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Utilisation of waste bread for fermentative succinic acid production

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ABSTRACT

A novel biorefinery concept of utilising waste bread as a sole nutrient source for the production of a nutrient rich feedstock for the fermentative succinic acid production by *Actinobacillus succinogenes* has been developed. Waste bread was used in the solid-state fermentations of *Aspergillus awamori* and *Aspergillus oryzae* that produce enzyme complexes rich in amylolytic and proteolytic enzymes, respectively. The resulting fermentation solids were added directly to a bread suspension to generate a hydrolysate containing over 100 g/L glucose and 490 mg/L free amino nitrogen (FAN). A first-order kinetic model was used to describe the effect of initial bread mass ratio on glucose and FAN profiles. The bread hydrolysate was used as the sole feedstock for *A. succinogenes* fermentations, which led to the production of 47.3 g/L succinic acid with a yield and productivity of 1.16 g SA/g glucose and 1.12 g/L h. This corresponds to an overall yield of 0.55 g succinic acid per g bread. This is the highest succinic acid yield compared from other food waste-derived media reported to date. The proposed process could be potentially utilised to transform no-value food waste into succinic acid, one of the future platform chemicals of a sustainable chemical industry.

1. Introduction

Food waste is one of the largest potions of municipal solid waste with a fraction falls between 12% and 39% among different countries, such as United States [1], Hong Kong [2] and Singapore [3], which causes severe environmental problems. Utilisation of food waste as the renewable feedstock in biorefinery for chemicals and energy production becomes a promising option to target both solid waste problem and the over-dependence on petroleum as the major source of chemicals and energy [4,5]. The nutrients contained within food waste can be converted by micro-organisms into desired products, such as biofuels [6–8], functional chemicals [9] and monomers of bioplastics [10].

Succinic acid (SA), as a precursor for many chemical substances [11] with a production capacity of 30,000 tonnes per year and a corresponding market value of $225 million [12], has attracted research interest in the development of fermentative SA production as an alternative to the current non-sustainable petrochemical route. This bio-based SA production has a lower energy consumption due to milder operation conditions and lower dependence on a single feedstock [13,14]. The common micro-organisms used in fermentative SA bio-production are *Actinobacillus succinogenes* [15], *Anaerobiospirillum succiniciproducens* [16], *Mannheimia succiniciproducens* [17] and recombinant *Escherichia coli* [18]. The possibility of using these micro-organisms to convert food waste into SA has been explored. Yu et al. [19] examined SA production from corncob by *A. succinogenes*. Li et al. [20] demonstrated SA production from orange peel by *Fibrobacter succinogenes*.

Nutrients in food are stored in the form of macromolecules such as starch and proteins. To facilitate the growth of micro-organisms, these large molecules have to be broken down into utilisable form such as sugars and amino acids. Usually, enzymatic hydrolysis using α-amylase and protease can efficiently facilitate this process [21]. Compared with methods for the hydrolysis of lignocellulosic based materials [22], enzymatic hydrolysis has several outstanding advantages. This includes mild reaction conditions, avoidance of using hazardous chemicals and reduced risks of generating fermentative inhibitors. Apart from using commercial available enzymes in the hydrolysis, solid-state fermentation [23] is a preferred option to produce enzymes with advantages such as high enzyme concentration due to absence of free water and low protein breakdown [24].

Among various micro-organisms used in solid state fermentations (SSF), filamentous fungi are widely exploited. Du et al. [25] developed a strategy of generating glucoamylase from *Aspergillus awamori* and protease from *Aspergillus oryzae* in SSF on wheat.

