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MICROBIOLOGY

Kinetics of Whole Cells and Ethanol Production from *Candida tropicalis* TISTR 5306 Cultivation in Batch and Fed-batch Modes Using Assorted Grade Fresh Longan Juice

CHATCHADAPORN MAHAKUNTHA, ALISSARA REUNGSANG, ROJAREJ NUNTA & NOPPOL LEKSAWASDI

Abstract: The kinetic profiles of *Candida tropicalis* TISTR 5306 cultivation based on modified yeast-malt (MYM), assorted grade fresh longan juice (AsgLG) and longan solid waste extract (LSWE) medium were evaluated in 1 l batch mode. The highest ethanol concentration level (25.5 ± 0.8 g/l) and ethanol yield – $Y_{p/s}$ of 0.491 ± 0.017 g ethanol/g consumed substrate, dried biomass concentration level (9.44 ± 0.05 g/l) and dried biomass yield – $Y_{x/s}$ of 0.533 ± 0.170 g dried biomass/g consumed substrate, specific pyruvate decarboxylase (PDC) activity (0.037 ± 0.003 U/mg protein) were achieved ($p \leq 0.05$) in AsgLG medium. Scores ranking strategy were employed and AsgLG medium was subsequently selected with in the highest total score ($p \leq 0.05$) of 698 ± 7 at 48 h. The cultivation in fed-batch mode with three rounds of pulse feeding (PF) in 1 l AsgLG medium was carried out. The apparent highest ethanol and dried biomass concentration levels with corresponding yields relative to time zero were (28.3 ± 0.5 g/l, 0.482 ± 0.012 g/g) at 120 h of PF2 and (9.39 ± 0.04 g/l, 0.110 ± 0.001 g/g) at 192 h of PF3. The maximum specific PDC activity was 0.057 ± 0.006 U/mg protein during PF1 feeding.

Key words: batch mode, fed-batch mode, *Candida tropicalis*, whole cells, ethanol, phenylacetylcarbinol.

INTRODUCTION

Longan is one of the tropical fruit and the important economic fruit of Thailand. Longan was exported in the forms of fresh longan and dried longan more than 90% of total production. Ten percents of longan production was consumed domestically with the rest being exported (Office of Agricultural Economics 2019, Sudswang et al. 2018). The production level and productivity of this fruit in Thailand during 2018 and 2019 were (1.077 and 1.011) million t as well as (5.92 and 5.40) t/ha, respectively, with the corresponding expected production figures of 1.044 million t and 5.54 t/ha in 2020 (Office of Agricultural Economics 2020, Nunta et al. 2019). On the global scale, the current production of longan in Thailand would account for nearly 30% based on the overall longan annual production of 3.445–3.600 million t (2015–2017 production data) worldwide (Altendorf 2018) with exportation values of 849 and 885 million USD in 2018 and 2019, respectively (Office of Agricultural Economics 2020, Nation Thailand 2019). The Royal Thai Government employed several strategies to prevent plummetted longan

market price from overproduction, for example, by promoting domestic consumption, supporting processed products to valorize longan, and supporting research which utilized longan for production of high value products (price in USD per g) such as fructo-oligosaccharide (0.00419), longan syrup (0.18), ethanol (0.00083), and phenylacetylcarbinol or PAC (0.146) — a precursor for nasal degestant and anti-asthmatic compounds (Global Petrol Prices 2019, IndiaMART 2019a,b, Made in Thailand 2019, Wattanapanom et al. 2019, Nunta et al. 2018, Tangtua et al. 2015, 2013).

The cytoplasmic pyruvate decarboxylase (PDC) is one of the key enzymes of yeast fermentative metabolism, such as *Bacillus subtilis Candida tropicalis*, *C. utilis*, *Kluyveromyces marxianus*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Aspergillus niger* (Andrews et al. 2016, Tangtua et al. 2013, Berłowska et al. 2009). PDC was the first active enzyme of the glycolytic pathway in many fermentative microorganisms and its role was generally recognized for ethanol production (Zhao et al. 2015, Ward & Singh 2000). The whole cells obtained from yeast cultivation after ethanol production could produce phenylacetylcarbinol (PAC) by addition of pyruvate and benzaldehyde through biotransformation process (Nunta et al. 2019, Tangtua et al. 2017, Andreu & del Olmo 2014, Khan et al. 2012). The current commercial PAC production processes utilizes yeast fermentation to produce sufficient biomass for the associated accumulation of intracellular pyruvate and inducing PDC synthesis with a fed-batch system (Miguez et al. 2014, Meyer et al. 2011). By employing whole cells in the biotransformation system, one might expect higher catalyst stability than the enzymatic counterpart with the possibility of cofactors regeneration as well as decreased cost of enzyme preparation (Gunawan et al. 2007, Chen et al. 2005, Matsumoto et al. 2001).

Nunta et al. 2018 identified *C. tropicalis* TISTR 5306 as the best strain over *S. cerevisiae* TISTR 5606 for producing PAC (27.2 ± 0.7 mM) but not ethanol (22.3 ± 1.1 vs 33.4 ± 3.2 g/l) from ammonium sulphate supplemented C-grade fresh longan juice — originally containing (in mg /g fresh fruit) 51.6 ± 0.5 total sugars, 0.021 ± 0.004 nitrogen, 0.025 ± 0.001 gallic acid, and 0.016 ± 0.001 ellagic acid — in batch processes (100 ml for ethanol and 30 ml for PAC production). Further studies by Nunta et al. 2019 revealed that the optimal C / N molar ratio of 21.88 ± 0.20 between C-grade fresh longan juice and supplemented ammonium sulfate for cultivation of *C. tropicalis* TISTR 5306 could result in 24.0 ± 1.1 g/l ethanol and specific PDC activity of 0.138 ± 0.001 U/mg protein. The subsequent studies in 100 L batch and 10 L continuous processes yielded the highest ethanol concentration (g/l) and volumetric PDC acitivities (U/ml) of (13.2 ± 0.2 at 108 h, 34.3 ± 0.5 with dilution rate of 0.0492 h^{-1}) and (0.107 ± 0.001 at 48 h, 0.081 ± 0.001 dilution rate of 0.0070 h^{-1}), respectively. Detailed investigation of the experimental data at a steady state using a set of mathematical model also indicated a higher affinity constant (K_S) for sucrose (123 ± 1 g/l) in comparison with fructose and glucose (34.1 ± 1.5 g/l). Wattanapanom et al. 2019 indicated that alkali and saturated steam pretreatment steps were not required to improve total sugars yield from longan solid waste powder digestion process as this type of waste powder contained relatively high content of starch, pectin, and other carbohydrates ($36.9 \pm 2.6\%$ (w/w). In fact, the inclusion of any pretreatment stage would actually result in further loss of starch and its related components thereby affecting final sugars yield. In addition, only single step digestion of commericial enzyme mixture (amylase, glucoamylase, cellulase, and xylanase) was necessary to attain the optimal specific overall sugars productivity of $(141 \pm 1.4) \times 10^{-4}$ g total sugars/g longan solid waste powder /digestion step/ h.

