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Contributed Paper

Kinetic Parameters of *Candida tropicalis* TISTR 5306 for Ethanol Production Process Using an Optimal Enzymatic Digestion Strategy of Assorted Grade Longan Solid Waste Powder

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Abstract

The optimal enzymatic digestion strategy for assorted grade whole fruit longan solid waste powder (WF–LSWP) to release the highest level of fermentable sugars for subsequent production of ethanol using *Candida tropicalis* TISTR 5306 has been reported for the first time in this study with several important fermentation kinetic parameters. WF–LSWP contained relatively low lignin content (5.79 ± 0.43 %(w/w)) with the presence of relatively high starch and pectin contents of 27.9 ± 0.86% (w/w) and 2.07 ± 0.16% (w/w), respectively. Pretreatment by alkali and saturated steam before enzymatic digestion step did not result in the improvement of overall sugars being released. The implementation of commercial enzyme mixture (amylase, glucoamylase, cellulase, and xylanase) for one step enzymatic digestion at 50°C for 48 h resulted in the statistical significantly highest ($p \leq 0.05$) specific overall sugars productivity of (141 ± 1.4) × 10^{-4} g total sugars/g WF–LSWP/digestion step/h. Cultivation of *C. tropicalis* TISTR 5306 in digested and concentrated WF–LSWP extract at concentration level of 90 g/l during 0 – 12 h resulted in the following statistical significantly highest ($p \leq 0.05$) kinetic parameters; specific growth rate (m) of 0.097 ± 0.001 h^{-1} and specific ethanol production rate (q_P) of 0.221 ± 0.010 gP/gX/h. Dried biomass yield ($Y_{X/S}$) and ethanol yield ($Y_{P/S}$) based on utilized sugars of 90 g/l WF–LSWP extract at 0.180 ± 0.018 gX/gS and 0.411 ± 0.044 gP/gS, respectively, were statistical significantly highest ($p \leq 0.05$) in comparison with those of 16 and 45 g/l WF–LSWP extracts.

Keywords: lignocellulosic materials, longan solid waste powder, *Candida tropicalis*, enzymatic digestion, pretreatment, ethanol

1. INRODUCTION

Longan is one of the important economic crops of Thailand with an average annual production volume for the past 5 years of more than 911,800 ± 53,186 tons [1]. The most abundant longan variety is *Dimocarpus longan* Lour. which accounts for 5.46% of all fruit plantation area in Thailand due to the ability to bloom and bear fruit regularly [2]. Nevertheless, the recurring longan overproduction problem has led to the regular cycle of selling price devaluation that affected a number of farmers [3, 4]. One strategy to tackle this problem is to process whole fresh longan fruit into longan syrup in large scale to extend shelf life and add values as evident from the technology transfer by our research group to private sector during the early period of 2016 [5]. Such process has generally produced a sizable solid waste (2,500 tons, unpublished data), in the form of lignocellulosic materials, to longan juice ratio of 2 to 1 by our estimate.

Three main constituent components of lignocellulosic materials are cellulose, hemicellulose, and lignin. Cellulose is homopolymer of glucose consisting of b – 1, 4 glycosidic bonds. Hemicellulose is heteropolymer of pentoses (xylose and arabinose), hexoses (glucose, fructose, galactose and mannose), and sugar acids (acetic acid). Lignin acts as a structural strengthener between cellulose and hemicellulose [6].

The pretreatment of agricultural residues (such as sugarcane bagasse) with $Ca(OH)_2$ solution is able to remove lignin which obstructs the enzyme activity. Furthermore, it supports carbohydrate digestion and removal of interfering chemicals which can inhibit the microorganism growth [6-8]. The advantages of $Ca(OH)_2$ utilization are due to the relatively safety, high recoverability, easily handling, inexpensiveness, and minor environmental effects of this compound [8].

Tangtua, *et al.* [9] screened 50 microbial strains for production of ethanol and *R* – phenylacetylcarbinol (PAC) with results indicating that *Candida tropicalis* TISTR 5350 and 5306 were the best producers of these compounds. The presence of pyruvate decarboxylase enzyme (PDC, EC 4.1.1.1) in the whole cells of both yeasts can catalyze the ligation reaction between pyruvate and benzaldehyde to produce PAC [9-12]. This secondary alcohol is a precursor for useful bronchial dilator (ephedrine) and nasal decongestant (pseudoephedrine) [13-14]. In addition, ethanol is a natural *in vivo* product during cultivation process from this yeast strain which can be used as an important constituent in petroleum–based product [15].

Several published articles relating to application of longan solid waste from peel, seed, or outer layer of seed focused on extraction of antioxidant phenolic compounds such as ellagic or gallic acids [16-18] while the study focusing on pretreatment and enzymatic digestion of longan solid waste is still lacking. The comprehensive study of how to properly carry out pretreatment and utilizing available commercial enzyme mixtures in the group of amylase, cellulase, and xylanase to (1) digest longan solid waste powder (LSWP) and (2) obtain the suitable quantity of total sugars for further step of fermentation to ethanol by *C. tropicalis* TISTR 5306 yielding the relevant kinetic parameters were elucidated for the first time in current study.