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Nomenclature

A	linear regression constant for Eq. (4) (g/L)
B	linear regression constant for Eq. (4) (g/L)
$C(t)$	glucose/FAN concentrations at time t (g/L for glucose; mg/L for FAN)
C_∞	saturation concentration of glucose/FAN (g/L for glucose; mg/L for FAN)
k	kinetic constant (h^{-1}).
FAN	free amino nitrogen
OD_{660}	optical density at 660 nm
SA	succinic acid
SSF	solid-state fermentation
t	time (h)
TS	total sugars (g/L)
x	initial bread mass ratio (w/v%)

These enzymes were utilised to hydrolyse gluten-free flour and gluten into carbon-rich and nitrogen-rich streams, respectively. Dorado et al. [26] devised another strategy of combining hydrolysis of starch and protein with fungal autolysis. In this strategy, *A. awamori* and *A. oryzae* SSF solids were added to wheat middlings and bran at 55 °C to produce a nutrient-complete stream, which was subsequently utilised for fermentative SA production. Melikoglu [27] developed a multi-enzyme solution of glucoamylase and protease during solid-state fermentation using *A. awamori*. This was used in subsequent hydrolysis reactions to produce nutrient rich hydrolysates from waste bread pieces and from wheat flour. Considering the similar composition of waste bread and wheat, it should be possible to utilise waste bread as a feedstock for the production of fermentation medium using a novel bioprocess as illustrated in Fig. 1.

2. Materials and methods

All chemicals involved in this study were obtained from Sigma–Aldrich, US except otherwise specified.

2.1. Micro-organisms

A. awamori ATCC 14331 was used for the production of amylolytic enzymes. An industrial strain of *A. oryzae* isolated from a soy sauce starter kindly provided by the Amoy Food Ltd., Hong Kong. It was utilised to produce proteolytic enzymes. Their storage and sporulation for inoculum preparation have been described in a previous publication [25]. *A. succinogenes* ATCC 55618 were utilised for succinic acid fermentations.

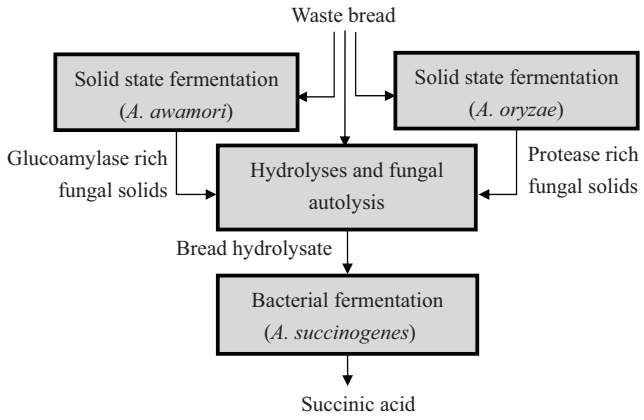

Fig. 1. Process flow diagram of the proposed waste bread biorefinery concept for succinic acid production. The biorefinery consists of three steps: (1) *A. awamori* and *A. oryzae* SSF were carried out on waste bread to obtain glucoamylase and protease-rich fungal solids. (2) The solids were added to a waste bread suspension to produce a nutrient-rich hydrolysate. (3) *A. succinogenes* fermentation using the waste bread hydrolysate for SA production.

2.2. Raw materials

Waste bread was kindly provided by the Coffee Shop at the Hong Kong University of Science and Technology (HKUST). Moisture and starch were analysed according to procedures described in Koutinas et al. [28].

2.3. Solid state fermentation (SSF)

Bread was cut into cubes of approximately 1 cm^3 in size and autoclaved for 15 min prior to cultivation. Ten grams of bread cubes were transferred into each Petri dish. One millilitre of cryopreserved spores of *A. awamori* (2.85×10^7 spores/mL) or *A. oryzae* (6.31×10^6 spores/mL) were diluted by 10-folds and spread evenly on bread pieces separately. The prepared samples were incubated at 30 °C for 4 days.

2.4. Enzymatic hydrolysis

Two 2.5 L bioreactors equipped with automatic temperature control water jacket and stirrers were used for enzymatic hydrolysis. Various amount of waste bread pieces were blended with 1 L water for 15 min. The resulting mixture was transferred into the bioreactors at 55 °C. Fungal marshes from SSF were added into the vessel. The reaction mixture pH was not controlled and was stirred at 300 rpm. Samples were taken every hour for 24 h. The resultant broth was centrifuged at 10,000 rpm for 15 min. The supernatant was subsequently filtered by vacuum filtration using Whatman No.1 filter paper. All experiments were carried out in duplicate.