The objectives of this research were to study the kinetic profiles of whole cells and ethanol production from *C. tropicalis* TISTR 5306 cultivation in batch mode with three media, namely, modified yeast-malt (MYM), assorted grade fresh longan juice (AsgLG) and longan solid waste extract (LSWE) medium. The most suitable medium type was the selected for subsequent fed-batch mode based on scores ranking process. The kinetic profile of fed-batch mode cultivation in AsgLG medium was then also elucidated and quantified for the first time by thrice pulse feeding so that the whole cells could be produced and later utilized for the previously mentioned biotransformation system.

MATERIALS AND METHODS

Microorganism and cultivation media preparation

The yeast *C. tropicalis* TISTR 5306 was purchased from Thailand Institute of Scientific and Technological Research (TISTR). Yeast Malt (YM) medium was used for inoculum media composed of (in one litre): 10.0 g glucose, 3.0 g yeast extract, 5.0 g malt extract, and 5.0 g peptone. The medium was sterilized at 121° C, 15 psi for 15 min with a portable pressure sterilizer (All American, Model No.1925x, Wisconsin, United States). Modified yeast malt (MYM) medium was used as one of cultivation medium composed of (in one litre): 100 g glucose, 3.0 g yeast extract, 5.0 g malt extract, and 5.0 g peptone. The pH of medium was adjusted to 6.0 with 10 M KOH and sterilized at 1210C, 15 psi for 15 min with a portable pressure sterilizer (Charoensopharat & Wechgama 2018). Assorted grade fresh longan juice (AsgLG) medium composed of (in one litre): 8.52 g/l $(NH_4)_2SO_4$ in longan juice for nitrogen supplement (Nunta et al. 2018). Concentrated AsgLG for pulse feeding (PF) in a fed-batch experiment of 745 g/l was prepared by vacuum evaporation at 500C to minimize caramel reaction between sugars species while Maillard's reaction remained minimal as the nitrogen content in AsgLG was relatively small (Nunta et al. 2018). After solution mixing, pH of medium was adjusted to 6.0 with 10 M KOH and sterilized with boiled water for 15 min (Gumienna et al. 2016). Longan solid waste extract (LSWE) medium was prepared by the enzymatic digestion of dried longan solid waste (LSW) with addition of 8.52 g/ll $(NH_4)_2SO_4$ as nitrogen supplement. The commercial enzyme was used which consisted of amylase and cellulase enzymes cocktail (Vland, China) with the mass : volume ratio (g/ml) for LSW to enzyme of 7 : 100 under digestion condition (50° C, 48 h, 200 rpm) as described previously (Wattanapanom et al. 2019). After solution mixing, pH of medium was adjusted to 6.0 with 10 M KOH and 200 ppm potassium metabisulphite (KMS) was used as a disinfectant (Wattanapanom et al. 2019).

Microbial propagation for whole cells and ethanol production in 1 l scale batch mode

C. tropicalis TISTR 5306 was cultivated in a 10 ml inoculation medium for later propagation in a 100 ml scale of cultivation medium. The seed inoculum of 100 ml (10% (v/v) inoculum at 6.1×10^7 CFU/ml) was added to MYM, AsgLG, and LSWE media and incubated at 30° C for 72 h without pH control between cultivation (Tangtua et al. 2013, Chen et al. 2005). The consecutive aeration were controlled at two levels, namely, 2.190 vvm during the first 24 h for whole cells production and 1.095 vvm during the next 48 h to induce ethanol formation (Wattanapanom et al. 2019).

Whole cells and ethanol production in 1 l scale fed-batch mode

Fed-batch cultivation with PF of the concentrated AsgLG was started as a batch with an initial total sugar concentration of 100 g/l. The initial substrate concentration of selected medium being fed was adjusted to 100 g/l total sugar concentration for each feeding every 48 h, which were carried out thrice (Kang et al. 2011, Cot et al. 2007) without pH control (Chen et al. 2005). The consecutive aeration were controlled at two levels as described previously in batch system but the ethanol induction stage was extended to 168 h.

Analytical methods

After sample collection, the cells pellet was separated from the supernatant by a centrifuge machine at 2,822 × g for 15 min. The separated cells pellet was washed with distilled water and dried at 105°C for 24 h (or until constant weight was reached) in order to determine dried biomass concentration. The supernatants were analyzed for sugars and ethanol concentration levels by HPLC using methods described previously. The volumetric and specific PDC activities were analyzed by carboligase assay (determination for the rate of PAC liberation between the reaction of benzaldehyde and pyruvate under previously published condition) and Bradford's assay (determination of soluble protein concentration at a physiological condition) (Wattanapanom et al. 2019, Nunta et al. 2018, Tangtua et al. 2013, 2015, Rosche et al. 2002).

Score and ranking methods

The highest value of raw data for each criterion (dried biomass concentration, ethanol concentration, protein concentration, volumetric PDC activity, and specific PDC activity) was assigned to a score of 100 whereas the highest values of protein productivity, volumetric PDC productivity, and specific PDC productivity were assigned a score of 50 (Nunta et al. 2018, Tangtua et al. 2013). The other data were calculated as proportional percentages of the highest score. The scores of all criteria from cultivation in each medium were then combined and calculated with an average percentage (Tangtua et al. 2013). The ranking list was then obtained by arranging the percentage in descending order.

Hypothesis testing

The overall experimental design was carried out in quintuplicate with the calculated mean, standard deviation, and standard error values. The reliabilities of the mean values among the samples were identified and assessed for significant difference based on the Duncan's Multiple Range Test (DMRT) procedure. The statistical analysis was employed statistical significance at $p \leq 0.05$ (Nunta et al. 2018, 2019).