2. MATERIALS AND METHODS

2.1 Microorganism

The ethanol producing yeast – *C. tropicalis* TISTR 5306 – was ordered from Thailand Institute of Scientific and Technological Research (TISTR, Pathum Thani, Thailand) and was subsequently propagated in 60% (v/v) glycerol stock prior to storage at -70°C. This yeast strain was used instead of the previously reported *C. tropicalis* TISTR 5350 in Yeast – Malt (YM) medium [9], [19-21] as the strain TISTR 5306 could produce the relatively higher ethanol concentration and

pyruvate decarboxylase activity in the presence of longan extract medium [22].

2.2 Commercial Enzymes and Chemicals

Five types of enzyme mixtures (EzM) from various sources were investigated in this study. EzM 1 consists of endo – 1, 4 – beta – xylanase, amylase, pentosanase, beta–glucanase, hemicellulases, and xylanase (DSM Nutritional Products). EzM 2 is a complex of multiple cellulase enzymes such as cellulase, exoglucanase, endoglucanase, xylanase, hemicellulase, cellobiase, and beta–glucosidase (DuPont). EzM 3 is a cocktail of amylase and cellulase enzymes (Vland). EzM 4 composes of glucoamylase, alpha–amylase and protease (NovoZyme). EzM 5 consists of endoamylase which hydrolyzes internal alpha – *D* – 1, 4 glucosidic bonds (NovoZyme). All chemicals were either AR or HPLC grades.

2.3 Longan Solid Waste Powder

Assorted grade fresh longan (*D. longan* Lour.) of E–dor variety (300 kg) was purchased from longan orchards in Saraphi District, Chiang Mai Province, Thailand. The fresh fruit were divided into three groups before removal of specific part(s) to attain the corresponding characteristics of designated group's name, namely, peel–only (PO) group, seed–only (SO) group, and whole fruit (WF) group. The last group was the solid waste obtained after longan juice extraction process. All groups were dried at 80°C for 8 h and ground by a hammer mill (Crompton Control Series 2000) with a 40 mesh size screen. The longan solid waste powder (LSWP) from each group was later assigned with the following names, PO–LSWP, SO–LSWP, and WF–LSWP. All LSWPs were subjected to proximate analysis and subsequent comparison. WF–LSWP was used as substrate for selection of the most appropriate pretreatment and enzymatic digestion strategy because this material was the main by–product obtained from longan syrup production factory [22] located in Lamphun Province, Thailand and thus had more potential for commercial value in zero waste process.

2.4 Assessment for the Necessity of Pretreatment prior to Enzymatic Digestion of WF–LSWP

In order to elucidate the effect of pretreatment, an alkali – 1.84% (w/v) $Ca(OH)_2$ [9, 23] was chosen as a pretreatment strategy for removal of lignin from WF–LSWP. This chemical was found to be ideal for pretreatment of switchgrass and corn stover as it was relatively inexpensive with less toxicity than several acids and alkalis. The removal of Ca^{2+} could also be carried out with ease by carbonating with CO_2 gas and screening out the $CaCO_3$ precipitate [23, 24]. Four experimental conditions (A) – (D) were set up to assess the effectiveness of using pretreatment. In the control condition (A), 7% (w/v) WF–LSWP was prepared in distilled water before heating at 50°C for 48 h under shaking condition of 200 rpm. Direct enzymatic digestion (B) was used as a comparative strategy in the situation where the alkali pretreatment was absence. In this case, 7% (w/v) WF–LSWP in either 10% (v/v) EzM 1 or EzM 2 were prepared and enzymatic digestion at 50°C for 48 h was carried out [23]. For condition (C) – treatment with saturated steam, 7% (w/v) WF–LSWP was prepared in distilled water before being treated in saturated steam condition at 121°C, 15 psi, for 4 h [24]. The filtration process was then followed by filtering pretreated mixture through two layers of muslin cloth. The resulting filter cake was then removed and dried at 80°C for 8 h or until there was no further weight change to obtain pretreated WF–LSWP. The subsequent enzymatic digestion was carried out by preparing 7% (w/v) pretreated WF–LSWP in 10% (v/v) either EzM1 or EzM2 prior to digestion at 50°C for 48 h. In the alkali

