed consortium at initial substrate concentration 5-10 g/L in microbial growth medium (M_{SE} value in order to 10^{-4}). Therefore, the Monod incorporated modified Luedeking-Piret model and the Monod incorporated Luedeking-Piret model with substrate inhibition have been selected to explain the β-galactosidase production kinetics at low (5-20 g/L) and high (> 20 g/L) lactose concentration in growth medium.

4.4 Selection of substrate utilization kinetics

In Table 2, the values of kinetic parameters of different substrate utilization models, such as, $Y_{X/lac}$, $Y_{\beta\text{-}gal}$ and m_{lac} are reported along with correlation coefficients (R^2). To describe the Model fit, a bar plot is constructed in Fig. 4 with the experimental and the simulated values of specific substrate utilization rate, q_{lac}, based on proposed substrate utilization models against concentration of lactose in abiotic phase.

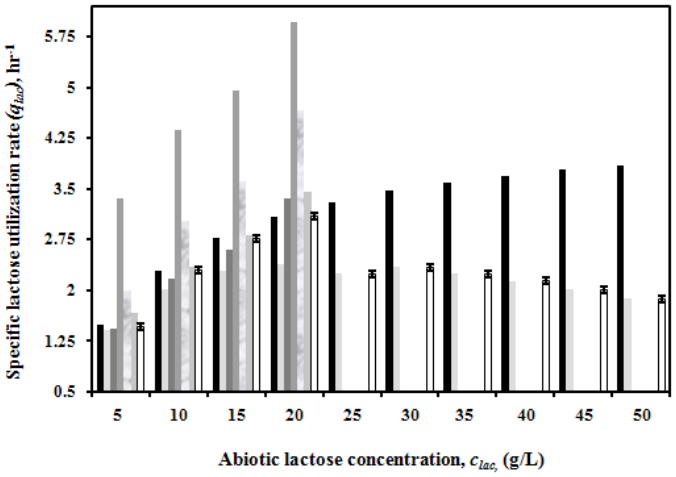

Fig. 4 Simulated, and experimental values of specific substrate utilization rate, q_{lac} for different lactose concentration in microbial growth medium. Simulated values of q_{lac} based on the classical Monod substrate utilization model (■), the substrate inhibited Monod substrate utilization model (□), the logistic substrate utilization model (▩), the logistic incorporated Luedeking-Piret substrate utilization model (▨), the logistic incorporated Mercier substrate utilization model (▩), the logistic incorporated modified Luedeking-Piret substrate utilization model (▨), and experimental (□).

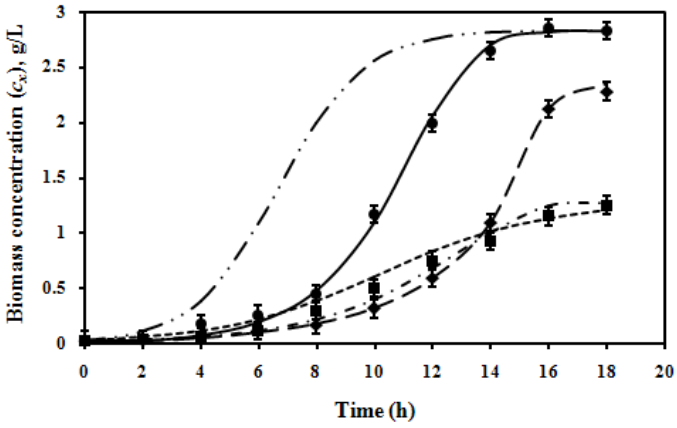

Fig. 5 Simulated (lines), and experimental (points) time histories of concentration of biomass, c_x, at optimum condition (incubation temperature 37°C, stirrer speed 170 r.p.m., and pH 7). Lines: for the classical Monod model (——), and for the logistic model (— · ·) at initial lactose concentration 20 g/L in the medium, for the classical Monod model (— · —), and for the logistic model (– – – –) at initial lactose concentration 10 g/L in the medium, for the substrate inhibited Monod model (— —) at initial lactose concentration 40 g/L in the medium; Points: for initial lactose concentration 20 g/L in the medium (●), for initial lactose concentration 10 g/L in the medium (■), for initial lactose concentration 40 g/L in the medium (♦).

