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ORIGINAL PAPER

Modeling and experimental studies on intermittent starch feeding and citrate addition in simultaneous saccharification and fermentation of starch to flavor compounds

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Abstract Simultaneous saccharification and fermentation (SSF) is a combined process of saccharification of a renewable bioresource and fermentation process to produce products, such as lactic acid and ethanol. Recently, SSF has been extensively used to convert various sources of cellulose and starch into fermentative products. Here, we present a study on production of buttery flavors, namely diacetyl and acetoin, by growing *Lactobacillus rhamnosus* on a starch medium containing the enzyme glucoamylase. We further develop a structured kinetics for the SSF process, which includes enzyme and growth kinetics. The model was used to simulate the effect of pH and temperature on the SSF process so as to obtain optimum operating conditions. The model was experimentally verified by conducting SSF using an initial starch concentration of 100 g/L. The study demonstrated that the developed kinetic was able to suggest strategies for improved productivities. The developed model was able to accurately predict the enhanced productivity of flavors in a three stage process with intermittent addition of starch. Experimental and simulations demonstrated that citrate addition can also lead to enhanced productivity of flavors. The developed optimal model for SSF was able to capture the dynamics of SSF in batch mode as well as in a three stage process. The structured kinetics was also able to quantify the effect of multiple substrates present in the medium. The study demonstrated that structured kinetic models can be used in the future for design and optimization of SSF as a batch or a fed-batch process.

Keywords *Lactobacillus rhamnosus* · Glucoamylase · SSF · Diacetyl · Acetoin · Lactic acid · Mathematical model · Citrate addition · Intermittent addition of starch

Introduction

Simultaneous saccharification and fermentation (SSF) is a combined process of enzymatic saccharification and microbial fermentation. Enzymatic processes involving conversion of a polysaccharide such as cellulose or starch to glucose are limited by product inhibition. Glucose, the product of saccharification, inhibits the enzyme activity, thus limiting the rate of saccharification. During SSF, glucose is converted to another product through microbial fermentation, thus eliminating the glucose inhibition on saccharification. The advantage of SSF being faster saccharification rates resulting in reduced reactor volume [1, 3].

Several conditions must be satisfied to effectively apply the SSF process to produce fermentation products from polysaccharides. Firstly, the conditions such as temperature and pH, for enzymatic saccharification and the fermentation process should be coincident. Secondly, the final product from the fermentation should not sufficiently inhibit the saccharification process. SSF can be effectively used to produce fermentation products from starch if the saccharification and fermentation rates are matched. SSF has been used for the conversion of cellulose or starch to various fermentative products. Several studies exist

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wherein the conversion of cellulose to ethanol has been reported. SSF of cellulose powder to produce lactic acid in a media containing cellulases and *Lactobacillus delbrueckii* cells resulted in high yields and faster rates [1].

Starch, as a bioresource to produce lactic acid, ethanol, butanol and succinate has been attempted using a SSF process. For example, recently D-lactic acid has been produced using rice bran with a yield of 78% with an optical purity of 95% [22]. Lactic acid has also been produced using corn cobs and *Lactobacillus rhamnosus* through a SSF process [17]. A maximum lactic acid concentration of 81 g/L was obtained using response surface methodology when 15% (w/v) cassava bagasse was treated using *L. delbrueckii* [18]. SSF of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol has also been reported [25]. A lactic acid concentration of 40 g/L was obtained using SSF of cellulosic biosludge generated in a kraft pulp mill with a total product yield of 35 g/L lactic acid/100 g biosludge [19]. An overall yield of 80.2% has been reported by using a high temperature enzymatic prehydrolysis process prior to SSF of steam pretreated corn stover for ethanol production [14]. Similarly, 80% yield was reported for ethanol production from steam pretreated barley straw at low enzyme loading [13]. A comparison between SSF and separate hydrolysis and fermentation of steam pretreated corn stover to product ethanol demonstrated an efficiency of SSF with 13% higher yields [15]. SSF has also been employed to produce hydrogen using crop straw by *Clostridium sp*. with a maximal hydrogen yield of 126 mL/g solids [12]. Recently, it is reported that butanol was successfully produced with a productivity of 0.31 g/L per hour using SSF of wheat straw [16]. It can be noted that in the recent past, SSF has been successfully used to produce various fermentative products using different starchy or cellulosic raw materials. SSF to produce flavor compounds, such as diacetyl and acetoin, has not been reported. *L. rhamnosus* is known to metabolize glucose and citrate to produce lactic acid, diacetyl and acetoin. Diacetyl and acetoin are buttery flavors that are used in food and pharmaceutical industries. Diacetyl and acetoin are a key component of buttery flavor in fermented foods. It is difficult to produce a flavor material with a buttery note without including diacetyl within the formulation. Fermentation process is used to manufacture diacetyl and acetoin mainly using lactic acid starter cultures [21]. Commercially available natural mixture of diacetyl and acetoin is obtained by steam distillation of fermentation broth of *Streptococcus lactis* or *S. cremoris* using a medium consisting of skim milk usually fortified with about 0.1% citric acid to increase flavor productivity.

Although many experimental studies on SSF have been reported, attempts at modeling the SSF process have also been very few. The few existing models were unstructured growth models, which are empirical in nature with inherent problems of limited use in the design and control of fermentation processes [2, 4, 5, 20]. Modeling SSF incorporates the kinetics of enzymatic saccharification and kinetics of growth and product inhibition due to cellular metabolism. The kinetics of saccharification is typically represented by Michelis–Menten type kinetics with a product inhibition term. The product inhibition could be competitive or noncompetitive in nature. Structured models attempt to capture the metabolic details to represent the kinetics of cell growth. For example, in a cybernetic model, cells are assumed to maximize the growth rate on alternative sources provided in the medium [7, 8, 10, 23]. Such models also include synthesis of intracellular growth enzyme that is essential for the growth of cells which captures the lag phase that exists before the exponential growth rate. The model assumes that the cell maximizes the growth rate on multiple substrates through control mechanisms existing at the genetic and metabolic levels. These mechanisms can yield simultaneous or sequential growth on multiple substrates based on the uptake rates on individual substrate.

The objective of the current study is to develop an optimal model to capture the dynamics of the SSF process for the conversion of starch to flavor compounds using *L. rhamnosus*. The organism also produces lactic acid in addition to diacetyl and acetoin, as flavor compounds. The model was able to capture the dynamics of SSF as observed in experiments including the formation of glucose and products. The model was used to represent the effect of temperature and pH by establishing the dependency of model parameters on these two variables. Further, the structured model was used to capture the kinetics of SSF with the addition of citrate into the starch medium. The model was also able to predict the effect of intermittent addition of starch during the SSF process to yield better productivities.