condition (D) – WF–LSWP was pressurized in the presence of 1.84% (w/v) $Ca(OH)_2$ using the similar process as described in (C). The exception was that 7% (w/v) WF–LSWP was prepared in 1.84% (w/v) $Ca(OH)_2$ [23] instead of distilled water before pretreatment, filtration process, and enzymatic digestion. The pretreated conditions (C) and (D) had immediate pH levels after pretreatment in the range of 10 – 14. Before enzymatic digestion, WF–LSWP was washed with tap water until the washed water had pH level of 7.0. The drying process of washed WF–LSWP was then followed with subsequent addition of 10 mM sodium acetate buffer and enzyme solution. The mass of dried and washed WF–LSWP to volumes of buffer + enzyme solution ratio was 7 : (90 + 10) or 1 : (12.86 + 1.43), in g/ml) and pH level adjustment (10 M H_2SO_4 / 10 M KOH) of the digestive mixture was performed to ensure that the initial pH was $5.00 \pm < 0.01$ [25]. Further analyses of WF–LSWP (B) and pretreated WF–LSWP in condition (C) and (D) were carried out for hemicellulose, and cellulose. The overall compositions of starch, pectin, and other carbohydrates (if any) were also analyzed for WF–LSWP based on mass balance. Detailed analyzes of starch and pectin were also carried out in condition (B) to quantify both components as described in analytical method section and other carbohydrates were subsequently elucidated by mass balance strategy. Each experimental condition was repeated in triplicate to evaluate random error and assess the necessity of alkali pretreatment based on score ranking method (score of 100 was the most preferable as it indicated the highest level of total sugars yield).

2.5 Effect of Enzymatic Digestion Sequence on WF–LSWP

The candidates of enzyme mixture to be used in the enzymatic digestion sequence on WF–LSWP were chosen by repeating the similar direct enzymatic digestion (B) in Section 2.4 for EzM 1 – EzM 5 in comparison with control. Three suitable types of EzM were selected (EzM 3, 4, and 5) for subsequent hydrolysis in sequential order by weight scoring of sugars production. The highest total sugars yield was assigned with the highest score of one hundred and the top three ranks were chosen for next experiment. The permutation and combinatorial theories [26] were applied to investigate all possible strategies for adding these three enzymes in sequential manner and performing digestion at 50°C. There were, thus, 3! or $3 \times 2 \times 1 = 6$ methods for digesting WF–LSWP with an individual enzyme mixture in three steps permutation sequence using 24 h digestion time in each step with the overall digestion time of $24 \times 3 = 72$ h. Other strategies were to combine two enzyme mixtures together in double / single or single / double combination sequences in two steps digestion with an overall number of $= {}^3C_2 \times {}^1C_1 + {}^1C_1 \times {}^3C_2 = (3 \times 1) + (1 \times 3) =$ 6 possible digestion methods. By using 24 h digestion time in each step, the overall digestion time of $24 \times 2 = 48$ h could be applied. The last strategy was to combined all three EzM together and carry out digestion process for 48 h. All experimental conditions have been tabulated in Table 5. The corresponding sugars productivity scores were then calculated based on the previous method of weight scoring for each step of enzymatic sequence being used within one h. The highest sugars production score per step per h or sugars productivity was assigned with the highest score of one hundred and the best candidate was selected for enzymatic digestion pretreatment prior to cells cultivation. The comparison of surface characteristic under electron micrograph was also made between WF–LSWP (as control) and digested WF–LSWP with EzM 3, 4, and 5.

2.6 Kinetic of *C. tropicalis* TISTR 5306 Cultivation in Erlenmeyer Flask

Cultivation media consisting of three initial total sugars concentration levels (in g/l), namely, 16 (digested WF–LSWP with EzM 1), 45 (digested WF–LSWP with EzM 3 + 4 + 5) and 90 (evaporated and digested WF–LSWP with EzM 3 + 4 + 5) were selected. The 16 and 45 g/l total sugars were the direct results of enzymatic digestion of WF–LSWP with EzM 1 and EzM (3 + 4 + 5) while the 90 g/l concentration level were obtained by further evaporation of 45 g/l total sugars mixture to investigate evaporation effect on microbial cultivation. Ammonium sulphate (8.52 g/l) [22] was added to each media as supplementary nitrogen source. Five ml of microbial inoculum, which was prepared as described in previous studies [9, 22], was transferred to 45 ml cultivation media to initiate microbial cultivation. The quality of microbial inocula was assessed based on cells counting method under microscope with a haemocytometer for both viable and total cells concentration as described elsewhere [9]. The total and viable cell concentration levels of *C. tropicalis* TISTR 5306 were $6.08 \pm 0.12 \times 10^7$ and $5.28 \pm 0.10 \times 10^7$ cells/ml, respectively. The samples were collected in triplicate at a regular interval of 12 h for 48 h with similar cultivating condition described previously.