Additionally, to indicate the strength of substrate utilization kinetics, M_{SE} values, calculated by comparing with experimental and theoretical results of specific substrate utilization rate, q_{lac}, have been evaluated for different initial lactose concentration in microbial growth medium (Table 2C, Supplementary section). Lower M_{SE} values of the classical Monod substrate utilization model (without substrate and product inhibition) (M_{SE} value in order to 10^{-4}) and the substrate inhibited lactose utilization model (M_{SE} value in order to 10^{-4}) for low, and high concentration regimes of substrate concentration in microbial growth medium respectively depict the best agreement with experimental data. This indicates that the substrate consumption is mostly associated with biomass growth, whereas, the Monod incorporated Luedeking Piret model is not able to predict the substrate consumption rate. Expectedly, it has been found that the logistic substrate utilization model can explain the substrate utilization at initial abiotic lactose concentration 5-10 g/L in microbial growth medium (M_{SE} value in order to 10^{-4}). Therefore, the Monod type substrate utilization model and that with substrate inhibition are selected to represent lactose utilization kinetics in low and high concentration of lactose respectively.

4.5 Model validation for 5L bioreactor

To validate the mathematical model for the large scale (5 L) bioreactor, experimental time histories of concentrations of biomass, abiotic lactose and β-galactosidase in the reactor have been compared with the simulated ones. The real casein whey was used in this bioreactor. Similar with previous, lactose concentrations in real whey were varied ranging from 5-50 g/L. In Figure 5, experimental and simulated time histories concentrations of biomass are depicted.

From the figure, it is evident that the concentration of biomass increases with the progress of batch time. As expected, growth curve of proposed consortium shows a typical sigmoidal nature involving a lag phase, an exponential phase and a stationary phase, similar to previous observations on microbial growth [37-39]. It reveals that the lag phase of *Bacillus safensis (JUCHE 1)* extends upto 3 hr, followed by the exponential phase which eventually reached the asymptote and finally the stationary phase.

In Figure 6, experimental and simulated time histories of concentrations of substrate (lactose) are depicted.

All the observations have good agreement with different substrate utilization models as discussed in earlier. The pattern of time histories of residual lactose concentration in microbial growth medium, as shown in Fig. 6, is in agreement with the trend of biomass concentration. It is observed that lactose concentration in microbial growth medium reduces monotonically with batch time progress.

In Figure 7, experimental and simulated time histories of concentrations of β-galactosidase are portrayed.

All the findings have in good agreement with the different β-galactosidase synthesis models as discussed in previous. It is observed that with the progress of time the concentration of β-galactosidase increases monotonically and the production of β-galactosidase is low for initial concentration of lactose 40 g/L in comparison with initial concentration of lactose 20 g/L in microbial growth medium. The pattern of time histories of concentration of β-galactosidase, as shown in Fig. 7, is in agreement with the trend of biomass concentration.

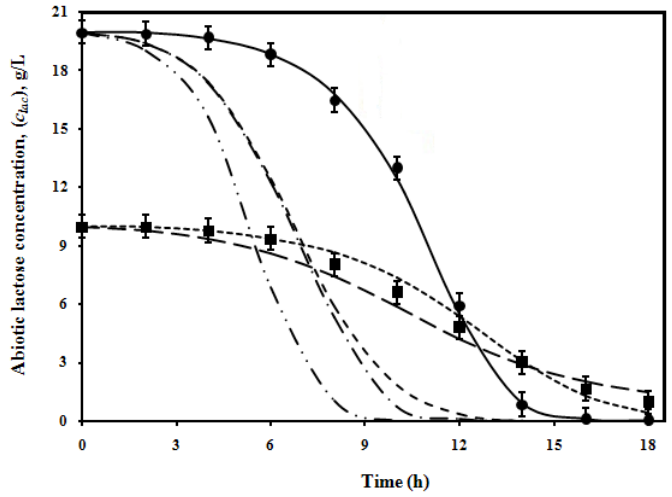

Fig. 6 Simulated (lines), and experimental (points) time histories of concentration of lactose in microbial growth medium, c_{lac}, at optimum condition (incubation temperature 37°C, stirrer speed 170 r.p.m., and pH 7). Lines: for the classical Monod substrate utilization model (——), for the logistic incorporated Luedeking-Piret substrate utilization model (— · ·), for the logistic incorporated Mercier substrate utilization model (— · —), and for the logistic substrate utilization model (— — —) at initial lactose concentration 20 g/L in the medium, for the classical Monod substrate utilization model (- - - - -), and for the logistic substrate utilization model (— —) at initial lactose concentration 10 g/L in the medium; Points: for initial lactose concentration 20 g/L in the medium (●), and for initial lactose concentration 10 g/L in the medium (■).