2.5. Bacterial fermentation

Two 2.5 L fermentors (Biostat, Germany) were used for fermentative SA production by *A. succinogenes*. Bacterial fermentation using bread hydrolysate was preformed and the inoculums size was 5%v/v. The initial glucose concentration of the broth was 40 g/L while the free amino nitrogen (FAN) concentration was 200 mg/L. The latter corresponds to the FAN content of a 4 g/L yeast extract solution. The hydrolysate was filtered by 0.2 μm PTFE membrane filter (Sartorius, Germany). Prior to autoclaving, 10 g/L magnesium carbonate ($MgCO_3$) was added to the fermentation medium as a neutral pH buffer. The pH of the fermentation broth was automatically controlled within 6.6–6.8 with the addition of 10 M NaOH and 0.05 M H_2SO_4. The broth was sparged with 0.5 vvm CO_2 and agitated at 300 rpm. Fermentation samples were taken every 3 h to measure optical density and metabolites concentration. Fermentations were considered to have ended when glucose was completely depleted.

2.6. Analytical techniques

2.6.1. Cell density measurement

Bacterial growth was determined by optical density (OD) measurements at 660 nm (spectrophotometer UV-1800, Shimadzu, Japan). Optical density (OD), glucose and fermentation metabolites were determined as described previously [17]. At an OD_{660} of 1.0 in TSB, *A. succinogenes* has a concentration of 0.626 g dry cell weight (DCW)/L [17].

Table 1
Waste bread composition per 100 g bread.

Moisture	22.3 g
Starch (dry basis)	59.8 g
Total organic nitrogen (dry basis)	1.56 g
Protein (TN × 5.7) (dry basis)	8.9 g
Total phosphorus	Trace

2.6.2. High performance liquid chromatography (HPLC)

High performance liquid chromatography (Waters, UK) equipped with BIO-RAD column (HPX-87H), refractive index (RI) detector (Waters, UK) and photodiode array (PDA) analyser (Waters, UK) were used to quantify the glucose and metabolite concentration of the samples. Five millimolar H_2SO_4 was used as the mobile phase. The flowrate was set to 0.6 mL/min. The column and RI detector were equilibrated to 65 °C and 35 °C, respectively. Injection volume of all samples and standards were set to 10 μL.

2.6.3. Free amino nitrogen (FAN) analysis

The FAN concentration of hydrolysis samples was analysed by the ninhydrin colorimetric method promulgated in the European Brewery Convention [29].

3. Result and discussions

3.1. Waste bread composition

Table 1 shows the composition of waste bread which is rich in organic carbon and nitrogen. Around 60% of the dry mass of waste bread is hydrolysed predominantly into glucose when significant activity of glucoamylase is used. According to Kent [30], 100 g of white bread contains around 50 g carbohydrate (where 47 g is in the form of starch), 37 g water, and around 8 g protein, which make 95% of the total weight. This composition makes bread an excellent source of nutrition.

3.2. Production of fermentation feedstock from waste bread

This section presents the feasibility of utilising SSFs of *A. awamori* and *A. oryzae* on waste bread for the production of enzyme complexes that convert bread pieces into a generic fermentation medium. As mentioned above, this strategy involves the subsequent hydrolyses of waste bread and fungal autolysis. The addition of *A. awamori* and *A. oryzae* SSF solids into the suspensions of waste bread led to the production of a generic fermentation feedstock that is rich in glucose and nitrogen, as well as other essential nutrients required for the subsequent SA fermentation.

Fig. 2 shows the glucose and FAN concentration profiles in the hydrolysate with an initial 10–30% (w/v) bread concentration. Both glucose and FAN productions almost reached their saturation concentrations after 18 h. Therefore, the enzymatic hydrolysis of waste bread for 24 h is sufficient to break down the nutrients completely. The duration of the hydrolysis reactions was similar to the hydrolysis of wheat flour milling by-products using a paddle reactor as reported in our previous study [26].