Model development

In SSF, since saccharification and fermentation are carried out simultaneously in the reactor, the glucose formed through saccharification is immediately consumed by the cells. Therefore, the net glucose accumulation rate is given as the difference between the glucose formation rate due to enzymatic saccharification (r_E) and glucose consumption rate due to fermentation (r_G). Thus, the net glucose accumulation rate is given by the following equation:

$$\frac{dG}{dt} = r_E - r_G. \quad (1)$$

Enzymatic Saccharification of starch is quantified by Michaelis–Menten kinetics including competitive inhibition by glucose. Thus,

$$r_E = v_m \frac{S}{K_m\left(1+\frac{G}{K_G}\right)+S} \quad (2)$$

where S and G are concentration of starch and glucose, respectively. Based on stoichiometry, the starch concentration at any given time, t is given as below

$$S = S_0 - \frac{G^*}{1.11}. \quad (3)$$

In the above expression, 1.11 is the stoichiometric conversion factor from starch to glucose accounting for the water molecule that is consumed in the reaction. The rate of saccharification (r_E) is given by combining the above two equations,

$$r_E = v_m \frac{\left(S_0 - \frac{G^*}{1.11}\right)}{K_m\left(1+\frac{G}{K_G}\right)+\left(S_0 - \frac{G^*}{1.11}\right)}. \quad (4)$$

It should be noted that G^* accounts for the stoichiometric conversion of starch to glucose in SSF without considering the consumption of glucose through fermentation. Thus,

$$\frac{dG^*}{dt} = r_E. \quad (5)$$

The value of G^* will be different from the glucose obtained purely through saccharification due to the reduced inhibition by glucose. Determination of G^* tracks the amount of glucose formed stoichiometrically from starch at any given instant.

Optimal model for the growth of *L. rhamnosus*

L. rhamnosus is capable of growing on glucose, citrate and lactate as energy sources. The organism while growing on glucose and citrate, the organism produces lactate and flavors (diacetyl + acetoin). In absence of glucose and citrate, the organism is capable of metabolizing lactate to acetate and the flavor compounds. *L. rhamnosus* can consume glucose and citrate simultaneously from a medium. Thus, the cells regulate the uptake of glucose, citrate and lactate as an energy source to match the maximum growth rate of the organism (μ_{max}) in a mixed culture environment. Thus, the growth of cells is represented as

$$\frac{dx}{dt} = \frac{\mu x}{(1+K_{IF}F^n)} \quad (6)$$

where μ is the growth rate of the *L. rhamnosus* in a mixture containing all the three substrates and x represents the mass of the cells. F represents the total flavors concentration and K_{IF} and n are inhibition constants. The term $(1 + K_{IF}F^n)$ captures the inhibition of flavors on the growth of *L. rhamnosus*. It should also be noted that the inhibition term also includes the inhibition by lactate. As both lactate and flavors are mainly growth associated products, mathematically their association is linear and the variables can be clubbed. Therefore, the net growth rate (μ) depends on the individual growth rates on the three substrates at any given instant and is given as below:

$$\mu = \alpha_G\mu_G + \alpha_C\mu_C + \alpha_L\mu_L. \quad (7)$$

Here, μ_G, μ_C and μ_L are growth rates on glucose, citrate and lactate, respectively. α_G, α_C and α_L are the control coefficients which represent the regulation of the uptake of these substrates and thus in turn regulate the growth rate (see Fig. 1 for the schematic). The expressions for μ_G, μ_C and μ_L are as follows:

$$\mu_G = \mu_G^{max}\frac{e_G}{e_{G,max}}\frac{G}{K_G + G + \frac{G^2}{K_{IG}}} \quad (8)$$

$$\mu_C = \mu_C^{max}\frac{e_C}{e_{C,max}}\frac{C}{K_C + C + \frac{C^2}{K_{IC}}} \quad (9)$$

$$\mu_L = \mu_L^{max}\frac{e_L}{e_{L,max}}\frac{L}{K_L + L + \frac{L^2}{K_{IL}}}. \quad (10)$$

The growth rates on individual substrates are a modified Monod equation including substrate inhibition term and the relative growth enzyme concentration ($e_i/e_{i,max}$). The relative growth enzyme concentrations for the three substrates are given as follows [8, 10, 22]:

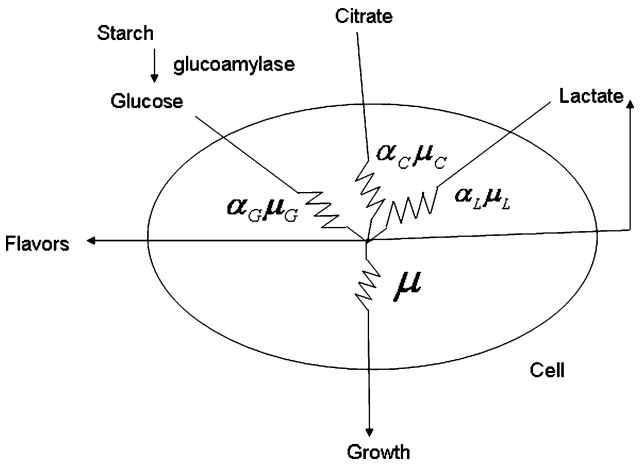

Fig. 1 Schematic diagram of the optimal model showing the extracellular degradation of starch by glucoamylase, uptake of glucose, citrate and lactate by *L. rhamnosus* and production of lactate and flavors through the metabolism of substrates. The organism activates only a fractional of the possible growth rate on a given substrate. Thus, the net growth rate, $\mu = \alpha_G\mu_G + \alpha_C\mu_C + \alpha_L\mu_L$, where α is the control coefficient and μ_G, μ_C and μ_L are the growth rate on glucose, citrate and lactate, respectively

$$\frac{d\left(\frac{e_i}{e_i^{max}}\right)}{dt} = \alpha_i(\mu_i^{max} + \beta_i)\left[\frac{S_i}{K_i + S_i}\right] - (\mu + \beta_i)\frac{e_i}{e_i^{max}} \quad (11)$$

where $i = G$, C or L represents glucose, citrate and lactate and S_G, S_C and S_L are equivalent concentrations of glucose, citrate and lactate, respectively. The first term represents the synthesis of the growth enzymes dependent on the specific substrate concentration and the control coefficients. The second term includes the dilution due to overall growth (μ) and the first order natural degradation of the enzymes. The parameter, β_i, represents the first order rate constant for degradation of the key enzymes. The uptake of the substrates (glucose, citrate and lactate) are also dependent on the value of the control coefficient and is expressed as follows:

$$r_G = -\frac{1}{Y_G}\alpha_G(\mu_G + m_G)x. \quad (12)$$

The net accumulation of glucose is given by Eq. 1, where r_G represents the consumption of glucose due to growth. The consumption of glucose is both due to growth (μ_G) and maintenance (m_G). It should be noted that the rate of consumption depends on the value of α_G, the control coefficient for glucose. Similarly, the uptake of citrate from the medium is given as follows:

$$\frac{dC}{dt} = -\frac{1}{Y_C}\alpha_C(\mu_C + m_C)x. \quad (13)$$

The rate of product formation is represented by Leudeking–Piret [11] type of relationship from both the metabolism of glucose and citrate. The rate of lactate formation is given by

$$\frac{dL}{dt} = [\alpha_G(l_{1G}\mu_G + l_{2G}) + \alpha_C(l_{1C}\mu_C + l_{2C})]x - \left(\frac{1}{Y_L}\right)\alpha_L\mu_L x \quad (14)$$

where l_{1G} and l_{1C} represents growth associated lactate formation constants on glucose and citrate, respectively, while l_{2G} and l_{2C} are non-growth associated lactate formation constants on glucose and citrate, respectively. Also, lactate is consumed as a substrate in the absence of glucose and citrate and is represented by the last term. Similarly, for acetate and flavors, the following product formation rates are represented.