RESULTS

Whole cells and ethanol production kinetics in batch mode

Whole cells (in the form of dried biomass concentration) and ethanol formation profiles of *C. tropicalis* TISTR 5306 cultivation in three media are given in Fig. 1. The cultivation using AsgLG medium resulted in

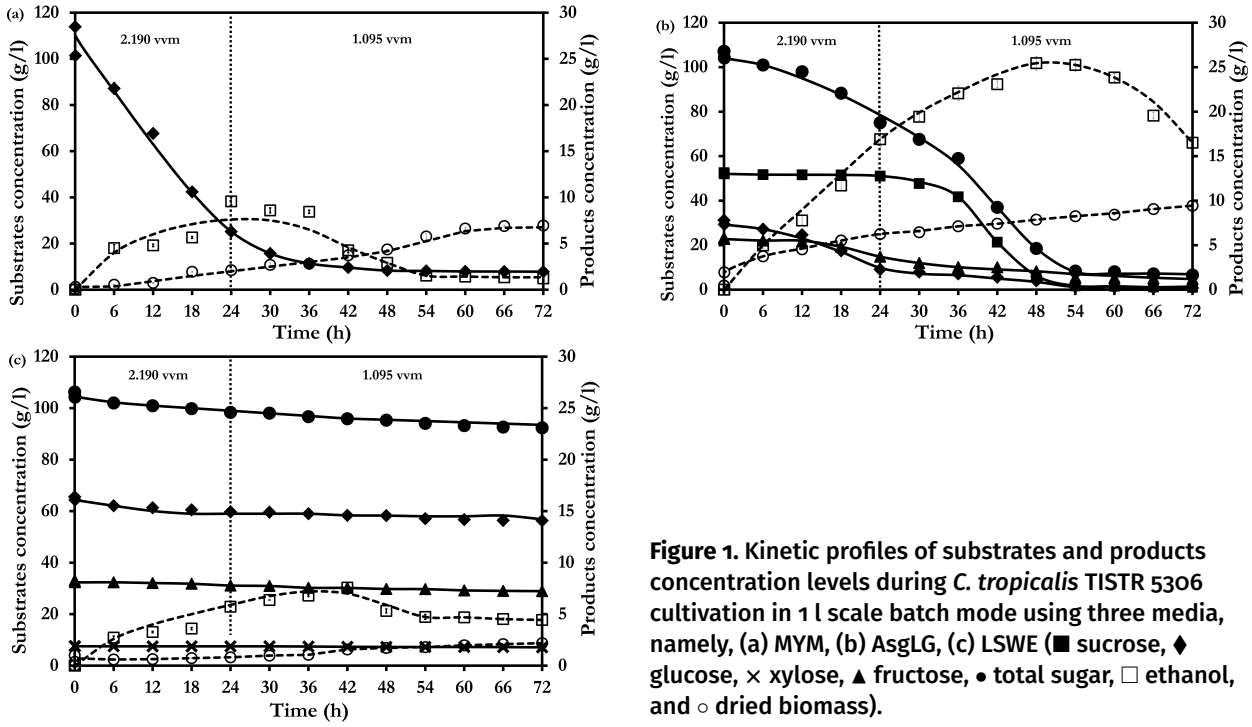

Figure 1. Kinetic profiles of substrates and products concentration levels during *C. tropicalis* TISTR 5306 cultivation in 1 l scale batch mode using three media, namely, (a) MYM, (b) AsgLG, (c) LSWE (■ sucrose, ♦ glucose, × xylose, ▲ fructose, ● total sugar, □ ethanol, and ○ dried biomass).

the highest ethanol concentration level of 25.5 ± 0.8 g/l after 48 h with corresponding highest ethanol production yield of 0.491 ± 0.017 g ethanol/g consumed substrate at 30 h and maximum specific ethanol formation rate of 0.395 ± 0.032 g ethanol/g dried biomass/h, respectively (Table I). In term of whole cells production, dried biomass concentration level and dried biomass yield were obtained with the corresponding values of 9.44 ± 0.054 g/l at 72 h and 0.533 ± 0.170 g dried biomass/g consumed total sugars at 6 h, respectively, for AsgLG medium. This was compared to the maximum specific growth rate of 0.216 ± 0.007 h^{-1} at 6 h in the same medium which was similar ($p > 0.05$) to that in MYM medium. For cultivation using MYM medium, the highest ethanol concentration (9.57 ± 0.41 g/l) was produced at 24 h while the maximum value of ethanol yield ($Y_{p/s}$) was 0.169 ± 0.022 g ethanol/g consumed total sugars at 6 h. In fact, the highest dried biomass concentration (6.95 ± 0.14 g/l at 72 h) and dried biomass yield ($Y_{x/s}$) of 0.065 ± 0.002 g/g at the same cultivation time obtained from MYM medium were also produced at the lower levels than those of LSWE medium. From Fig. 1(c), cultivation of this yeast in LSWE medium revealed slight overall decrease of all sugars (glucose, fructose, and xylose) concentration levels during the cultivation period in comparison to other types of cultivation media, although the initial μ_{max}, $q_{s,max,ts}$, and $q_{p,max}$ during the first 6 h appeared to be in the middle range when compared to other media (Table I). The trends of ethanol production, protein concentration, volumetric PDC activity, and specific PDC activity from the cultivation of *C. tropicalis* TISTR 5306 in three media are shown in Fig. 2. The highest volumetric PDC activity was detected in yeast cells which was cultivated in LSWE medium at 60 h of 0.075 ± 0.001 U/ml while yeast cells from other carbon sources (MYM and AsgLG) produced volumetric PDC activity of 0.026 ± 0.002 (at 66 h) and 0.025 ± 0.001 (at 6 h) U/ml, respectively. The highest specific PDC activity was obtained from cultivation in MYM medium at 6 h of 0.109 ± 0.015 U/mg protein. The yeast cultivated in AsgLG and LSWE medium had specific PDC activity of 0.037 ± 0.003 (at 6 h) and 0.026 ± 0.001 (at 60 h) U/mg protein, respectively.

In comparison to the studies by Nunta et al. 2018, Wattanapanom et al. 2019, current study provided kinetic profiles at a larger scale for AsgLG medium (1 l instead of 100 ml) and smaller scale for LSWE medium (1 l instead of 10 l) with the relatively higher initial total sugars concentration levels (up to 100 g/l instead of 50–60 g/l for the previous studies).