In order to develop a model that can predict the reaction rate at different initial bread concentrations, the reaction order of the starch hydrolysis and protein hydrolysis by glucoamylase and protease were firstly determined by fitting the glucose and FAN profiles with zeroth-order (Eq. (1)), first-order (Eq. (2)) and second-order (Eq. (3)) reaction rate equations. The model that best fit the observed results will have the coefficients of determination (R^2) value closer to 1. In this study, the saturation glucose and FAN concentrations, C_∞ are determined by averaging the last four data points in the profile. Using 30% (w/v) bread mass ratio as an example, the comparison of the R^2 values for the models (Table 2) shows that the enzymatic hydrolysis follows a first-order reaction kinetic.

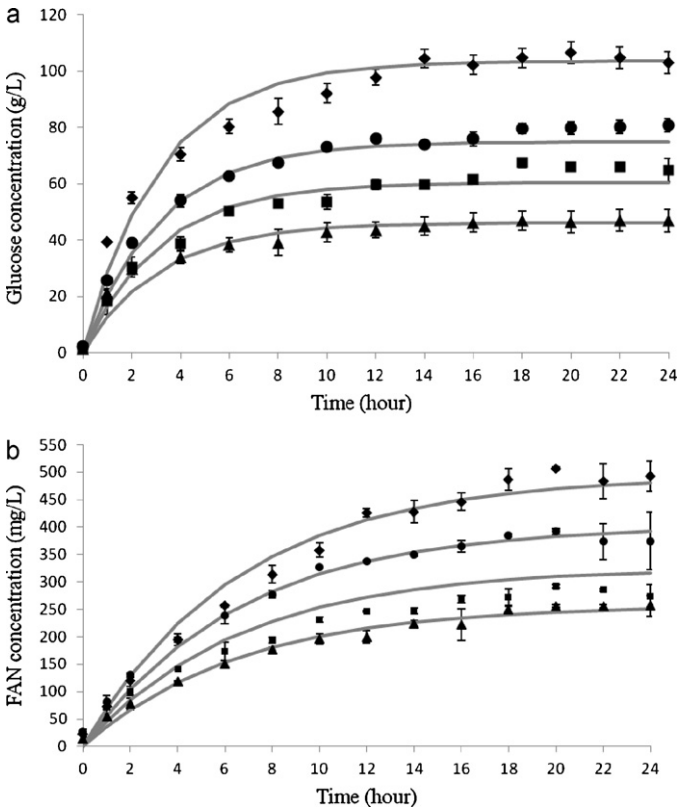

Fig. 2. (a) Glucose production from simultaneous hydrolytic/autolytic reactions using 10%, 15%, 20% and 30% (w/v, dry basis) (b) FAN production from combined hydrolytic/autolytic reactions using 10%, 15%, 20% and 30% (w/v, dry basis). 10%(▲); 15%; (■); 20% (w/v) (●); 30% (w/v) (♦) at 55 °C. The first-order kinetic model fitting of corresponding conditions was shown as solid grey line. *A. awamori* and *A. oryzae* cultivated bread from solid-state fermentation were added at time = 0. Hydrolysis was carried out at 55 °C. Average values and error bars of the duplicate experiments of each condition are shown.

$$C(t) = kt \quad (1)$$

$$C(t) = C_\infty(1 - e^{-kt}) \quad (2)$$

$$C(t) = C_\infty - \frac{1}{(1/C_\infty) + kt} \quad (3)$$

where $C(t)$ is the glucose/FAN concentrations at time t, C_∞ is the saturation concentration of glucose/FAN and k is the kinetic constant (1/s).

Among the three reaction rate equations, the first-order reaction rate equation (Eq. (2)) fitted both glucose and FAN profiles, with R^2 values for the experimental and modelled profiles closest to 1. Therefore, the first-order kinetic model (Eq. (2)) is selected to describe the enzymatic hydrolysis reported in this study.