2.7 Analytical Methods

The assessment of total carbohydrate and energy contents were carried out based on Compendium of Methods for Food Analysis [27] by Central Laboratory (Thailand). The recommended methods by Association of Official Analytical Chemists (AOAC) were applied with assigned reference number in bracket for quantification of crude protein (991.20), crude fat (948.15), ash (923.03 and 920.153), and moisture content (925.10 and 950.46) [28] by Central Laboratory (Thailand). The compositions of cellulose and hemicellulose were measured by the sequential method of Van Soest *et al.* [29]. The lignin content was evaluated by acetyl bromide method [30] which contained a cleansing step for removal of interfering components such as protein and fat. Microwave – assisted extraction method was used for determination of pectin contents in %(w/w) and modified perchloric acid method was used for determination of starch contents in %(w/w) [31-32]. In addition, the morphological structures of the undigested and digested longan waste powder using EzM 3, 4, and 5 were compared by passing the materials through a gold–coater machine for 20 min. The surface characteristic was observed under a scanning electron microscope (SEM). Photomicro–graphs by SEM were taken at 1,200 × magnification using JSM – IT300 SEM from the Central Science Laboratory, Department of Chemistry, Faculty of Science, Chiang Mai University. The quantification of sugars (glucose, xylose and fructose), acetic acid, and ethanol concentration levels were performed with High Performance Liquid Chromatography (HPLC) using Aminex® Hi–Plex column (BioRad, Hercules, California, USA) as described previously [9]. The published analytical methods for pH and dried biomass concentration levels were also followed [9]. All measurements were carried out in triplicate and quantification of standard error (SE) was carried out as described previously [22]. The analyses of score ranking in section 2.4 and 2.5 had already been described in respective section and examples of calculation had been made in the footnotes of Table 2, 4, and 5. Calculation of the relevant kinetics parameters including specific growth rate (μ), doubling time (t_d), specific total sugar consumption rate (q_s), specific ethanol production rate (q_p), ethanol yield on produced biomass ($Y_{p/x}$), ethanol yield on sugar consumption ($Y_{p/s}$), as well as biomass yield ($Y_{x/s}$) on three time intervals (0 – 12 h,

0 – 24 h, 0 – 36 h, and average of all three) were done based on the previously published works [22, 33].

2.8 Hypothesis Testing

Statistical analysis for reliability measurement of the average among treatments were identified and assessed for significant difference based on the Duncan procedure. The statistical analysis was employed by SPSS for Windows®, with statistical significance at $p \leq 0.05$ as mentioned in previous work [22]. Strategies for determining errors propagation of experimental values had also been described elsewhere [22].

3. RESULTS AND DISCUSSION

3.1 Assessment for the Necessity of Pretreatment prior to Enzymatic Digestion of WF–LSWP

The main component of PO–LSWP, SO–LSWP, and WF–LSWP was carbohydrate at 80.7 ± 0.3, 84.8 ± 0.4, and 79.7 ± < 0.1 g / 100 g LSWP, respectively as shown in Table 1 with relatively small amount of ash (1.69 – 6.39%). The pretreatment processes (condition C and D) were examined for removal of lignin so that subsequent inhibition of enzyme mixture (EzM 1 and 2) activity in the next stage could be minimized or avoided [6-8]. According to Table 2, the direct enzymatic digestion using EzM 1 and 2 without pretreatment could produce the highest total sugars yields at 0.206 ± 0.002 and 0.210 ± 0.010 g/g WF–LSWP, respectively. In contrast, the enzymatic digestion with pretreatment using saturated steam (C) or alkali (D) resulted in sugars yield ranges of merely (0.048 ± 0.001 – 0.058 ± 0.001) and (0.024 ± 0.001 – 0.060 ± 0.001) g/g WF–LSWP, respectively. These were statistical significantly lower ($p \leq 0.05$) than the direct enzymatic digestion without pretreatment or even the control (0.127 ± 0.001 g/g WF–LSWP) due to loss of raw materials which contained relatively high content of starch, pectin and other carbohydrates (Table 3) through washing process. It should be noted that washing process after pretreatment step was originally designed for lignocellulosic materials for removal of lignin content with insignificant amount of other carbohydrates besides hemicellulose and cellulose which was not the case for WF–LSWP. In addition, the obtained total sugars concentration levels from the enzymatic digestion without pretreatment (in range of 14.4 – 14.7 g/l) were similar to the enzymatic digestion with pretreatment (in range of 6.07 – 15.5 g/l). Further analyses of WF–LSWP in Table 3 for (1) cellulose, (2) hemicellulose, (3) lignin, as well as (4) starch,

Table 1. Proximate analysis of longan peel (P), longan seed (S), whole fruit (W) of longan solid waste powder (LSWP) per 100 g of each material.