$$\frac{dA}{dt} = [\alpha_G(a_{1G}\mu_G + a_{2G}) + \alpha_C(a_{1C}\mu_C + a_{2C})]x \quad (15)$$

$$\frac{dF}{dt} = [\alpha_G(f_{1G}\mu_G + f_{2G}) + \alpha_C(f_{1C}\mu_C + f_{2C})]x \quad (16)$$

It should be noted that F represents the total concentration of diacetyl and acetoin. The control coefficients α_G, α_C and α_L are estimated based on the maximizing criterion of overall growth rate. Thus,

$$\max(\alpha_G\mu_G + \alpha_C\mu_C + \alpha_L\mu_L) \quad (17)$$

$$\text{S.t. } \alpha_G\mu_G + \alpha_C\mu_C + \alpha_L\mu_L \le \mu^{max} \quad 0 \le \alpha_G \le 1$$
$$0 \le \alpha_C \le 1 \quad 0 \le \alpha_L \le 1 \quad (18)$$

where μ^{max} represents the maximum possible growth rate for the organism on any medium. Prior information regarding the simultaneous uptake of glucose and citrate is necessary for calculating the values of the control coefficient. The solution for the above optimization depends on the growth rate on each of the substrate at any given instant. For example, if $\mu_G > \mu_C$ and $\mu_G + \mu_C > \mu^{max}$, then α_G will be assigned a value 1 and $\alpha_C = \frac{\mu^{max} - \mu_G}{\mu_C}$. If $\mu_G + \mu_C < \mu^{max}$, then the value for the control coefficient for glucose and citrate would be $\alpha_G = \alpha_C = 1$. To determine the value of α_L, if the value of μ_G and $\mu_C > 0$, then $\alpha_L = 0$ and takes a value of 1 if μ_G and $\mu_C = 0$, and this condition represents the catabolite repression.

The above set of equations were solved using MATLAB ode45 (Mathworks Inc., USA) under a set of initial conditions for the multiple substrate environment. The model parameters were obtained using experimental data by fitting the model solution using least square method. The model parameters are listed in the supplementary information. Parameters are also evaluated giving separately on glucose and citrate, respectively at various temperature and pH values (data not shown). The parameter dependency on temperature and pH were fitted by an appropriate empirical function and are listed in supplementary information. Some parameters which depended on initial starch concentration were re-estimated using SSF data. It should be noted that the optimum conditions indicated for the SSF process are local optima obtained in the subset of experimentally measured conditions.

Materials and methods

Materials

Hetrofermentative *L. rhamnosus*, a strain producing mainly L-(+)-lactic acid, was obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory (NCL), Pune, India. Cultures were maintained at 4°C on slants containing 3% glucose along with other essential nutrients. Lactic acid bacteria were revived by two successive propagations at 45°C for 12–18 h in the modified MRS broth. Commercial α-amylase and glucoamylase from Novo Nordisk, Denmark, were employed in this present study.

Fermentation medium

Shake flask experiments were carried out using MRS broth containing yeast extract (0.5%), urea (0.5%), potassium dihydrogen phosphate (0.1%), sodium acetate

(0.5%), magnesium sulphate (0.03%) and varying glucose concentrations.

SSF medium

The SSF medium consisted of yeast extract (0.5%), urea(0.5%), dipotassium hydrogen phosphate (0.1%), potassium phosphate (0.1%), sodium acetate (0.5%), magnesium sulphate (0.03%) and varying quantities of 10, 30, 100, 150 and 250 g/L of liquefied analytical grade potato starch (containing 20% moisture). Citrate (40 g/L) was also added into the SSF medium containing 100 g/L of starch at a pH of 5 and temperature of 30°C in a separate experiment.

Methods

Saccharification

Potato starch slurry (50 g/L) was used as substrate and pH adjusted to 6 using dilute HCl. α-amylase was added [0.1% on dry substrate basis] and 200 ppm of calcium added to stabilize α-amylase activity. The starch suspension was charged into a reactor kept in a water bath at 100°C. The reaction was carried out for 2 h. After liquefaction, saccharification was carried out using glucoamylase after changing the pH to 4.2. The temperature was maintained at 60°C. Experiments were carried out to determine the effects of citric acid and lactic acid on saccharification kinetics at 60°C and pH 4.2. The inhibitors were added at required concentrations after the liquefaction step. The concentrations studied were 10, 20 and 40 g/L. The pH for saccharification was adjusted after adding the inhibitor in appropriate concentration. Samples were withdrawn every hour and frozen immediately to arrest the enzyme action. The experiment demonstrated that lactic acid and citrate imparts lesser inhibition than glucose on the saccharification rate (results not shown). Saccharification experiments were performed in triplicate and the average is reported. The maximum deviation observed was about 7%.

Fermentation with L. rhamnosus

Batch experiments (100 mL) were carried out in 250 mL flasks, shaken at 250 rpm on a Neolab shaker at the required temperature. Samples of 2 mL of broth were removed for analysis every 2 h and pH was checked every 30 min. The pH was controlled by the addition of slurry of $CaCO_3$. The samples were centrifuged and washed after which they were analyzed for biomass, glucose and lactate content. The effect of pH was studied at a temperature of 45°C, by conducting fermentations at a pH of 4, 5, 5.5 and 6.5. The effect of temperature was studied at a pH of 5.6, by conducting fermentations at 40, 45, 50, 55 and 60°C. Fermentation kinetics was studied at substrate concentrations of 10, 30 and 100 g/L. The fermentation studies were done in triplicate and the average data is presented. The maximum deviation observed was about 11%.