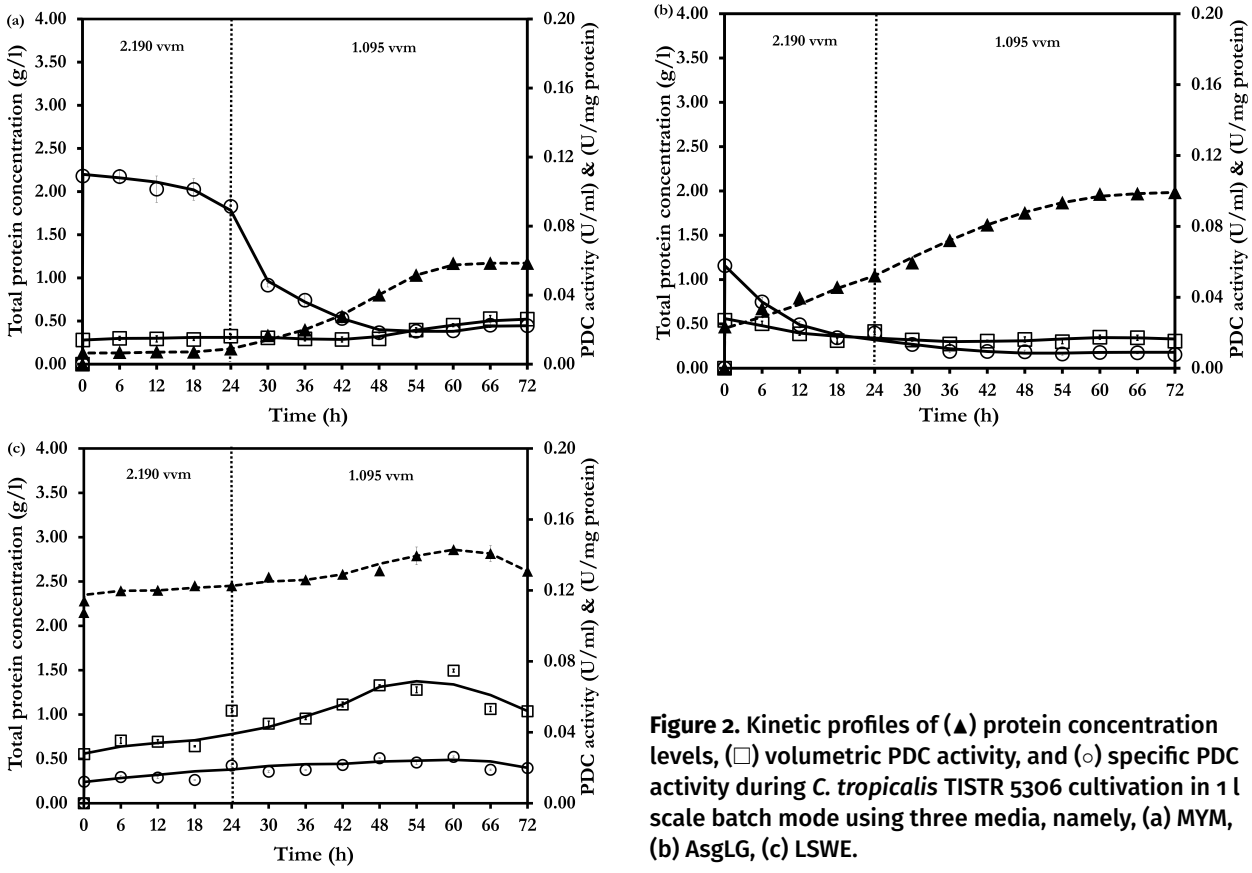

Figure 2. Kinetic profiles of (▲) protein concentration levels, (□) volumetric PDC activity, and (○) specific PDC activity during *C. tropicalis* TISTR 5306 cultivation in 1 l scale batch mode using three media, namely, (a) MYM, (b) AsgLG, (c) LSWE.

Whole cells and ethanol production kinetics in fed-batch mode

The ethanol concentration trend after pulse feeding was relatively stable after PF1 addition with increased concentration level after PF2. The steady ethanol concentration level was achieved again after PF3 addition as shown in Fig. 3. The highest ethanol concentration of the pulse feeding were 22.4 ± 0.2 g/l (at 72 h of PF1), 28.3 ± 0.5 g/l (at 120 h of PF2), and 21.3 ± 0.3 g/l (at 168 h of PF3). At the end of yeast cultivation at 192 h, ethanol concentration level of 17.1 ± 0.2 g/l was resulted with the highest dried biomass concentration level of 9.39 ± 0.04 g/l. Furthermore, the addition of the concentrated AsgLG medium until PF3 could generate the highest volumetric PDC activity ($p \leq 0.05$) with the range of 0.051 ± 0.002 – $0.057 \pm <0.001$ U/ml during 144–160 h cultivation period. The highest specific PDC activity ($p \leq 0.05$) from fed-batch mode was detected with PF1 feeding sequence within the range of 0.046 ± 0.004 – 0.095 ± 0.013 U/mg protein during at 48.05–72 h cultivation period (not shown in Table or Figure).

Table I. Comparison of investigated kinetic parameters of *C. tropicalis* TISTR 5306 cultivation during 72 h with three media at 1 l scale.