Longan components	Carbohydrate		Protein		Fat^{NS}	Ash		Moisture		Energy (kCal)	
PO–LSWP	80.7 ± 0.3	B	6.67 ± 0.10	B	2.73 ± 0.06	**6.39 ± 0.01**	**A**	3.53 ± 0.18	B	374 ± 0.4	B
SO–LSWP	**84.8 ± 0.4**	**A**	**8.20 ± 0.34**	**A**	2.01 ± 0.15	1.69 ± 0.05	C	3.35 ± 0.12	B	**390 ± 0.4**	**A**
WF–LSWP	79.7 ± < 0.1	B	**7.19 ± 0.17**	**AB**	2.51 ± 0.35	3.69 ± 0.005	B	**6.91 ± 0.23**	**A**	370 ± 2.6	B

Notes:

- Values with different capital alphabets (A – C) in the same column indicated significant difference ($p \leq 0.05$).
- Experimental results with the highest statistical values ($p \leq 0.05$) were bolded and underlined.

Table 2. Yields (mass basis on WF–LSWP) of individual sugars (glucose, xylose, fructose) and total sugars with numbers in the bracket representing concentration levels in g/l as well as weight scoring of total sugars yield (g / g WF–LSWP) after pretreatment condition (where applicable) and enzymatic digestion with enzyme mixtures EzM 1 and EzM 2.

Pretreatment conditions	Enzyme mixture (EzM)	Yield in g sugar(s)/g WF–LSWP (average concentration in g/l hydrolysate)								Total sugars score	
		Glucose		Xylose		Fructose		Total sugars			
(A) Control		0.052 ± <0.001 (3.61)	D	0.017 ± <0.001 (1.16)	B	0.059 ± 0.001 (4.12)	B	0.127 ± 0.001 (8.89)	B	60.6 ± < 0.1	C
(B) Direct enzymatic digestion	EzM 1	**0.185 ± 0.001 (12.9)**	**A**	**0.022 ± 0.003 (1.51)**	**A**	0.000 ± 0.000 (0.00)	C	**0.206 ± 0.002 (14.4)**	**A**	98.4 ± < 0.1	B
	EzM 2	0.128 ± 0.003 (8.93)	B	**0.019 ± <0.001 (1.33)**	**AB**	**0.063 ± 0.002 (4.41)**	**A**	**0.210 ± 0.010 (14.7)**	**A**	**100 ± < 0.1**	**A**
(C) Saturated steam	EzM 1	0.053 ± <0.001 (13.2)	D	0.006 ± <0.001 (1.37)	C	0.000 ± 0.000 (0.00)	C	0.058 ± 0.001 (14.6)	C	28.0 ± < 0.1	E
	EzM 2	0.046 ± <0.001 (11.6)	E	0.002 ± <0.001 (0.48)	C	0.000 ± 0.000 (0.00)	C	0.048 ± 0.001 (12.0)	D	23.1 ± < 0.1	F
(D) 1.84% (w/v) $Ca(OH)_2$	EzM 1	0.021 ± <0.001 (5.50)	F	0.002 ± <0.001 (0.56)	C	0.000 ± 0.000 (0.00)	C	0.024 ± 0.001 (6.07)	E	11.2 ± < 0.1	G
	EzM 2	0.058 ± <0.001 (15.0)	C	0.002 ± <0.001 (0.50)	C	0.000 ± 0.000 (0.00)	C	0.060 ± 0.001 (15.5)	C	28.7 ± < 0.1	D

Notes:

– Pretreatment conditions (C) and (D) resulted in the significant loss of initial WF–LSWP mass by 3.57 – 3.69 times which were later used as additional correction factors to normalize the calculation of sugar(s) yield in g / g WF–LSWP so that the comparison with condition (A) and (B) could be made. The relatively lower values of average sugar yields, for example, 0.053 g / g WF–LSWP for (C) saturated steam with EzM 1 or 0.058 g / g WF–LSWP for (D) 1.84% (w/v) $Ca(OH)_2$ with EzM 2 during pretreatment steps could still result in relatively high average sugar concentration of 13.2 g glucose / L and 15.0 g glucose / l during the enzymatic digestion step, respectively, as the dried biomass from either control or pretreated processes were subjected to the similar correction factors.

– Values with different capital alphabets (A – G) in the same column indicated significant difference ($p \leq 0.05$).

– Experimental results with the highest statistical values ($p \leq 0.05$) were bolded and underlined.

– Examples calculation for total sugars score for – (1) (B) EzM 2 is (0.210 g / g ÷ 0.210 g/g) ×100 = 100; (2) (C) EzM 2 is (0.048 g/g ÷ 0.210 g/g) × 100 = 23.1; (3) (D) EzM 2 is (0.060 g/g ÷ 0.210 g/g) ×100 = 28.7.

– Standard error of an average experimental value in each bracket could be determined from the same ratio of the preceding experimental yield since the standard error in WF–LSWP concentration (g/l) was relatively small and could thus be neglected.

Table 3. Mass ratio percentage of cellulose, hemicellulose, as well as corresponding starch, pectin, and other carbohydrates in WF–LSWP after each pretreatment condition.