SSF of starch with L. rhamnosus

The medium containing starch was autoclaved along with α-amylase (0.15 mL/100 g starch) at 121°C and 15 psi for 15 min. The starch concentration was varied from 10 to 250 g/L. Yeast extract and urea were proportionally increased to avoid nitrogen limitation. The medium was inoculated with the second generation of *L. rhamnosus* cultivated at 45°C for about 14–18 h. The inoculum size was fixed at 10% and SSF was carried out after adding 0.15% (based on dry starch) of glucoamylase. SSF was also operated by intermittent addition of starch. Using this strategy SSF was operated in three stages. In the first stage, SSF was carried out using 30 g/L starch by the same procedure outlined above. In the second and third stage, starch was added at a concentration of 30 and 40 g/L, respectively with the corresponding amounts of enzyme (0.15% glucoamylase). The intermittent starch was added as thick slurry (close to 400 g/L), so as to minimally affect the volume. This ensured that only less than 5% volume change was observed, which did not alter the product concentration. This was deliberately done so as to have a proper comparison between a normal SSF and the intermittent addition. However, when thick slurry of starch was autoclaved, some amount of glucose was formed in the process and that had to be accounted for in the model, which resulted in a jump in the glucose concentration. The pH was controlled every 30 min and samples were with drawn every hour. In this case, flavor concentration was monitored through out and the sterile starch–nutrient medium was fed at the time point when flavor concentration started to decrease. In the SSF experiments, the biomass was very difficult to estimate as the biomass could not be separated from the starch medium. This resulted in errors in the estimate of biomass as OD as well as dry weight. Thus, biomass values are not reported. All experiments were conducted as a triplicate and the maximum deviation observed was 15%.

Sample analysis

Samples were withdrawn from either fermentation or SSF medium were centrifuged at 10,000 rpm for 15 min, the supernatant frozen for further analysis and the pellet analyzed for biomass. Biomass was determined by measuring the absorbance using the standard curve of absorbance against dry cell weight. Absorbance was measured at 600 nm in a Shimadzu spectrophotometer (model UV 160).

Lactate and glucose concentrations were determined by the LDH and *o*-toluidine methods, respectively. Flavors (diacetyl and acetoin) were determined using Westerfeld method [24]. It should be noted that, the data for acetate and pyruvate in the extracellular broth are not presented although they were measured in the current study. While negligible quantity of pyruvate was measured, acetate was formed mainly from lactate after glucose concentration was reduced to zero.

Results and discussion

The model parameters for predicting SSF were independently got by carrying out saccharification and fermentation experiments. The optimum pH and temperature for saccharification was 4.2 and 60°C, whereas the optimum pH and temperature for diacetyl production by fermentation was 5.5 and 30°C. It was seen that there was a decrease of 72% in saccharification rate at the optimum conditions for diacetyl production. Although there was a decrease in saccharification rate, it was found to be substantial to operate SSF at the above stated conditions. Based on the parameters obtained from saccharification studies and fermentation experiments, performance of SSF were predicted at different temperatures and pH. As expected, the model could not predict the SSF accurately, although the profiles demonstrated similar trends. Certain parameters were estimated based on SSF experiments. It appeared that the product formation rates were altered in the presence of starch medium. Therefore, the growth associated parameters (l_{1i}, l_{2i}, a_{1i}, a_{2i}, f_{1i}, f_{2i}) were re-evaluated to fit SSF data. All the parameters are listed in the supplementary information. The fermentation data obtained at different pH and temperature values were fitted to obtain model parameters values at various conditions of pH and temperature. These parameter values were further fitted to empirical functions of temperature and pH, to predict the effect of these variables on the performance of SSF.

The SSF process was simulated at various pH and temperature values using 100 g/L of initial starch concentration. Table 1 shows the product concentrations and yield values at a fixed pH value of 5.5 and different values of temperature. It can be noted that a maximum lactic acid concentration was observed at 45°C with a yield of 0.525 g lactate/g glucose. The net flavor was maximally produced at 30°C with a yield of 0.068 g flavor/g glucose. On varying pH (see Table 2), it was noted that lactic acid was optimally produced at pH 6.5, while flavor were optimal at pH 5.5. Thus, SSF was operated at pH 5.5 and 30°C to maximally produce flavors. Although, this resulted in a sub-optimal production of lactic acid, the amount of lactic acid produced was still substantial.

Figure 2a shows the glucose profile at different starch concentrations. A maximum of glucose concentration of 24 g/L accumulated in the broth for an initial starch concentration of 150 g/L. This concentration was less than the K_G value (glucose inhibitory constant of 33 g/L) for the enzyme, thus indicating that SSF reduced the extent of glucose inhibition. The glucose profiles (see Fig. 2a) indicated that there was an initial phase where the saccharification rates were greater than the fermentation rates, wherein glucose accumulated. During this phase, the cells were in the lag phase which resulted in lower consumption of glucose for growth. The structured model was able to capture this initial lag phase through the specific growth enzyme concentration (e/e_{max}). However, at a later stage, more of the cells were in the exponential growth phase, the glucose concentration dropped, and thus demonstrating a peak concentration in the glucose concentration. The maximum glucose concentration that accumulated in the reactor was 5, 7, 22 and 24 g/L for SSF with an initial starch concentration of 10, 30, 100, 150 g/L, respectively.

Figure 2b shows the comparison between model prediction and experimental data for variation of lactate with time for SSF carried out with different initial starch concentrations. It can be seen from the figure that there are two distinct rates of lactic acid formation. The initial high rate of lactic acid production was in the exponential growth phase. The lower rate in the late logarithmic and stationary phase of growth may be due to cell maintenance. Maximum yields as high as 56% (5.2 g/L), 77% (24.8 g/L), 54% (60 g/L), 72% (119 g/L) were obtained for SSF with an

Table 1 Model prediction of SSF performance at different temperature values for a fixed pH value of 5.5

Temperature (°C)	25	30	35	45
Lactate yield (g lactate/g glucose)	0.328	0.452	0.5	0.525
Flavors yield (g flavors/g glucose)	0.037	0.068	0.048	0.034
Maximum lactate concentration (g/L)	41.89	57.63	63.91	67.03
Final flavors concentration (g/L)	4.72	8.66	6.19	4.34

Lactate and flavor yields were maximum at 45 and 30°C, respectively

Table 2 Model prediction of SSF performance at different pH values for a fixed temperature of 30°C

pH	4	5	5.5	6.5
Lactate yield (g lactate/g glucose)	0.283	0.28	0.452	0.49
Flavors yield (g flavors/g glucose)	0.045	0.047	0.068	0.039
Maximum lactate concentration (g/L)	36.12	35.66	57.63	63.74
Final flavors concentration (g/L)	5.79	6.09	8.66	5.03