Investigated parameters (unit)	Media																	
	MYM			AsgLG			LSWE			MYM			AsgLG			LSWE		
	Highest Ethanol Production									Highest Dried Biomass Production								
Cultivation time (h)	0 – 24			0 – 48			0 – 42			24 – 72			48 - 72			42 - 72		
[Ethanol] (g/l)	9.57 ± 0.41	B	(at 24 h)	25.5 ± 0.8	A	(at 48 h)	7.58 ± 0.34	C	(at 42 h)	1.23 ± 0.04	c	(at 72 h)	16.5 ± 0.5	a	(at 72 h)	4.44 ± 0.10	b	(at 72 h)
[Dried biomass] (g/l)	2.12 ± 0.11	B	(at 24 h)	7.87 ± 0.05	A	(at 48 h)	1.58 ± 0.08	C	(at 42 h)	6.95 ± 0.14	b	(at 72 h)	9.44 ± 0.05	a	(at 72 h)	2.19 ± 0.03	c	(at 72 h)
μ_{max} (h^{-1})	0.261±0.036	A,B	(0 - 6 h)	0.261±0.007	B	(0 – 6 h)	0.333±0.053	A	(0 – 6 h)	0.047±0.016	a	(36 - 42 h)	0.012±0.001	b	(60 - 66 h)	0.016±0.006	b	(54 - 60 h)
$t_{d,min}$ (h)	2.66 ± 0.36	A,B	(0 - 6 h)	2.66 ± 0.08	B	(0 – 6 h)	2.08 ± 0.33	A	(0 – 6 h)	14.8 ± 5.2	a	(36 - 42 h)	57.8 ± 6.4	b	(60 - 66 h)	43.7±17.0	b	(54 - 60 h)
$q_{s,max,s}$ (g_s/g_x/ h)	N/a			-0.468±0.043	A	(36 - 42 h)	N/a			N/a			-0.095±0.003	a	(48 - 54 h)	N/a		
$q_{s,max,g}$ (g_g/g_x/h)	-14.6 ± 2.3	A	(0 - 6 h)	-0.319±0.137	C	(0 – 6 h)	-1.93±0.42	B	(0 – 6 h)	-0.655±0.088	a	(24 - 30 h)	-0.056±0.008	b	(48 – 54 h)	-0.112±0.149‡	b	(at 48 -54 h)
$q_{s,max,f}$ (g_f/g_x/h)	N/a			-0.126±0.016	B	(18 – 24 h)	-0.397±0.249‡	A,B	(0 – 6 h)	N/a			-0.059±0.005	a	(48 - 54 h)	**-0.043±0.036‡**	a	(54 - 60 h)
$q_{s,max,xyl}$ (g_{xyl}/g_x/h)	N/a			N/a			-0.043±0.047‡	A	(0 – 6 h)	N/a			N/a			-0.006±0.007‡	a	(66 -72 h)
$q_{s,max,ts}$ (g_{ts}/g_x/h)	- 14.6 ± 2.3	A	(0 - 6 h)	-0.503±0.048	C	(36 – 42 h)	-2.37±0.53	B	(0 – 6 h)	-0.655±0.088	a	(24 - 30 h)	-0.210±0.012	b	(48 - 54 h)	-0.118±0.161	b	(48 - 54 h)
$q_{p,max}$ (g_p/g_x/h)	2.46 ± 0.35	A	(0 - 6 h)	0.395±0.032	C	(0 – 6 h)	1.51±0.24	B	(0 – 6 h)	Not being produced			Not being produced			Not being produced		
Highest $Y_{p/s}$ (g_p/g_{ts})	0.169±0.022	B	(at 6 h)	0.491±0.017*	A	(at 30 h)	N/a*			0.088±0.003	c	(at 30 h)	0.256±0.007	b	(at 54 h)	0.486±0.077	a	(at 48 h)
Highest $Y_{x/s}$ (g_x/g_{ts})	0.026±0.001	C	(at 18 h)	0.533±0.170	A	(at 6 h)	0.152±0.011	B	(at 42 h)	0.065±0.002	c	(at 72 h)	0.089±0.001	b	(at 72 h)	0.158±0.009	a	(at 72 h)

- The numbers with the same alphabet (A - C) and (a - c) indicated significant difference ($p \leq 0.05$) for comparison between different columns of the same row in the same group (highest ethanol or whole cells concentration levels).
- * as the relatively small quantity of ethanol was generally produced at the early stage of fermentation and often resulted in higher yield above the theoretical value (0.511), the reported maximum of $Y_{p/s}$ for this time point was chosen with additional condition of standard error $\leq$ 5%.
- ‡ these numbers have large errors as relatively low sugar consumption (< 1.5 g/l) was detected, the relative errors were thus large.
- N/a indicated not availability of data due to the nature and condition of each specific medium.

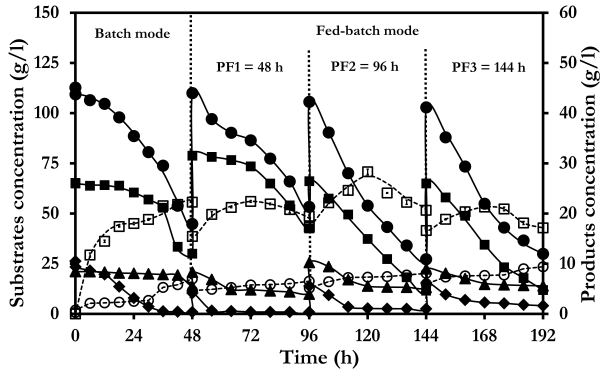

Figure 3. Kinetic profiles of substrates and products concentration levels during *C. tropicalis* TISTR 5306 cultivation using AsgLG medium in 1 l scale fed-batch mode (■ sucrose, ♦ glucose, ▲ fructose, ● total sugar, □ ethanol, and ○ dried biomass).

DISCUSSION

Whole cells and ethanol production kinetics in batch and fed-batch modes The analyses of kinetic parameters for sugars consumption, dried biomass formation, and ethanol production concentration levels during 72 h of *C. tropicalis* TISTR 5306 cultivation in 1 l scale batch mode using three media are shown in Table I. The sugars concentration levels were significantly decreased ($p \leq 0.05$) during yeast cultivation in three media. Minimum doubling times were also presented in Table I and signified the minimal cultivation time period that *C. tropicalis* TISTR 5306 required to be doubled in number from the formula 0.693/μ_{max} (Nunta et al. 2018). The maximum specific growth rate in MYM medium of 0.261 ± 0.036 h^{-1} and minimum doubling time ($t_{d,min}$) of 2.66 ± 0.36 h were significantly higher ($p \leq 0.05$) than AsgLG but were similar ($p > 0.05$) to LSWE media during the first 6 h of cultivation period. Comparing to Tangtua et al. 2013, the cultivation of *C. tropicalis* TISTR 5306 using yeast malt medium in 10 l scale for 72 h with also relatively high initial glucose concentration level as a carbon source resulted in the μ_{max} of 0.379 ± 0.020 h^{-1} and $t_{d,min}$ of 1.85 ± 0.13 h were faster than the current MYM medium by 1.44 folds. The maximum specific rates of total sugars consumption ($q_{s,max,ts}$) and ethanol production ($q_{p,max}$) during 24 h cultivation period in MYM medium were 14.6 ± 2.3 g/g/h and 2.46 ± 0.35 g/g/h, respectively which were significantly higher ($p \leq 0.05$) than cultivation in AsgLG and LSWE media. In case of cultivation in AsgLG and LSWE media during the period which generated the highest ethanol production, ethanol yield ($Y_{p/s}$) and dried biomass yield ($Y_{x/s}$) were higher ($p \leq 0.05$) than cultivation in MYM medium. For cultivation using LSWE medium, μ_{max} of 0.016 ± 0.006 h^{-1} and $t_{d,min}$ of 43.7 ± 17.0 h were obtained during 54 – 60 h which indicated retarded growth. Wattanapanom et al. 2019 performed kinetic studies of *C. tropicalis* TISTR 5306 in a 10 l predigested extract of whole fruit longan solid waste powder (WF-LSWP) with the initial total sugars concentration at 16, 45, and 90 g/l with the corresponding average μ_{max} of 0.036 ± 0.003, 0.056 ± 0.002, and 0.062 ± 0.001 h^{-1} which were in the same magnitude but was still significantly higher ($p \leq 0.05$) than the maximum μ_{max} obtained in this study (0.030 ± 0.004 h^{-1}) with the initial total sugars concentration slightly above 100 g/l. The much slower maximum specific growth rate for cultivation of *C. tropicalis* TISTR 5306 in LSWE medium compared to AsgLG medium by 3.53 ± 0.48 folds was probably due to the inhibitory effect of lignin and other bioactive compounds as evident from the study of Wattanapanom et al. 2019 who revealed the mass percentage ratios between 4.56 ± 0.68 – 5.79 ± 0.43 of this compound in either pretreated WF-LSWP or control. The pretreatment strategies by alkaline or saturated steam only slightly mitigated the presence of lignin but rather resulted in the significant removal of starch, pectin