As shown in Eq. (2), the saturation glucose and FAN concentrations C_∞ are required in order to predict the glucose and FAN profiles of hydrolysis. In a similar scenario, Delgado et al. [31] demonstrated that a linear relationship between saturation

Table 2
R^2 values for different kinetic models and the experimental result of hydrolysis at 30% (w/v) bread mass ratio.

	Coefficient of determination (R^2)		
	Zeroth-order	First-order	Second-order
Glucose concentration	0.00	0.96	0.95
FAN concentration	0.76	0.98	0.89

Table 3
Kinetic model parameters for fitting glucose and FAN productions in enzymatic hydrolysis.

	Parameters for Eq. (4)			R^2 for various bread mass ratio, x			
	A (g/L)	B (g/L)	k	10%	15%	20%	30%
Glucose	2.87	17.5	0.32	0.92	0.96	0.98	0.97
FAN	11.8	149.0	0.15	0.98	0.90	0.97	0.98

concentration (C_∞) and hydrolysis temperature can be deduced by performing experiments in various temperatures. This approach can be applied to correlate the initial bread mass ratio of hydrolysis, x with their corresponding saturation concentration, C_∞ by linear regression. In this case, the saturation concentration, C_∞ is replaced with a linear expression ($Ax + B$) as shown in Eq. (4).

$$C(t) = (Ax + B)(1 - e^{-kt}) \quad (4)$$

$C(t)$ = glucose/FAN concentration at time (t) (g/L), x = bread mass ratio (w/v%), k = reaction rate constant (h^{-1}), A and B = empirical constants from linear regression depends on initial substrate (g/L)

The model was fitted to the final glucose and FAN concentrations of enzymatic hydrolysis with different initial bread concentrations, x ranging from 10% (w/v) to 30% (w/v). As shown in Fig. 2, glucose and FAN concentrations produced from bread hydrolysate can sufficiently fulfil the nutrient requirement of a typical SA fermentation [32], which are around 100 g/L glucose and 200–300 mg/L FAN. Fig. 3 shows the kinetic model together with the experimental results for comparison.

By substituting the saturation concentration (C_∞) in Eq. (2) using the linear expression ($Ax + B$), the first-order kinetic model for glucose and FAN productions under different initial bread mass ratio were constructed (Table 3). The R^2 values close to 1.0 indicate that the modified first-order kinetic model by linear regression is applicable for the prediction of the bread hydrolysis at various initial bread mass ratios, x. As shown in Fig. 2, high consistencies between the first-order kinetic model and the experimental results were observed.

Table 4 shows the amount of sugars and FAN yields from the hydrolysis of waste bread and other food waste substrates. This method resulted 0.47 g glucose/g bread with 90.8% starch to glucose conversion, and 2.55 mg FAN/g bread. Stoichiometric coefficient of 1.11 has been included in the calculation of starch to glucose conversion to take into account of the additional mass of water taken during hydrolysis.

Lignocellulosic biomass consists of hemicelluloses which can be converted into sugar monomers such as xylose, arabinose, mannose, galactose and rhamnose [21]. Therefore, total sugars (TS) is used in Table 4 for the comparison of production yield of different types of food waste. Wheat, wheat flour milling by-product and bread consist high starch content in which starch can be hydrolysed either by chemical or enzymatic methods. Therefore, the total sugar yield is significantly higher as compared to corncob and rapeseed meal which compose of lignocellulosic materials. *A. succinogenes* has been reported to be capable of utilising various types of sugars such as glucose, maltose, xylose and cellobiose for SA production [19,26], while FAN is essential for cell growth [26]. In such cases, substances with higher total sugar production yield should be used in *A. succinogenes* fermentation. Wang et al. [32] also investigated the use of rapeseed meal for FAN production by *A. oryzae*. Protease produced from *A. oryzae* was used to release FAN from the rapeseed meal and over 500 mg/L FAN was released. This study suggested that rapeseed meal would be an alternative substrate for the efficient production of SA by *A. succinogenes* without additional nitrogen source supplementation such as yeast extract.