Lactate and flavor yields were maximum at pH values of 5.5 and 6.5, respectively

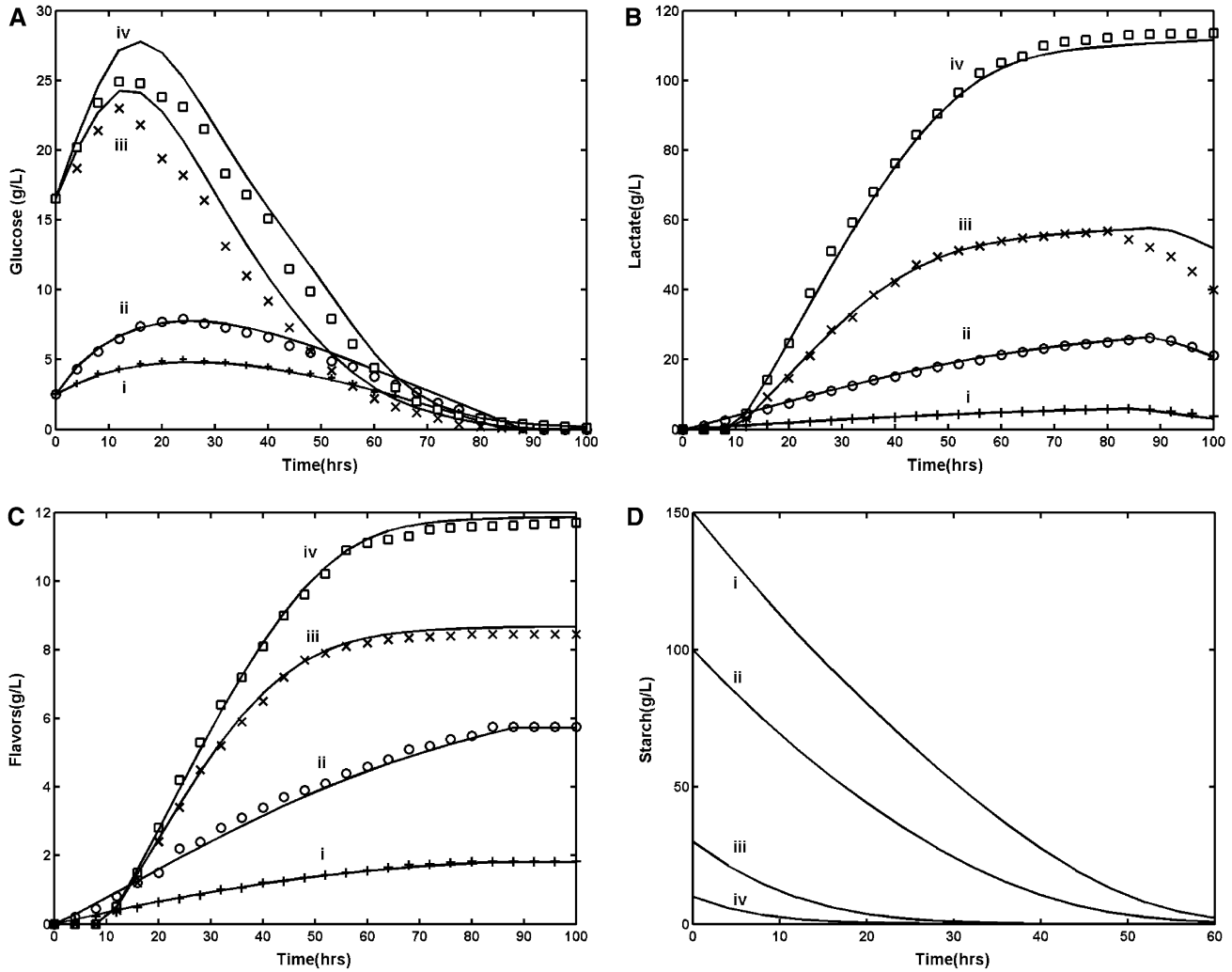

Fig. 2 Comparison of model prediction and experimental data for SSF performance on different starch concentration. **a** Glucose concentration, **b** lactic acid concentration, **c** flavor concentrations, **d** starch concentration. Model profiles for starch concentrations are indicated in the figure as follows: *i* 10 g/L, *ii* 30 g/L, *iii* 100 g/L, *iv* 150 g/L. Experimental data is represented by symbols: *plus* 10 g/L, *open circle* 30 g/L, *cross* 100 g/L, *open square* 150 g/L

initial starch concentration of 10, 30, 100 and 150 g/L, respectively. Although lactate is the end product of the fermentation, it can be further metabolized to acetate and CO_2. Therefore, the level of lactate dropped due to metabolism when the apparent glucose concentration reached close to zero concentration. At this stage, the cells did not perceive glucose in the broth and started to metabolize lactate for maintenance, thus resulting in the decrease of lactic acid concentration. At the end of 100 h of SSF, the lactate concentration dropped to 3.85, 24.8, 56.2 and 107 g/L for an initial starch concentration of 10, 30, 100 and 150 g/L, respectively.

Figure 2c shows the comparison between model prediction and experimental data for the variation of flavors (diacetyl and acetoin) concentration with time for the SSF carried out with different initial starch concentrations. Maximum yields for flavor were 11% (1.9 g/L), 11.1% (5.8 g/L), 4.7% (8.2 g/L) and 3.5% (11.8 g/L) for SSF with initial starch concentrations of 10, 30, 100 and 150 g/L of starch, respectively. Diacetyl is a primary metabolite produced only during the growth phase, while acetoin is produced both during growth and stationary phases. However, the profile of the net flavor produced demonstrates saturation at the end of fermentation indicating that diacetyl was converted to acetoin in the stationary phase. The extracellular pyruvate concentrations were estimated during the course of SSF. The extracellular pyruvate levels do not vary much as a function of time. The average extracellular pyruvate concentration was 8.8, 11.1, 19.8 and 21.3 mM for SSF with 10, 30, 100 and 150 g/L initial starch concentrations, respectively. The cell can tolerate about 10–20 mM of intracellular pyruvate concentration [22]. Therefore, the excess pyruvate formed either needs to be converted to diacetyl/acetoin or need to be expelled out of cells. This is

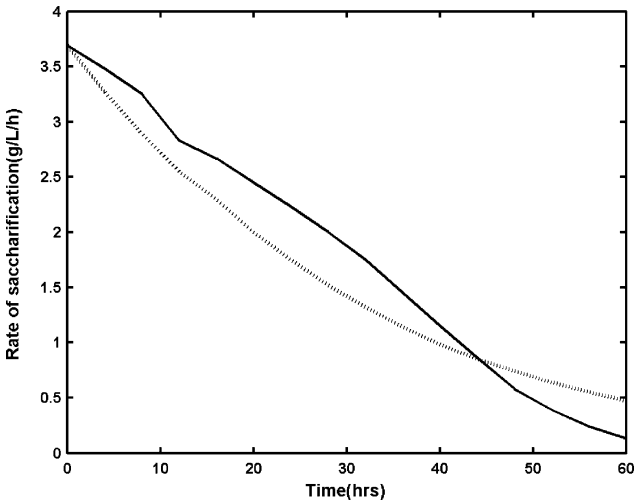

Fig. 3 Comparison of the rate of saccharification (g/L per h) between simple saccharification and SSF for $S_0 = 100$ g/L

one of the mechanisms used by the cell for detoxification. It was observed that the extracellular concentration of pyruvate was higher at higher initial starch concentration. This corresponds to an excess of pyruvate inside the cell and explains the result that the flavors accumulated at higher starch concentrations, as suggested by Hugenholtz [9].