Pretreatment conditions	Cellulose		Hemicellulose^{NS}	Starch, pectin and other carbohydrates (if any)*		Lignin	
(B) Direct enzymatic digestion	22.4 ± 0.1	C	20.4 ± 2.8	**36.9 ± 2.6**$^{\xi}$	**A**	**5.79 ± 0.43**	**A**
(C) Saturated steam	**29.0 ± 0.7**$^{\#}$	**A**	16.4 ± 6.0	21.4 ± 2.6	B	4.95 ± 0.20	B
(D) 1.84% (w/v) $Ca(OH)_2$	25.0 ± <0.1$^{\#}$	B	20.5 ± 1.7	20.3 ± 2.8	B	4.56 ± 0.68	B

Notes:

– * Mass ratio percentage of starch, pectin and other carbohydrates (excluding cellulose and hemicellulose) was calculated as a whole using mass balance with reference to total carbohydrate of 79.7 ± 0.05% (w/w) for (B) and average total sugars loss (from both EzM 1 and EzM 2 enzymatic digestion) relative to (B) in Table 2 (column of total sugars) of 100 × (0.208 – 0.053) = 15.5 ± 0.2% (w/w) for (C) (36.9 – 15.5 = 21.4% (w/w)) and 100 × (0.208 – 0.042) = 16.6 ± 1.0% (w/w) for (D) (36.9 – 16.6 = 20.3% (w/w)).

– $^{\xi}$ This was later determined experimentally to contain 27.9 ± 0.9 g starch, 2.07 ± 0.16 g pectin, and 6.93 ± 2.74 g other carbohydrates (by mass balance).

– $^{\#}$ The increases in mass ratio percentage of cellulose for (C) and (D) relative to (B) after pretreatment processes were possible as some starch, pectin and other carbohydrates were removed.

– Mean values with different capital alphabets (A – C) in the same column indicated significant difference ($p \leq 0.05$).

– Experimental results with the highest statistical values ($p \leq 0.05$) were bolded and underlined.

pectin and other carbohydrates indicated that the latter group was the most prevalent at 36.9 ± 2.6 % (w/w) of WF–LSWP with the presence of relatively small quantity of lignin at 5.79 ± 0.43 % (w/w) of WF–LSWP. Subsequent pretreatments in case of (C) and (D) might result in higher mass ratio percentage between 2.6 – 6.6 % (w/w) of WF–LSWP but there was no significant difference statistically ($p > 0.05$) for hemicellulose and the mitigation of lignin was also minute with the removal range in mass ratio percentage of only 0.84 – 1.23 % (w/w) of WF–LSWP in relation to the condition (B) where pretreatment process was omitted. Sun and Cheng [34] mentioned that the pretreatment process would be beneficial, in general, for the situation in which raw materials contained lignin at the level of more than 15% (w/w). Evidently, the pretreatment of WF–LSWP before enzymatic digestion was unnecessary and could thus be excluded.

The presence of relatively high starch, pectin and other carbohydrates in WF–LSWP – which were later quantified to be 27.9 ± 0.9 (possibly from the crushed longan seed in WF–LSWP), 2.07 ± 0.16, and 6.93 ± 2.74 % (w/w) of WF–LSWP, respectively (see footnote of Table 3) – thus necessitated the inclusion of cellulase, glucoamylase, and α – amylase (EzM 3 – 5) to enhance total sugars productivity for the WF–LSWP hydrolysis in the next section. Furthermore, Jadhav and Singhal [35] reported that co – conjugation between α – amylase and glucoamylase could increase the highest release of glucose to digestive medium. In fact, Dhital *et al.* [36] also warned that the presence of cellulose migh retard the activity of α – amylase by a certain extent.

3.2 Effect of Enzymatic Digestion Sequence on WF–LSWP

Firstly, five EzMs from various sources, namely, EzM 1 – 5 were investigated to find the group of most effective enzymes for studying the effect of enzymatic digestion sequence on WF–LSWP. The obtained total sugars concentration levels from individual enzymatic digestion are shown in Table 4. EzM 3 and 4

Table 4. Yields of individual sugars (glucose, xylose, fructose) and total sugars with numbers in bracket representing sugars concentration levels in hydrolysate as well as sugars production scores after enzymatic digestion with various enzyme mixtures at 50°C for 48 h in absence of pretreatment.