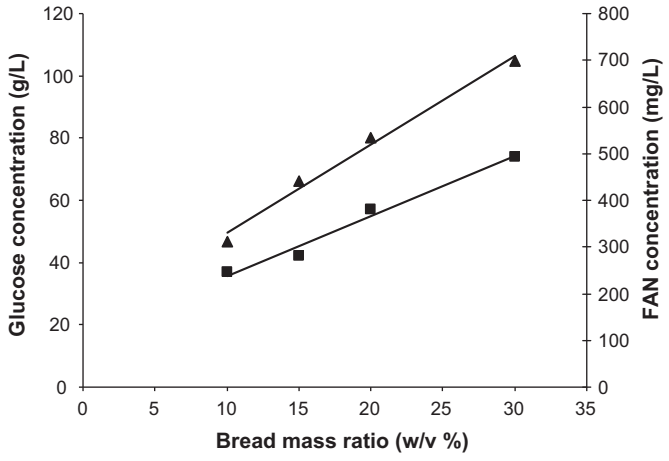

Fig. 3. The linear regression of saturation glucose (▲) and FAN (■) concentrations with various initial bread mass ratio.

3.3. Succinic acid fermentation using bread hydrolysate

The potential for utilising the bread hydrolysate in SA fermentation was investigated and is reported in this section. Fig. 4 presents the fermentation profiles for glucose consumption, cell growth and SA formation. 10 g/L $MgCO_3$ was added into the medium as a pH buffer. Without the additional supplement of sugars and FAN into the bread hydrolysate, *A. succinogenes* growth in the hydrolysate was observed in 12 h, which is shown by significant increase in OD_{660} from 0 to 2.98. This indicates that bread hydrolysate contains sufficient nutrients to support the cell growth. SA production began during the beginning of the exponential growth phase at 15 h and continued until 45 h. During stationary phase, the average OD_{660} was around 6.8, which was 50% higher than fermentation using wheat milling by-product hydrolysate as fermentation feedstock [26]. After 60 h 47.3 g/L SA was produced which corresponds to a yield and productivity of 1.16 g SA/g glucose and 1.12 g SA/L h, respectively. The overall conversion of waste bread into SA was 0.55 g SA/g bread.

SA production using various food waste reported in the literature is summarised in Table 4. Among other food waste-derived media, SA yield achieved using waste bread (1.16 g SA/total sugar) is the highest, to date. In this study, the amount of acid by-products formed in *A. succinogenes* fermentation was significantly less, in which 0.2 g/L acetic acid was produced with no formic nor pyruvic acids peaks were found. Whereas in the earlier research carried out by our group using wheat or wheat flour milling by-products [25,26], less than 10 g/L of acid by-products (e.g. acetic, pyruvic and formic acids) were produced in similar operating conditions. Considering the bread and wheat-milling by-products hydrolysates are composed mainly of glucose, which is the preferred carbon source of *A. succinogenes*. While the hydrolysates derived from corncob [19], orange peel [20], rapeseed meal [33] contain arabinose, galactose, mannose, rhamnose and xylose. Our earlier study has demonstrated that *A. succinogenes* can utilise xylose [34]. Similarly, McKinlay et al. [32] reported that sugars other than glucose would need additional processes by the cells into utilisable forms by *A. succinogenes*, and this requires extra energy. Therefore, the SA yield per total sugar in fermentation using bread hydrolysate is higher than using hydrolysates produced from lignocellulosic

Table 4
Comparison of total sugars, FAN and succinic acid yields in hydrolysis and batch fermentations using different food waste substrates.