Figure 3 shows the comparison of the saccharification rates in the simple saccharification and SSF of starch for an initial starch concentration of 100 g/L. It can be noted from the figure that, the rate of saccharification was higher in case of SSF as compared to simple saccharification upto 45 h. Since the amount of glucose accumulation was higher in case of simple saccharification, the rates were lower. Thus, SSF using *L. rhamnosus* could increase the saccharification rates by about 25% in the first 45 h, by eliminating the inhibition caused by glucose. Beyond 45 h, the saccharification rates reduced in the case of SSF due to limiting concentration of starch (see Fig. 2d). In case of simple saccharification, the limiting concentration of starch was reached only beyond 60 h (results not shown).

The yields and productivities for lactic acid and flavors from the SSF process was compared with that obtained from a two step process of saccharification followed by subsequent fermentation (see Table 3). It can be observed that the productivities and yield for both the products were higher for SSF for all the initial starch concentrations. The increase in productivities for flavors in SSF over the two step process was 2-, 2.7-, 1.3- and 1.23-folds for initial starch concentration of 10, 30, 100 and 150 g/L, respectively. The increase in the yields based on total equivalent glucose for SSF compared to two step process were 1.625-, 1.6-, 0.62- and 0.59-fold for initial starch concentration of 10, 30, 100, 150 g/L, respectively. Thus, at low starch concentration, SSF offers a clear advantage in terms of both productivity and yield for the production of flavors. In case of lactic acid as the final product, the fold increase in productivities in SSF as compared to the two step process were 1.17, 1.89, 0.65, 2.1 for initial starch concentration of 10, 30, 100 and 150 g/L, respectively. Thus, a higher starch concentration has to be used for producing lactic acid as the final product. However, for both the products, SSF allows an advantage at higher starch concentrations, which is obvious, as at lower starch concentrations, the glucose inhibition on the saccharification step is negligible. Due to lowering of glucose inhibition, the SSF process inherently becomes faster and thus overall productivity increases as compared to a two step process resulting in higher productivity for lactate and flavors. However, the yield saturates as the starch concentration is increased due to lower growth rate caused by product accumulation. Further, the acetate concentration also increases at higher starch concentration reducing the yield towards lactate and flavor.

SSF was also operated by adding 100 g/L of starch in a three intermittent stages. The starches added were in the concentrations of 30, 30 and 40 g/L at instances, where the diacetyl concentration started to decrease. This occurred at 38 and 67 h during the SSF process. Figure 4a shows the glucose concentration profile for such a three stage process with intermittent substrate feed strategy. It can be seen that the maximum glucose concentration reached was 9 g/L, whereas it was 23 g/L in a batch process with an initial starch concentration of 100 g/L (see Fig. 2a). Thus, the

Table 3 Comparison of productivity and yield of lactic acid and flavor between SSF process and two step saccharification and fermentation process at various initial starch concentrations

S_0	SSF_PF	SSF_YF	SSF_PL	SSF_YL	2SP_PF	2SP_YF	2SP_PL	2SP_YL
10	0.02	0.13	0.07	0.44	0.01	0.08	0.06	0.54
30	0.065	0.16	0.3	0.74	0.024	0.1	0.16	0.69
100	0.098	0.068	0.8	0.6	0.076	0.11	0.43	0.62
150	0.12	0.065	1.11	0.61	0.1	0.11	0.54	0.64

SSF simultaneous saccharification and fermentation, *2SP* two stage process, first stage saccharification and second stage fermentation, *PL* lactate productivity (g lactate/L per h), *YL* lactate yield (g lactate/g glucose), *PF* flavor productivity (g flavors/L per h), *YF* flavor yield (g flavors/g glucose)

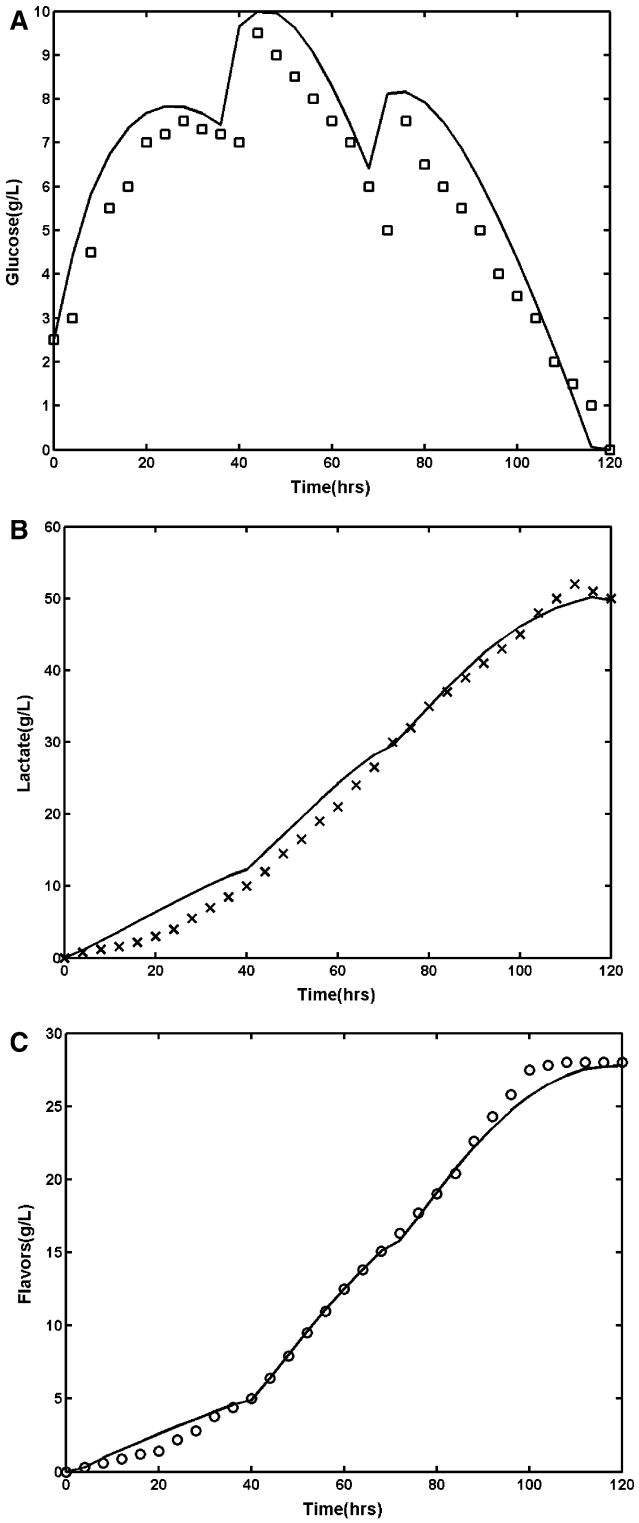

Fig. 4 Comparison of model prediction and experimental data for the three stage SSF process with intermittent addition of starch. Starch was added in three stages with 30, 30 and 40 g/L in the first, second and third stages, respectively. The starch was added at end of 38 and 70 h. **a** Glucose concentration, **b** lactic acid concentration, **c** flavor concentrations. *Solid lines* show the model prediction and symbols show: *open square* glucose concentration, *cross* lactic acid, *open circle* flavors