and other carbohydrates in WF-LSWP. The antimicrobial effects of polyphenolic bioactive compounds, for example, gallic acid, ellagic acid, tannic acid, as well as corilagin which are relatively abundant on the surface of the longan seed and peel may also contribute to the retarded specific growth rate of microbes (Wattanapanom et al. 2019, Nunta et al. 2018).

The comparison of investigated kinetic parameters determined from *C. tropicalis* TISTR 5306 cultivation period for 72 h with three media based on the optimal ethanol and whole cells production (by separating into two columns as indicated in Table I) revealed mitigated levels of all parameters and constants between two periods. In addition, the cultivation of *C. tropicalis* TISTR 5306 in AsgLG medium in this study and those in C-grade fresh longan juice medium by Nunta et al. 2018 revealed that μ_{max} of 0.261 ± 0.007 h^{-1} and $q_{p,max}$ of 0.395 ± 0.032 g/g/h obtained here at 48 h were higher and lower than Nunta et al. 2018 who reported the corresponding values of 0.028 ± 0.003 h^{-1} and 0.465 ± 0.031 g/g/h, respectively during 24–48 h cultivation period.

The ranking of relevant cultivation data for whole cells and ethanol production could be computed by proportional score percentages from the results of cultivation time, media preparation time, media cost, ethanol concentration, dried biomass concentration, protein concentration, volumetric PDC activity, specific PDC activity, protein productivity, volumetric PDC productivity, and specific PDC productivity as shown in Table II. The AsgLG medium was the most appropriate medium for ethanol and whole cells production of *C. tropicalis* TISTR 5306 cultivation at 48 h as evident from the highest total score and was thus selected for subsequent experiment. Such proportional score percentages comparison had been performed previously based on the results of ethanol, dried biomass, and protein concentrations, volumetric PDC activity, specific PDC activity, protein productivity, PDC productivity, and specific PDC productivity (Nunta et al. 2018, Tangtua et al. 2013). Nunta et al. 2018 could select C-grade fresh longan juice medium as the best cultivation medium for *C. tropicalis* TISTR 5306 as it had the highest score based on similar scores ranking strategy.

Dried biomass and product concentration levels were significantly increased ($p \leq 0.05$) during *C. tropicalis* TISTR 5306 cultivation in fed-batch mode (Fig. 3). The specific growth rate (μ) during cultivation in AsgLG medium for 192 h was $0.009\pm$ < 0.001 h^{-1} while the doubling time (t_d) and biomass yield ($Y_{x/s}$) were $77.0\pm$ < 8.6 h and 0.047 ± 0.001 g dried biomass/g consumed total sugars. Nunta et al. 2019 performed batch cultivation of the same yeast in a 100 l scale with average μ, t_d, and $Y_{x/s}$ of 0.020 ± 0.002 h^{-1}, 34.6 ± 3.5 h, and 0.248 ± 0.016 g dried biomass/g consumed total sugars, respectively. In fact, *C. tropicalis* TISTR 5306 could proliferate better than other systems during cultivation in a continuous 10 l scale bioreactor using dilution rate and therefore μ (at steady state) of 0.049 ± 0.003 h^{-1} or t_d of 14.1 ± 0.9 h with $Y_{x/s}$ of 0.102 ± 0.006 g dried biomass/g consumed total sugars (Nunta et al. 2019). In current study, *C. tropicalis* TISTR 5306 could produce the apparent highest ethanol concentration levels of 24.6 ± 0.8 and 28.3 ± 0.5 g/l at 112 and 120 h, respectively (Fig. 3). The apparent ethanol yield ($Y_{p/s}$) was 0.482 ± 0.012 g ethanol/g consumed total sugars during 0–120 h cultivation period with the specific rate of ethanol production (q_p) of 0.062 ± 0.001 g ethanol/g dried biomass/h. Nunta et al. 2019 reported the highest ethanol concentration, $Y_{p/s}$, and q_p (g/l, g ethanol/g consumed total sugars, g ethanol/g dried biomass/h) for cultivation of this yeast in a 100 l scale batch bioreactor of (13.2 ± 0.2 at 108 h, 0.287 ± 0.105 during 16 – 40 h, 0.040 ± 0.009 during 0–16 h) and (34.3 ± 0.5, 0.483 ± 0.026, 0.265 ± 0.023) at dilution rate of 0.049 ± 0.003 h^{-1} for cultivation in a 10 l scale cultivation tank, respectively. The comparison between three systems could thus be

Table II. Score ranking percentages of the relevant cultivation data from *C. tropicalis* TISTR 5306 in three media at 1 l scale.