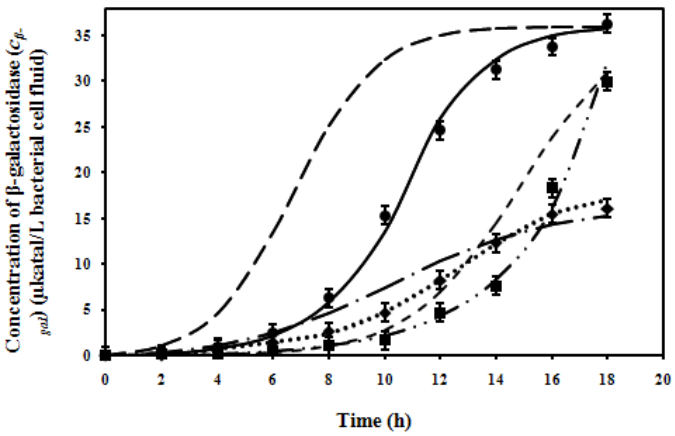

Fig. 7 Simulated (lines), and experimental (points) time histories of concentration of β-galactosidase, $c_{β\text{-}gal,}$ (μkatal/L) at optimum incubation temperature 37°C, stirrer speed 170 r.p.m., and pH 7. Lines: for the Monod incorporated modified Luedeking-Piret model (——), the Mercier model (— · —), and for the logistic incorporated modified Luedeking-Piret model (— — —) at initial lactose concentration 20 g/L in the medium, for the logistic incorporated modified Luedeking-Piret model (- - - - -), and for the Monod incorporated modified Luedeking-Piret model (— · ·) at initial abiotic lactose concentrations 10 g/L in the medium, and for the Monod incorporated Luedeking-Piret model (— —) at initial abiotic lactose concentrations 40 g/L in the medium; Points: for initial lactose concentration 10 g/L in the medium (■), for initial lactose concentration 20 g/L in the medium (●), and for initial lactose concentration 40 g/L in the medium (■).

Low M_{SE} values for selected kinetic model signify that accuracy of choice of kinetic model equations to describe the system dynamics. It is expected that the proposed methodology for the production of β-galactosidase could be viable in industrial scale and would minimize the environmental pollution from dairy disposal. The valid mathematical model can be utilized to scale up the bioreactor to industrial scale and to decide the control strategies of the reactor for production of β-galactosidase from casein whey using *Bacillus safensis (JUCHE 1)*.

5 Conclusions

Under the present investigation three bacterial strain IB1, IB2 and IB3 capable of β-galactosidase synthesis have been isolated from casein whey. Among them, *Bacillus safensis (JUCHE 1)* has been screened from all three bacteria based on its high tolerance limits of temperature and pH shocks, as well as heavy metal resistance. Different models for biomass growth, substrate utilization and β-galactosidase production have been attempted to explain the experimental data of small bioreactors (Erlenmeyer flasks) run by batch operation. At lower range of initial lactose concentration (5-20 g/L) in growth medium, the classical Monod model (without substrate and product inhibition) and the Monod incorporated modified Luedeking-Piret model can adequately describe the biomass formation, and production of β-galactosidase respectively. It was observed that the Monod substrate utilization model has good agreement to describe the substrate utilization kinetics at the entire range of substrate concentration. At higher range of initial lactose concentration (> 20 g/L) in growth medium, the substrate inhibited Monod model and the Monod incorporated Luedeking-Piret model can effectively explain the biomass formation and production of β-galactosidase respectively. In the interim, at initial lactose concentration 5-10 g/L (< Ks) in growth medium the logistic growth model, the logistic substrate utilization model and the logistic incorporated modified Luedeking-Piret model have the good agreement with experimental ones. Based on the successful comparison with experimental data of Erlenmeyer flask, the kinetic model for biomass growth, substrate utilization and β-galactosidase production have been successfully applied to understand the system dynamics of 5 L bioreactor.