intermittent feed strategy relieves even the slightest glucose inhibition on saccharification rates. Further, due to the three stages, the glucose concentration reached zero in about 120 h, as compared to 70 h in case of the batch process. This ensures that the cells remain in the exponential growth phase for a longer duration of time as compared to that in a batch process. Figure 4b shows the lactate profile for the three stage process. The maximum lactate concentration was 51 g/L at the end of 120 h as compared to 60 g/L in case of the batch process. Further, since glucose concentration was zero, due to the absence of the catabolite repression, lactate was consumed in the batch process, while lactic acid accumulated till the end of the fermentation. The net flavor concentration of 27 g/L was observed in the intermittent feed strategy (see Fig. 4c) at the end of 100 h, with a productivity of 0.27 g/L per hour. The productivity was 2.7-fold greater than that observed in the batch process. This may be due to longer exponential growth phase that was observed in the three stage process and it is known that the product diacetyl is growth associated. Further, the decreased lactic acid concentration in the three stage process also enhances flavor production, since it is reported that lactic acid has a negative effect over flavor formation [9]. The pyruvate concentration in the medium also increased to 30 mM, indicating activation of diacetyl and acetoin synthesis [6, 9]. Thus, intermittent addition of starch enhanced the production of flavor substantially.

Citrate is metabolized by *L. rhamnosus* and is known to enhance the yields for flavor production. Thus, the model was used to simulate the fermentation profiles for SSF with addition of citrate into the starch medium. It was established that for 75 g/L of citrate in the medium, the amount of diacetyl produced would inhibit the fermentation reducing the overall growth and product yields [7]. Further, citrate also inhibits glucoamylase reducing the rates of saccharification [3]. Incorporating both the inhibitions, it was determined that the optimum citrate concentration that can be used for SSF was 40 g/L. Figure 5 shows the profiles for various metabolites during the SSF process using a medium containing 100 g/L of starch and 40 g/L of citrate. It can be observed that the starch was completely hydrolyzed in 60 h with a final product concentration of 63 and 15 g/L of lactic acid and flavors, respectively. The 40 g/L of citrate was also consumed in 60 h. This results in a productivity of 1.05 and 0.25 g/L per hour for lactic acid and flavors, respectively. As in the case of SSF without citrate addition, the glucose concentration was kept below the inhibition constant of 33 g/L throughout the fermentation time of 60 h. Therefore, the addition of citrate enhanced flavor production, offering a further advantage of reducing fermentation times. In case of citrate addition, the fermentation time (60 h) was lower with a final flavor

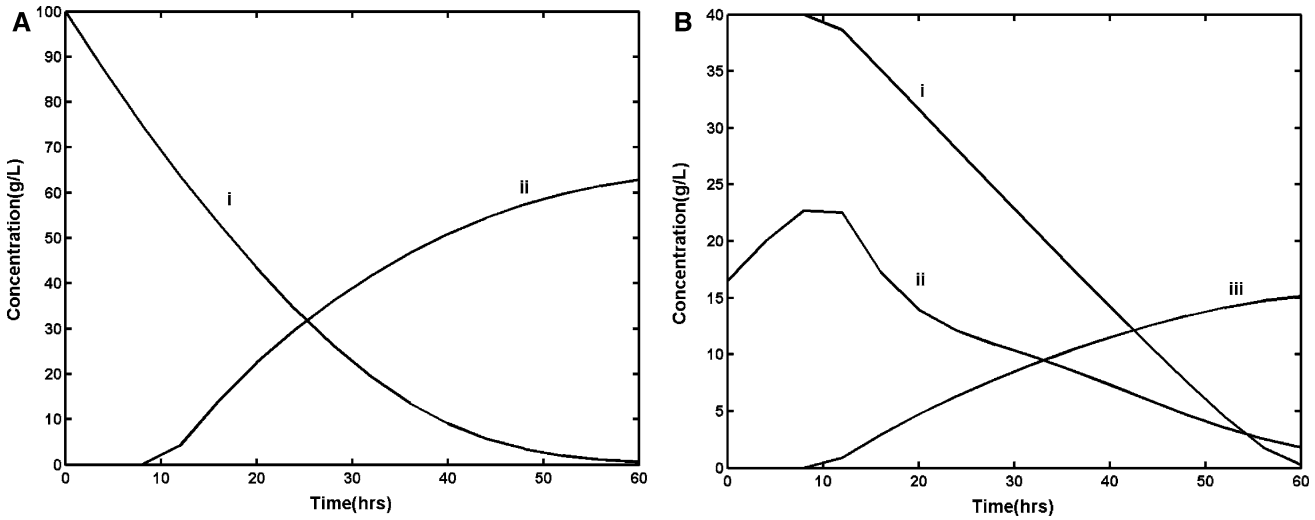

Fig. 5 Model prediction of SSF performance with addition of 40 g/L of citrate to a medium containing 100 g/L of starch. **a** Starch concentration (*i*), lactic acid concentration (*ii*), **b** citrate concentration (*i*), glucose concentration (*ii*), flavor concentration (*iii*). Experiments were conducted with these conditions and the final lactate and flavor concentration at the end of 60 h were determined to be 65 and 15 g/L, respectively. These values closely match the predicted values

concentration of 15 g/L, while in case of the three stage process, the fermentation time was longer (100 h) with a higher flavor concentration of 27 g/L. Thus, the productivity for flavor production due to citrate addition was comparable to the productivity obtained in a three stage intermittent addition of starch. Experiments were conducted with these conditions and the final lactate and flavor concentration at the end of 60 h were determined to be 65 and 15 g/L, respectively and these values closely matched the predicted values.

Conclusion

A structured kinetic model was developed for SSF of starch to flavor compounds and compared with experimental data. The models for saccharification of starch and growth of *L. rhamnosus* on glucose and citrate were independently obtained to predict the performance of SSF. The model parameters were also obtained at different pH and temperatures to predict the performance of SSF. It was noted that parameters related to the product formation had to be altered to fit the SSF data, although the trends were similar. The model demonstrated that the flavors could be produced optimally at pH 5.5 and at a temperature of 30°C. The model was able to predict the experimental observations of the dynamic profiles for glucose, lactic acid and flavor compounds.