Cultivation data	Media and selected cultivation time											
	MYM at 60 h				AsgLG at 48 h				LSWE at 60 h			
1. Cultivation time	79.8	±	0.1	B	**99.8**	**±**	**0.1**	**A**	79.8	±	0.1	B
2. Media preparation time	**97.5**	**±**	**1.4**	**A**	78.0	±	0.9	B	6.8	±	0.1	C
3. Media cost	49.9	±	0.3	B	**98.2**	**±**	**1.0**	**A**	24.5	±	0.1	C
4. Ethanol concentration	5.3	±	0.1	C	**94.2**	**±**	**2.9**	**A**	17.4	±	0.1	B
5. Dried biomass concentration	**98.1**	**±**	**1.1**	**A**	92.1	±	1.1	B	24.9	±	1.5	C
6. Protein concentration	75.9	±	3.5	B	**96.8**	**±**	**1.6**	**A**	43.3	±	3.7	C
7. Volumetric PDC activity	29.6	±	1.1	B	21.3	±	1.9	C	**97.9**	**±**	**1.1**	**A**
8. Specific PDC activity	71.2	±	1.8	B	34.2	±	3.6	C	**96.4**	**±**	**1.9**	**A**
9. Protein productivity	30.4	±	1.4	B	**48.4**	**±**	**0.8**	**A**	17.3	±	1.5	C
10. Volumetric PDC productivity	14.8	±	0.6	B	13.3	±	1.2	C	**49.0**	**±**	**0.6**	**A**
11. Specific PDC productivity	35.6	±	0.9	B	21.4	±	2.2	C	**48.2**	**±**	**0.9**	**A**
12. Total scores	**588.1**	**±**	**7.2**	**B**	**697.6**	**±**	**6.7**	**A**	**505.7**	**±**	**5.2**	**C**

The numbers with the same alphabet (A - C) indicated significant difference ($p \leq 0.05$) for comparison between different columns of the same row and the proportional scoring percentages for relevant cultivation data items 9. – 11. were set at 50 instead of 100 to mitigate the bias towards protein and PDC production as previously tabulated for items 6. - 8. The listed total scores in the last row were based on total values of 950. Example calculation: Row 4 for AsgLG, score for [ethanol] was the percentage ratio based on the maximum value of a replicate, i.e., 27.07 g/l for this case, thus 100 × (25.5 ± 0.79)/27.07 = 94.2 ± 2.9.

made with fed batch system positioned in the middle between continuous and batch system in term of apparent ethanol concentration being produced. Although apparent $Y_{p/s}$ of fed batch system was as comparable to that of continuous system ($p > 0.05$). The value of q_p for fed batch was actually at the lowest level among the others. In fact, by taking into account of the dilution factors by PF1 and PF2 as well as the increased concentration of total sugars due to PF, the equivalent $Y_{p/s}$ during 0 – 120 h and $Y_{x/s}$ during 0–192 h would be 0.147 ± 0.003 g ethanol/g consumed total sugars and $0.033 \pm < 0.001$ g dried biomass/g consumed total sugars, respectively. As the specific rate of ethanol production was determined by dividing the concentration levels of ethanol and dried biomass, both of which were equally affected by similar dilution factor after addition of PF, the word "apparent" was thus omitted for this parameter. The determined q_p can thus be directly compared to the other batch or continuous systems. Kinetic parameters determined during batch and fed-batch modes were significantly different ($p \leq 0.05$) during *C. tropicalis* TISTR 5306 cultivation. Some parameters including μ_a, t_d, $Y_{p/s}$, and q_p during cultivation in batch mode for 48 h were all significantly higher ($p \leq 0.05$) than three cultivation intervals during fed-batch mode (PF1, PF2, and PF3). The impact of feeding strategy in fed-batch mode was thus clearly shown on relevant kinetic parameters as tabulated in Table III.

Table III. Comparison of kinetic parameters (μ, $q_{s,s}$, $q_{s,g}$, $q_{s,f}$, $q_{s,tot}$, q_p, $Y_{p/s}$, and $Y_{x/s}$) of cultivation processes for 1 l scale during 0 – 48 h (batch cultivation process) and 1 l scale during 48.05 – 96, 96.05 – 144, and 144.05 - 192 h of fed-batch cultivation periods for *C. tropicalis* TISTR 5306 in AsgLG medium.

Investigated parameter (unit)	Batch mode				Fed-batch mode											
	0 – 48 h				48.05 – 96 h				96.05 – 144 h				144.05 – 192 h			
μ (h^{-1})	**0.038**	**±**	**<0.001**	**A**	0.007	±	<0.001	B	0.009	±	<0.001	B	0.009	±	<0.001	B
$q_{s,s}$ (g_s/g_x/ h)	**0.202**	**±**	**0.003**	**A**	0.136	±	0.004	C	0.172	±	0.003	B	0.144	±	0.002	C
$q_{s,g}$ (g_g/g_x/h)	**0.145**	**±**	**0.002**	**A**	0.036	±	<0.001	B	0.036	±	<0.001	B	0.031	±	0.001	B
$q_{s,f}$ (g_f/g_x/h)	**0.042**	**±**	**0.001**	**A**	0.041	±	0.001	A	0.039	±	0.001	A	0.023	±	0.002	B
$q_{s,ts}$ (g_{ts}/g_x/h)	**0.390**	**±**	**0.003**	**A**	0.212	±	0.006	C	0.247	±	0.006	B	0.198	±	0.005	C
q_p (g_p/g_x/h)	**0.128**	**±**	**0.004**	**A**	0.015	±	<0.001	B	0.011	±	0.003	B	0.001	±	0.001	C
$Y_{p/s}$ (g_p/g_{ts})	**0.328**	**±**	**0.011**	**A**	0.071	±	0.005	B	0.043	±	0.011	C	0.007	±	0.005	D
$Y_{x/s}$ (g_x/g_{ts})	**0.098**	**±**	**0.001**	**A**	0.033	±	0.001	C	0.037	±	0.001	C	0.047	±	0.001	B

The numbers with the same alphabet (A - D) indicated significant difference ($p \leq 0.05$) for comparison between different columns of the same row.

Proposed mathematical model for simulation and optimization

The determined kinetic parameters in Tables I and III will be useful for prebcribing initial values of parameters in the development of mathematical model for batch growth-related differential equations for microbial cultivation such as those given in Eq. (A) with accompanying matrix of constants. This set of differential equations can be used for two non-dissociated monosaccharide substrates such as glucose ($i = 2.1$) and fructose ($i = 2.2$) with specific rates (r_i), substrate limitation constants ($K_{S\Phi_i}$), substrate inhibition constants ($K_{i\Phi_i}$), and maximum ethanol concentration ($P_{m\Phi_i}$) on cells growth, substrate consumption, and product formation (adapted from Yuvadetkun et al. 2017, Boonmee et al. 2003, Leksawasdi et al. 2001). Further consideration of disaccharide substrate such as sucrose which can dissociate into glucose and fructose using the law of mass action (Lund 1965) resulting in proposed Eq. (B.1) with sucrose dissociation constant (k) based on the action of invertase enzyme. The general form of modified differential equation from Eq. (A) ($i = 2.1$ and $i = 2.2$) for monosaccharide consumption rate is given in Eq. (B.2) with addition of Eq. (B.1) for sugars species $n = 2.1$ and $n = 2.2$ which are equivalent to $i = 2.1$ and $i = 2.2$ in Eq. (A). The negative sign indicates the consumption of sucrose in Eq. (B.1) while the positive sign in Eq. (B.2) shows the formation of either glucose or fructose resulting from the hydrolysis reaction of sucrose on the active site of invertase.