Acknowledgements

The first author acknowledges Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing the research fellowship. The work reported in this Article is part of an Indo-Australian project (vide sanction letter no. BT/PR9547/ICD/16/754/2006 of DBT/Indo-Aus/01/35/06 dated July 02, 2007), jointly funded by DEST (Australia) and DBT (India).

Nomenclature

$[c_s]$	Substrate concentration in microbial growth medium	g/L
$\left[c_{s_o}\right]$	Substrate concentration in microbial growth medium at initial moment	g/L
$[c_{lac}^*]$	Critical concentration of lactose in microbial growth medium	g/L
$[c_{lac}]$	Lactose concentration in microbial growth medium	g/L
$\left[c_{lac_0}\right]$	Lactose concentration in microbial growth medium at initial moment	g/L
$[c_{\beta\text{-}gal}]$	β-galactosidase concentration	µkatal/L intracellular fluid
$[c_{\beta\text{-}gal}]_{max}$	Maximum β-galactosidase concentration	µkatal/L intracellular fluid
$[c_{\beta\text{-}gal}]_r$	Constant of the Mercier equation	hr^{-1}
$[c_x]$	Biomass concentration	g/L
$\left[c_{x_0}\right]$	Biomass concentration at initial moment	g/L
$[c_x]_\infty$	Carrying capacity	g/L
k	Constant of the Logistic eq	hr^{-1}
K_s	Monod half saturation constant	g/L
$K_{s_{lac}}$	Monod half saturation constant depends upon lactose	g/L
m	Power constant of substrate inhibition	dimensionless
m_{lac}	Maintenance coefficient for lactose	hr^{-1}
$q_{\beta\text{-}gal}$	Specific β-galactosidase synthesis rate.	(L/(g biomass. hr))(µkatal/L bacterial cell fluid)
q_{lac}	Specific substrate utilization rate	hr^{-1}
t	Time	hr
t_{lag}	Lag period of microbial growth	hr
$Y_{X/lac}$	Yield coefficient (biomass formation with respect to lactose utilization)	g dry cell weight/ g lactose
$Y_{\beta\text{-}gal/lac}$	Yield coefficient (β-galactosidase formation with respect to lactose utilization)	(µkatal/L bacterial cell fluid)/ (g lactose /L microbial growth medium)

Greek letters

$\alpha_{\beta\text{-}gal}$	Constant of growth associated enzyme synthesis	(µkatal/L bacterial cell fluid).(L/(g biomass)
$\beta_{\beta\text{-}gal}$	Constants of non growth associated enzyme synthesis	(µkatal/L bacterial cell fluid).(L/(g biomass. hr))
μ	Specific growth rate	hr^{-1}
$\mu_{\max_{lac}}$	Maximum specific growth rate depends upon lactose	hr^{-1}

References

[1] Prazeres, A. R., Carvalho, F., Rivas. J. "Cheese whey management: A review." *Journal of Environmental Management*. 110. pp. 48-68. 2012. DOI: 10.1016/j.jenvman.2012.05.018

[2] Illanes, A. "Whey upgrading by enzyme biocatalysis." *Electronic Journal of Biotechnology*. 14. pp. 1-28. 2011. DOI: 10.2225/vol14-issue6-fulltext-11

[3] Nath, A., Chakraborty, S., Bhattacharjee, C., Chowdhury, R. "Studies on the separation of proteins and lactose from casein whey by cross-flow ultrafiltration." *Desalination and Water Treatment*. 54 (2). pp. 481-501. 2015. DOI: 10.1080/19443994.2014.888685

[4] Siso, G. M. L. "The biotechnological utilization of cheese whey: A review." *Bioresource Technology*. 57. pp. 1-11. 1996. DOI: 10.1016/0960-8524(96)00036-3

[5] Gosling, A., Stevens, G.W., Barber, A. R., Kentish, S. E., Gras, S. L. "Recent advances refining galactooligosaccharide production from lactose." *Food Chemistry*. 121 (2). pp. 307–318. 2010. DOI: 10.1016/j.foodchem.2009.12.063

[6] Hwang, C-F., Chang, J-H., Houng, J-Y., Tasi, C-C., Lin, C-K., Tsen, H-Y. "Optimization of medium composition for improving biomass production of Lactobacillus plantarum Pi06 using the Taguchi array design and the Box-Behnken method." *Biotechnology and Bioprocess Engineering*. 17. pp. 827-834. 2012. DOI: 10.1007/s12257-012-0007-4

[7] Ismail, S. A-A., Mohamady, Y. E., Helmy, W. A., Abou-Romia, R., Hashem, A. M. "Cultural condition affecting the growth and production of β-galactosidase by lactobacillus acidophilus NRRL 4495." *Australian Journal of Basic and Applied Sciences*. 4 (10). pp. 5051-5058. 2010.