Enzyme Mixture	Yield in g sugar(s)/g WF–LSWP (average concentration in g/l hydrolysate)								Sugars production scores	
	Glucose		Xylose		Fructose		Total sugars			
Control	**0.052 ± <0.001 (3.61)**	F	0.017 ± <0.001 (1.16)	D	0.059 ± 0.001 (4.12)	D	0.127 ± 0.001 (8.89)	D	21.7 ± < 0.1	F
EzM 1	0.185 ± 0.001 (12.9)	C	0.022 ± 0.003 (1.51)	D	0.000 ± 0.000 (0.00)	F	0.206 ± 0.002 (14.4)	C	35.2 ± < 0.1	E
EzM 2	0.128 ± 0.003 (8.93)	D	0.019 ± <0.001 (1.33)	D	0.069 ± 0.002 (4.86)	C	0.216 ± 0.005 (15.1)	C	36.9 ± < 0.1	D
EzM 3	0.245 ± 0.003 (17.1)	B	0.077 ± 0.001 (5.38)	B	**0.263 ± 0.002 (18.4)**	**A**	**0.585 ± 0.002 (40.9)**	**A**	**100.0 ± <0.1**	**A**
EzM 4	**0.269 ± 0.007 (18.8)**	**A**	**0.251 ± 0.001 (17.6)**	**A**	0.048 ± 0.002 (3.39)	E	0.568 ± 0.008 (39.8)	A	97.2 ± < 0.1	B
EzM 5	0.108 ± 0.001 (7.59)	E	0.053 ± 0.001 (3.68)	C	0.147 ± 0.005 (10.3)	B	0.308 ± 0.007 (21.6)	B	52.6 ± < 0.1	C

Notes:

– Values with different capital alphabets (A – F) in the same column indicated significant difference ($p \leq 0.05$).
– Experimental results with the highest statistical values ($p \leq 0.05$) were bolded and underlined.
– Examples calculation for sugars production scores for – (1) EzM 3 is (0.585 g/g ÷ 0.585 g/g) ×100 = 100; (2) EzM 4 is (0.568 g/g ÷ 0.585 g/g) ×100 = 97.2; (3) EzM 5 is (0.308 g/g ÷ 0.585 g/g) ×100 = 52.6.
– Standard error of an average experimental value in each bracket could be determined from the same ratio of the preceding experimental yield since the standard error in WF–LSWP concentration (g/l) was relatively small and could thus be neglected.

consisted of amylase, cellulase, and glucoamylase enzymes. Therefore, the highest total sugars concentration levels were produced by EzM 3 and EzM 4 at 40.9 ± 0.1 and 39.8 ± 0.6 g/l with total sugars yields at 0.585 ± 0.002 and 0.568 ± 0.008 g/g WF–LSWP, respectively. Moreover, the obtained total sugars concentration level from the digestion of WF–LSWP using EzM 5 came in the third place at 21.6 ± 0.5 g/l with corresponding sugars yield of 0.308 ± 0.007 g/g WF–LSWP which was statistical significantly higher ($p \leq 0.05$) than either yields by EzM 1 or 2 hydrolyses. The relatively low sugar yields obtained from EzM 1 or 2 mixtures were due to their limiting activities in cleaving alpha and beta – 1, 4 glycosidic bonds, which were widely available in starch and pectin of WF–LSWP, but rather beta–1,4 xylan and terminal of non–reducing beta – *D* – glucosyl residues with release of xylose and beta – *D* – glucose as previously described in section 2.2. Comparison of sugars production scores from all conditions revealed the scores of EzM 3 – 5 in range of 52.6 – 100 which was statistical significantly higher ($p \leq 0.05$) than EzM 1 and EzM 2 (35.2 – 36.9) as well as control (21.7 ± < 0.1). Therefore, EzM 3 – 5 were chosen for the further study on WF–LSWP regarding the effect of enzymatic digestion sequence.

According to Table 5, different types of enzymatic digestion sequence influenced the yields of sugars being formed from WF–LSWP. In case of the permutation, EzM (3→5→4), (5→3→4), and (5→4→3) produced the highest yields of total sugars in the range of 0.592 – 0.620 g / g WF–LSWP with corresponding average concentration of total sugars in the range of 41.5 – 43.4 g / l hydrolysate. Additionally, the highest yields for individual sugars (glucose, xylose, and fructose) were also obtained from these enzyme sequences. Li and Mitchinson [37] mentioned that glucoamylase could cleave α – (1, 4) and α – (1, 6) glycosidic bonds from non–reducing ends of maltodextrins resulting

Table 5. Comparison of yields for individual sugars, namely, glucose, xylose, fructose as well as total sugars yields with corresponding concentration levels in bracket representing volumetric concentration in g/l and weight scoring of sugars productivity in hydrolyzed WF–LSWP using enzymatic hydrolyses at 50°C for 24 – 72 h in each sequence (permutation, double/single combination, and single/double combination) as designated by an arrow symbol.