$$\frac{d\Phi_i}{dt} = r_i \left(\frac{\Phi_2}{K_{S\Phi_i} + \Phi_2}\right)\left(\frac{K_i\Phi_i}{K_{i\Phi_i} + \Phi_2}\right)\left(1 - \frac{\Phi_3}{P_{m\Phi_i}}\right)\Phi_i \tag{A}$$

i	Φ_i	r_i	$K_{S\Phi_i}$	$K_{i\Phi_i}$	$P_{m\Phi_i}$
1	x	μ_{max}	K_{Sx}	K_{ix}	P_{mx}
2.1	$s.1$	$-q_{s.1,max}$	$K_{Ss.1}$	$K_{is.1}$	$P_{ms.1}$
2.2	$s.2$	$-q_{s.2,max}$	$K_{Ss.2}$	$K_{is.2}$	$P_{ms.2}$
3	p	$q_{p,max}$	K_{Sp}	K_{ip}	P_{mp}

$$\left(\frac{d\Phi_{2.3}}{dt}\right) = -k\Phi_{2.3} \tag{B.1}$$

$$\left(\frac{d\Phi_n}{dt}\right) = k\Phi_{2.3} + \left(\frac{d\Phi_{n(A)}}{dt}\right) \tag{B.2}$$

The multiple substrates (three substrates in this case, namely, sucrose, glucose, and fructose) model (Eq. (A) for $i = 1, 2$; Eq. (B.1); as well as Eq. (B.2) for $n = 2.1, 2.2$) proposed here would be useful for simulation and optimization applications in batch (Yuvadetkun et al. 2017), fed batch (current study), and continuous processes (Nunta et al. 2019). The mathematical model could be incorporated into the mass balance equations for a specific production process with intermittent, regular pulse, or continuous feeding strategies (Cheung et al. 2018). The kinetic parameters in the model could be thoroughly searched and determined based on a written Visual Basic for Applications (VBA) source code in Microsoft® Excel (Nunta et al. 2019, Cheung et al. 2018, Khemacheewakul et al. 2018, Leksawasdi et al. 2005a,b, 2003) after implementing numerical integration strategies of Eq. (A), (B.1), and (B.2) to

regress multiple experimental data sets in a series of batch cultivation system with various levels of initial substrates concentration (pure substrates or variety of longan related products). The validation of the proposed mathematical model based on the quality of fit assessed by prediction error plot and/or statistical parameters including the coefficient of determination (R^2), residual sum of sqaure (RSS), as well as mean sum of square (MS) could then be carried out (Yuvadetkun et al. 2017, Pulsawat et al. 2003, Leksawasdi et al. 2006) by verifying with the independently determined kinetic profiles such as those given in Fig. 1 and 3.

CONCLUSION

AsgLG medium was the most suitable medium for whole cells and ethanol production by *C. tropicalis* TISTR 5306 based on scores ranking strategy with the highest total score of 698 ± 7 at 48 h. The ethanol and dried biomass concentration levels were at the highest levels of 25.5 ± 0.8 g/l at 48 h and 9.44 ± 0.05 g/l at 72 h, respectively. Fed-batch mode could improve ethanol concentration levels within the range of 24.6 ± 0.8 – 28.3 ± 0.5 g/l during 112–120 h cultivation time during the second feed addition. The highest dried biomass concentration levels in the range of 8.99 ± 0.07 – 9.39 ± 0.04 g/l were achieved during 184–192 h of the third feed addition. The highest specific PDC activities obtained were within range of 0.042 ± 0.002 – 0.095 ± 0.013 U/mg protein during 48.05–56 h cultivation period in the first feeding. The implementation of further feeding sequences to optimize ethanol and whole cells production can be considered based on the proposed mathematical model as an alternative to improve this process and facilitate production of high value chemicals such as phenylacetylcarbinol in the future study.

Nomenclature

Φ	biomass, sugars, or ethanol species in the microbial cultivation system
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
AsgLG	assorted grade fresh longan juice medium
HPLC	high pressure liquid chromatography
i	identifiers of species in microbial cultivation system; 1 is biomass, 2.1 is glucose, 2.2 is fructose, 2.3 is sucrose, 3 is ethanol
k	sucrose dissociation constant (h^{-1})
K_S	substrate limitation constant (g substrate / l)
K_i	substrate inhibition constant (g substrate / l)
KMS	potassium metabisulphite
LSW	longan solid waste
LSWE	longan solid waste extract medium
MYM	modified yeast malt medium
n	identifiers for modified form of differential equations for glucose ($n = 2.1$) and fructose ($n = 2.2$) consumption rates
PAC	phenylacetylcarbinol
PDC	pyruvate decarboxylase

PF	pulse feeding addition
P_m	maximum ethanol concentration (g ethanol / l)
$q_{p,\max}$	maximum specific ethanol production rate (g ethanol / g biomass / h)
$q_{s,\max,f}$	maximum specific fructose consumption rate (g fructose / g biomass / h)
$q_{s,\max,g}$	maximum specific glucose consumption rate (g glucose / g biomass / h)
$q_{s,\max,s}$	maximum specific sucrose consumption rate (g sucrose / g biomass / h)
$q_{s,\max,ts}$	maximum specific total sugars consumption rate (g total sugars / g biomass / h)
r	specific rate of biomass and substrates consumption as well as product formation (g species / g biomass / h)
TISTR	Thailand Institute of Scientific and Technological Research
vvm	aeration rate (volume air per min) per volume of liquid in a bioreactor
YM	yeast malt medium
$Y_{p/s}$	yield of ethanol produced over total sugars consumed (g ethanol / g total sugar)
$Y_{x/s}$	yield of biomass produced over total sugars consumed (g biomass / g total sugar)

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C.M. performed the experiments and write manuscript draft. A.R. and R.N. provided comments, suggestions, and revised the manuscript. N.L. supervised the experiments, verified experimental data, model development, refined and finalized the revised manuscript.