[8] Kumar, D. J. M., Sudha, M., Devika, S., Balakumaran, M. D., Kumar M. R., Kalaichelvan, P. T. "Production and Optimization of β-galactosidase by Bacillus Sp. MPTK 121, Isolated from Dairy Plant Soil." *Annal Biological Research*. 3 (4). pp. 1712-1718. 2012.

[9] Princely, S., Saleem Basha, N., John Kirubakaran, J., Dhanaraju, M. D. "Biochemical characterization, partial purification, and production of an intracellular beta-galactosidase from Streptococcus thermophilus grown in whey." *European Journal of Experimental Biology*. 3 (2). pp. 242-251. 2013.

[10] Natarajan, J., Christobell, C., Mukesh Kumar, D. J., Balakumaran, M. D., Ravi Kumar, M., Kalaichelvan, P. T. "Isolation and Characterization of β-Galactosidase Producing Bacillus sp. from Dairy Effluent." *World Applied Sciences Journal*. 17 (11). pp. 1466-1474. 2012.

[11] Choonia, H. S., Lele, S. S. "Kinetic modeling and implementation of superior process strategies for β-galactosidase production during submerged fermentation in a stirred tank bioreactor." *Biochemical Engineering Journal*. 77. pp. 49-57. 2013. DOI: 10.1016/j.bej.2013.04.021

[12] Bailey, M.J., Linko, M. "Production of β-galactosidase by *Aspergillus oryzae* in submerged bioreactor cultivation." *Journal of Biotechnology*. 16 (1-2). pp. 57-66. 1990. DOI: 10.1016/0168-1656(90)90065-J

[13] Akcan, N. "High level production of extracellular β-galactosidase from Bacillus licheniformis ATCC 12759 in submerged fermentation." *African Journal of Microbiology Research*. 5 (26). pp. 4615-4621. 2011. DOI: 10.5897/AJMR11.716

[14] Barberis, S., Segovia, R. "Maximum volumetric production of β-galactosidase by Kluyveromyces fragilis in fed-batch culture with automatic control." *Journal of Chemical Technology and Biotechnology.* 77 (6). pp. 706-710. 2002. DOI: 10.1002/jctb.626

[15] Rech, R., Ayub, M. A. Z. "Simplified feeding strategies for fad-batch cultivation of Kluyveromyces marxianus in cheese whey." *Process Biochemistry.* 42. pp. 873-877. 2007. DOI: 10.1016/j.procbio.2007.01.018

[16] Willey, J., Sherwood, L., Woolverton, C. "*Laboratory Exercise in Microbiology*." New York: McGraw-Hill Publishing Company. 2010.

[17] Shimizu, H., Mizuguchi, T., Tanaka, E., Shioya, S. "Nisin production by a mixed-culture system consisting of Lactococcus lactis and Kluyveromyces marxianus." *Applied and Environmental Microbiology*. 65 (7). pp. 3134-3141. 1999.

[18] Das, R., Sen, D., Sarkar, A., Bhattacharya, S., Bhattacharjee, C. "A comparative study on the production of galacto-oligosaccharide from whey permeate in recycle membrane reactor and in enzymatic batch reaktor." *Industrial Engineering Chemistry Research*. 50 (2). pp. 806-816. 2011. DOI: 10.1021/ie1016333

[19] Miller, J. "*Experiments in Molecular Genetics*." New York: Cold Spring Harbor Laboratory. 1972.