Substrate	Total sugars yield (g TS/g substrate)	FAN yield (mg FAN/g substrate)	SA yield (g SA/g TS)	Overall SA yield (g SA/g substrate)	References
Wheat	0.984	5.3	0.40	0.40	[25]
Wheat flour milling by-product	0.384	1.27	1.02	0.087	[26]
Bread	0.47	2.55	1.16	0.55	This work
Potatoes	0.30	N/A	N/A	N/A	[30]
Corncob	0.241	N/A	0.58	N/A	[19]
Rapeseed meal[a]	0.19	N/A	0.115	N/A	[33]
Rapeseed meal[b]	0.03	24.9	N/A	N/A	[31]
Orange peel	N/A	N/A	0.58	Negligible	[20]

N/A: Not available.
[a] Rapeseed meal is treated by diluted sulphuric acid hydrolysis and subsequent enzymatic hydrolysis of pectinase, celluclast and viscozyme.
[b] Rapeseed meal is treated by enzymatic hydrolysis using *A. oryzae*.

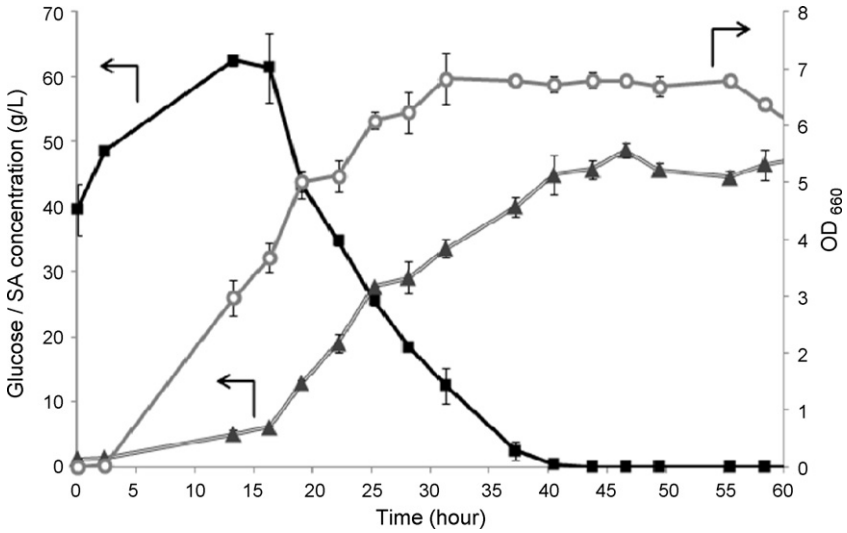

Fig. 4. Succinic acid fermentation using bread hydrolysate, with 10 g/L $MgCO_3$. Glucose (■), SA (▲) and OD_{660} (○). The average concentrations and error bars of the duplicated experiments are shown.

materials. These results clearly showed that waste bread pieces can be used for the production of value-added products. Consequently, all the steps of the bioprocess, represented in Fig. 1, were experimentally validated with this study.

4. Conclusions

This paper has demonstrated the feasibility of utilising waste bread as a genetic feedstock for the production of SA as a value-added product. The results showed that solid-state fermentation of waste bread pieces can be successfully used to process waste bread for the production of nutrient rich hydrolysates. Experimental studies showed that the empirical first-order kinetic model could be used to predict the amount of glucose and FAN liberated during the simultaneous hydrolysis and fungal autolysis reactions.

Bread hydrolysate was subsequently used for the fermentative production of succinic acid by *A. succinogenes* as part of the proposed bioprocess. The results demonstrated that the bread hydrolysate contains sufficient nutrients to support *A. succinogenes* growth and SA production. The resultant SA concentration was 47.3 g/L SA and an overall yield of 0.55 g SA/g bread, which is the highest among other food waste-derived media reported. Although SA was selected as the test case, thousands of other value-added products could be produced from waste bread. This could reduce the dependence of petroleum for chemical production. Therefore, it would be beneficial to explore other profitable products in future studies. Finally, it can be suggested that the utilisation of waste bread for the production of value-added products should be seriously considered by local governments as part of their strategy for tackling the MSW problem and for the environmentally friendly production of chemicals, materials and fuels.

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