As it was observed that there was sufficient accumulation of glucose under high feed concentration of starch, a process with three stages with intermittent addition of starch was also operated. The model was able to predict the three stage process accurately and demonstrated that the formation of flavor compounds can be considerably enhanced. This was mainly due to the maintenance of low glucose concentration (<9 g/L) for a longer duration as compared to that in a batch process which enhanced flavor synthesis. Since *L. rhamnosus* could metabolize citrate, another strategy that was used to enhance flavor synthesis was the addition of citrate into the SSF medium. The organism could metabolize citrate in presence of glucose and the excess pyruvate synthesized was directed towards the synthesis of flavors [6]. The structured model incorporated the consumption of multiple substrates and could predict the SSF process with addition of citrate in to the medium. Addition of citrate enhanced flavor synthesis as reported and also reduced the fermentation times due to increased growth.

SSF is a promising technology for converting renewable bioresource such as starch and cellulose to fermentative products. In the recent past, many studies have been reported wherein saccharification and fermentation has been combined to yield high productivities of products, such as lactic acid, ethanol, butanol, hydrogen, etc. Here, we have reported two strategies, one biochemical (addition of citrate) and another process related (three stage process) to enhance productivities using SSF process. The kinetic model developed could capture the lag phase, preference of multiple substrates as energy source by the organism, effect of process variables such as substrate, pH and temperature and feeding strategy used to operate the SSF process. Thus, the model developed can now truly be used to design and optimize a SSF process for flavor production in batch as well as a fed-batch process.

References

1. Abe S, Takaji M (1991) Simultaneous saccharification and fermentation of cellulose to lactic acid. Biotechnol Bioeng 37:93–96. doi:10.1002/bit.260370113
2. Kroumor AD, Modenes AN, Tait MCA (2006) Development of new unstructured model for simultaneous saccharification and fermentation of starch to ethanol by recombinant strain. Biochem Eng J 28(3):243–255. doi:10.1016/j.bej.2005.11.008
3. Anuradha R (1999) Simultaneous saccharification and fermentation of starch. Ph.D. Thesis, Indian Institute of Technology, Bombay
4. Anuradha R, Suresh AK, Venkatesh KV (1999) Simultaneous saccharification and fermentation of starch to lactic acid. Process Biochem 35(3):367–375. doi:10.1016/S0032-9592(99)00080-1
5. Asenjo JA, Sun WH, Spencer JL (1990) Optimization of batch processes involving simultaneous enzymatic and microbial reactions. Biotechnol Bioeng 37(11):1087–1094. doi:10.1002/bit.260371114
6. Cardenas IL, Ledesma OV, Oliver G (1989) Effect of lactic acid on diacetyl and acetoin production by *Lactobacillus casei* subsp. *Rhamnosus* ATCC 7469. Curr Microbiol 18:351–354. doi:10.1007/BF01571127
7. Dixit JB, Suresh AK, Venkatesh KV (2003) An optimal model for representing the kinetics of growth and product formation by *Lactobacillus rhamnosus* on multiple substrates. J Biosci Bioeng 96(5):481–486
8. Doshi P, Venkatesh KV (1998) An optimal model for microbial growth in a multiple substrate environment: simultaneous and sequential utilization. Process Biochem 33(6):663–670. doi:10.1016/S0032-9592(98)00031-4
9. Hugenholtz J (1993) Citrate metabolism in lactic acid bacteria. FEMS Microbiol Rev 12:165–178. doi:10.1111/j.1574-6976.1993.tb00017.x
10. Kompala DS, Ramkrishna D, Tsao GT (1984) Cybernetic modeling of microbial growth on multiple substrates. Biotechnol Bioeng 26:1272–1281. doi:10.1002/bit.260261103
11. Leudeking R, Piret EL (1959) A kinetic study of the lactic acid fermentation batch process at controlled pH. J Biochem Microbiol Technol Eng 1:393–405. doi:10.1002/jbmte.390010406
12. Li D, Chen H (2007) Biological hydrogen production from steam-exploded straw by simultaneous saccharification and fermentation. Int J Hydrogen Energy 32(12):1742–1748. doi:10.1016/j.ijhydene.2006.12.011
13. Linde M, Galbe M, Zacchi G (2007) Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. Enzyme Microb Technol 40(5):1100–1107. doi:10.1016/j.enzmictec.2006.08.014
14. Ohgren K, Bengtsson O, Gorwa-Grauslund MF, Galbe M, Hahn-Hägerdal B, Zacchi G (2006) Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with Saccharomyces cerevisiae TMB3400. J Biotechnol 126(4):488–498. doi:10.1016/j.jbiotec.2006.05.001
15. Ohgren K, Bura R, Lesnicki G, Saddler J, Zacchi G (2007) A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochem 42:834–839. doi:10.1016/j.procbio.2007.02.003
16. Qureshi N, Saha BC, Cotta MA (2008) Butanol production from wheat straw by simultaneous saccharification and fermentation using *Clostridium beijerinckii*: Part II. Fed-batch fermentation. Biomass Bioenergy 32(2):176–183. doi:10.1016/j.biombioe.2007.07.005
17. Rivas B, Moldes AB, Domínguez JM, Parajó JC (2004) Lactic acid production from corn cobs by simultaneous saccharification and fermentation: a mathematical interpretation. Enzyme Microb Technol 34(7):627–634. doi:10.1016/j.enzmictec.2004.01.011
18. Rojan PJ, Rajeev KS, Madhavan NK, Ashok P (2007) Statistical optimization of simultaneous saccharification and l (+)-lactic acid fermentation from cassava bagasse using mixed culture of lactobacilli by response surface methodology. Biochem Eng J 36(3):262–267. doi:10.1016/j.bej.2007.02.028
19. Romani A, Yáñez R, Garrote G, Alonso JL (2008) SSF production of lactic acid from cellulosic biosludges. Bioresour Technol 99(10):4247–4254
20. Roy S, Gudi RD, Venkatesh KV, Shah SS (2001) Optimal control strategies for simultaneous saccharification and fermentation of starch. Process Biochem 36:713–722. doi:10.1016/S0032-9592(00)00270-3
21. Sharpell FH Jr (1985) Microbial flavors and fragrances. In: Moo-Young M (ed) Comprehensive biotechnology: the principles, applications and regulations in industry, agriculture and medicine, vol 3. Pergamon Press, New York
22. Tanaka T, Hoshina M, Tanabe S, Sakai K, Ohtsubo S, Taniguchi M (2006) Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation. Bioresour Technol 97(2):211–217. doi:10.1016/j.biortech.2005.02.025
23. Venkatesh KV, Doshi P, Rengaswamy R (1997) An optimal strategy to model microbial growth in a multiple substrate environment. Biotechnol Bioeng 56(6):635–644. doi:10.1002/(SICI)1097-0290(19971220)56:6<635::AID-BIT6>3.0.CO;2-O
24. Westerfeld WW (1945) A colorimetric determination of blood acetoin. J Biol Chem 162:495–502
25. Wilkins MR, Widmer WW, Grohmann K (2007) Simultaneous saccharification and fermentation of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol. Process Biochem 42(12):1614–1619