[20] Dybkaer, R. "Unit "Katal" for Catalytic Activity (IUPAC Technical Report." *Pure and Applied Chemistry.* 73. pp. 927-931. 2001. DOI: 10.1351/pac200173060927

[21] Nath, A., Sarkar, S., Maitra, M., Bhattacharjee, C., Chowdhury, R. "An experimental study on production of intracellular β-galactosidase at different conditions by batch process using isolate Bacillus safensis (Juche 1) characterization of synthesized β-galactosidase." *Journal of Institute of Engineers (India): Series E*. 93 (2). pp. 55-60. 2013. DOI: 10.1007/s40034-013-0011-z

[22] Nath, A., Sarkar, S., Maitra, M., Bhattacharjee, C., Chowdhury, R. "Production, Purification and Characterization of of β-galactosidase Synthesized by Bacillus safensis (JUCHE 1)." In: *3rd International Conference on Advances in Biotechnology and Pharmaceutical Sciences (ICABPS'2013)*. Planetary Scientific Research Centre, Malaysia, DATE. pp. 7-16. 2013.

[23] Shuler, M. L., Kargi, F. "*Bioprocess Engineering: Basic Concept*." New Jersey: Prentice-Hall Inc. 2002.

[24] Okpokwasili, G. C., Nweke, C. O. "Microbial growth and substrate utilization kinetics." *African Journal of Biotechnology*. 5 (4). pp. 305-317. 2005.

[25] Han, K., Levenspiel, O. "Extended monod kinetics for substrate, product and cell inhibition." *Biotechnology and Bioengineering*. 32 (4). pp. 430-447. 1988. DOI: 10.1002/bit.260320404

[26] Sivakumar, A., Srinivasaraghavan, T., Swaminathan, T., Baradarajan, A. "Extended monod kinetics for substrate inhibited system." *Bioprocess Engineering*. 11. pp. 185-188. 1994. DOI: 10.1007/bf00369628

[27] Lin, S. K. C., Du, C., Koutinas, A., Wang, R., Webb, C. "Substrate and product inhibition kinetics in succinic acid production by Actinobacillus succinogenes." *Biochemical Engineering Journal*. 41 (2). pp. 128-135. 2008. DOI: 10.1016/j.bej.2008.03.013

[28] Mercier, P., Yerushalmi, L., Rouleau, D., Dochain, D. "Kinetics of lactic acid fermentation on glucose and com by Lactobacillus amylophilus." *Journal of Chemical Technology and Biotechnology.* 55 (2). pp. 111-121. 1992. DOI: 10.1002/jctb.280550204

[29] Kafarov, V., Kuzetson, B. "*Cybernatic Methods in Chemistry & Chemical Engineering*." Moscow: Mir Publication. 1976.

[30] Constantinides, A. "*Applied Numerical Methods with Personal Computers* (Chemical Engineering Series)." New York: Mc Graw-Hill International Editions. 1987.

[31] Satomi, M, La Duc, M. T., Venkateswaran, K. "*Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces." *International Journal of Systematic and Evolutionary Microbiology.* 56. pp. 1735–1740. 2006. DOI: 10.1099/ijs.0.64189-0

[32] Kothari, V. V., Kothari, R. K., Kothari, C.R., Bhatt, V. D., Nathani, N. M., Koringa, P. G., Joshi, C. G., Vyas, B. R. M. "Genome Sequence of Salt-Tolerant *Bacillus safensis* Strain VK, Isolated from Saline Desert Area of Gujarat, India." *Genome Announcements*. 1 (5). e00671-13. 2013. DOI: 10.1128/genomeA.00671-13

[33] Kargi, F. "Re-interpretation of the logistic equation for batch microbial growth in relation to Monod kinetics." *Letters in Applied Microbiology*. 48 (4). pp. 398-401. 2009. DOI: 10.1111/j.1472-765X.2008.02537.x

[34] Benthin, S., Nielsen, J., Villadsen, J. "Galactose expulsion during lactose metabolism in Lactococcus lactis subsp. Cremoris FD1 due to dephosphorylation of intracellulare galactose 6-phosphate." *Applied and Environmental Microbiology.* 60 (4). pp. 1254-1259. 1994.

[35] Görke, B., Stülke, J. "Carbon catabolite repression in bacteria: many ways to make the cost out of nutrients." *Nature Reviews Microbiology.* 6. pp. 613-624. 2008. DOI: 10.1038/nrmicro1932

[36] Nelson, D. L., Cox, M. M. "*Lehninger Principles of Biochemistry*." New York: W.H. Freeman and Company. 2008.

[37] Prakasham, R. S., Subba Rao, C., Sarma, P. N. "Green gram husk- an inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation." *Bioresource Technology*. 97. pp. 1449-1454. 2006. DOI: 10.1016/j.biortech.2005.07.015

[38] Subba Rao, C., Sathish, T., Mahalaxmi, M., Suvarna Laxmi, G., Sreenivas Rao, R., Prakasham, R. S. "Modelling and optimization of fermentation factors for enhancement of alkaline protease production by isolated Bacillus circulans using fad-forward neural network and genetic algorithm." *Journal of Applied Microbiology*. 104 (3). pp. 889-898. 2008. DOI: 10.1111/j.1365-2672.2007.03605.x

[39] Johnvesly, B., Naik, G. R. "Studies on production of novel thermostable alkaline protease from thermophilic and alkaliphilic Bacillus sp. JB99 in a chemically defined medium." *Process Biochemistry*. 37. pp. 139-144. 2001. DOI: 10.1016/s0032-9592(01)00191

Supplementary section

Table 2A Statistical evaluation of the goodness of model equations for specific growth rate, μ for different initial lactose concentration in growth medium ($t > t_{lag}$).

$[c_{lac}]_0$, g/dm3	MSE		
	Classical Monod model (Without substrate, and product inhibition)	Substrate inhibited Monod model	Logistic model
5	4.47×10^{-4}	4.45×10^{-2}	7.68×10^{-4}
10	4.46×10^{-4}	2.85×10^{-2}	8.45×10^{-4}
15	1.12×10^{-5}	3.48×10^{-2}	0.195
20	2.01×10^{-5}	1.13×10^{-3}	0.367
25	0.277	1.18×10^{-4}	-
30	0.505	1.17×10^{-4}	-
35	0.799	1.752×10^{-5}	-
40	2.234	1.846×10^{-5}	-
45	2.831	1.442×10^{-5}	-
50	5.632	1.12×10^{-5}	-

Table 2B Statistical evaluation of the goodness of model equations for specific β-galactosidase production rate, $q_{\beta\text{-}gal}$ for different initial lactose concentration in growth medium ($t > t_{lag}$).

$[c_{lac}]_0$, g/dm^3	M_{SE}				
	Monod incorporated modified Luedeking-Piret model	Monod incorporated Luedeking-Piret model	Logistic incorporated modified Luedeking-Piret model	Logistic incorporated Luedeking-Piret model	Mercier model
5	1.425×10^{-5}	1.58	3.92×10^{-4}	0.231	0.38
10	1.357×10^{-5}	3.45	2.77×10^{-4}	1.51	1.586
15	1.554×10^{-5}	16.56	4.09	9.2	9.49
20	1.624×10^{-5}	40.2	16.9	54.8	23.40
25	83.9	1.465×10^{-5}	-	-	-
30	170.2	1.526×10^{-5}	-	-	-
35	270.1	1.552×10^{-5}	-	-	-
40	404.8	1.646×10^{-5}	-	-	-
45	611.1	1.442×10^{-5}	-	-	-
50	879.6	1.676×10^{-5}	-	-	-

Table 2C Statistical evaluation of the goodness of model equations for specific substrate utilization rate, q_{lac} for different initial lactose concentration in growth medium ($t > t_{lag}$).

$[c_{lac}]_0$, g/dm^3	M_{SE}					
	Classical Monod substrate utilization model	Substrate inhibited Monod substrate utilization model	Logistic substrate utilization model	Logistic incorporated modified Luedeking-Piret substrate utilization model	Logistic incorporated Luedeking-Piret substrate utilization model	Logistic incorporated Mercier substrate utilization model
5	1.005×10^{-3}	0.71	2.001×10^{-3}	1.36	0.82	2.2×10^{-2}
10	3.001×10^{-4}	1.73	5.06×10^{-3}	1.71	5.36	0.76
15	1.121×10^{-4}	3.25	1.25	5.52	12.76	2.54
20	1.007×10^{-4}	6.62	20.27	35.38	37.99	24.57
25	31.39	1.865×10^{-4}	-	-	-	-
30	59.1	1.526×10^{-4}	-	-	-	-
35	110.3	1.752×10^{-5}	-	-	-	-
40	191.2	1.846×10^{-5}	-	-	-	-
45	242.1	1.442×10^{-5}	-	-	-	-
50	287.5	1.876×10^{-5}	-	-	-	-