Permutation/ combination sequence	Enzymatic digestion sequence	Yields in g sugar(s)/g WF–LSWP (average concentration in g/l hydrolysate)								Sugars productivity $\times 10^{-4}$ g / g / h / step (scores with no unit)	
		Glucose		Xylose		Fructose		Total sugars			
Permutation (3 steps, 72 h)	EzM 3→ 5→ 4	**0.346 ± 0.039 (24.2)**	**ABC**	**0.075 ± 0.007 (5.26)**	**A**	0.199 ± 0.002 (13.9)	BCD	**0.620 ± 0.044 (43.4)**	**AB**	28.7 ± 2.0 (20.4)	G
	EzM 3→ 4→ 5	0.231 ± 0.007 (16.1)	DE	0.061 ± 0.001 (4.24)	BC	0.179 ± 0.002 (12.5)	CDE	0.470 ± 0.004 (32.9)	CDE	21.8 ± 0.2 (15.5)	H
	EzM 5→ 3→ 4	0.328 ± 0.030 (22.9)	BC	**0.070 ± 0.003 (4.91)**	**AB**	**0.220 ± 0.019 (15.4)**	**ABC**	**0.617 ± 0.047 (43.2)**	**AB**	28.6 ± 2.2 (20.3)	G
	EzM 5→ 4→ 3	0.315 ± 0.026 (22.0)	BC	**0.075 ± 0.005 (5.25)**	**A**	**0.202 ± 0.038 (14.2)**	**ABCD**	**0.592 ± 0.069 (41.5)**	**AB**	27.4 ± 3.2 (19.5)	G
	EzM 4→ 5→ 3	0.204 ± 0.017 (14.3)	E	0.055 ± 0.001 (3.86)	CD	0.144 ± 0.006 (10.1)	E	0.403 ± 0.024 (28.2)	E	18.6 ± 1.1 (13.2)	H
	EzM 4→ 3→ 5	0.207 ± 0.005 (14.5)	E	0.055 ± 0.001 (3.84)	CD	0.171 ± 0.003 (11.9)	DE	0.433 ± 0.009 (30.3)	E	20.0 ± 0.4 (14.2)	H
Double/single combination (2 steps, 48 h)	EzM (3+5)→ 4	**0.346 ± 0.001 (24.2)**	**ABC**	0.061 ± <0.001 (4.24)	BC	**0.225 ± 0.004 (15.8)**	**AB**	**0.632 ± 0.005 (44.2)**	**AB**	65.8 ± 0.5 (46.7)	C
	EzM (3+4)→ 5	0.287 ± 0.037 (20.1)	CD	**0.064 ± 0.005 (4.46)**	**ABC**	**0.217 ± 0.011 (15.2)**	**ABC**	**0.568 ± 0.053 (39.7)**	**ABC**	59.1 ± 5.5 (42.0)	DE
	EzM (4+5)→ 3	**0.366 ± 0.016 (25.6)**	**AB**	**0.070 ± 0.005 (4.91)**	**AB**	**0.244 ± 0.009 (17.0)**	**A**	**0.679 ± 0.029 (47.6)**	**A**	70.8 ± 3.1 (50.3)	B
Single/double combination (2 steps, 48 h)	EzM 4→ (3+5)	0.333 ± 0.008 (23.3)	BC	0.041 ± 0.003 (2.87)	E	0.179 ± 0.006 (12.5)	CDE	0.553 ± 0.018 (38.7)	BCD	57.6 ± 1.9 (40.9)	E
	EzM 5→ (3+4)	0.235 ± 0.002 (16.4)	DE	0.052 ± 0.003 (3.62)	CDE	0.170 ± 0.004 (11.9)	DE	0.457 ± 0.009 (32.0)	DE	47.6 ± 0.9 (33.8)	F
	EzM 3→ (4+5)	**0.354 ± 0.001 (24.8)**	**ABC**	0.048 ± <0.001 (3.33)	DE	0.199 ± 0.003 (13.9)	BCD	**0.600 ± 0.004 (42.0)**	**AB**	62.5 ± 0.4 (44.4)	CD
Triple combination (1 step, 48 h)	EzM (3+4+5)	**0.402 ± 0.003 (28.1)**	**A**	0.054 ± 0.004 (3.77)	CD	0.220 ± 0.001 (15.4)	ABC	**0.676 ± 0.007 (47.3)**	**A**	**141 ± 1.4 (100)**	**A**

Notes:

– Total digestion times were 24 + 24 + 24 = 72 h for permutation; 24 + 24 = 48 h for both double/single or single/double combination; and 48 h for triple combination sequences.

– Values with different capital alphabets (A – H) in the same column indicated significant difference ($p \leq 0.05$).

– Experimental results with the highest statistical values ($p \leq 0.05$) were bolded and underlined.

– Standard error of an average experimental value in each bracket could be determined from the same ratio of the preceding experimental yield since the standard error in WF–LSWP concentration (g/l) was relatively small and could thus be neglected.

– Examples calculation for sugars productivity scores for – (1) EzM 4→ 3→ 5 is [(0.433 g/g × 1 step × 48 h) ÷ (0.676 g/g × 3 steps × 72 h)] × 100 = 14.2; (2) EzM 4→ (3+5) is [(0.553 g/g × 1 step × 48 h) ÷ (0.676 g/g × 2 steps × 48 h)] × 100 = 40.9; (3) EzM (3+4+5) is [(0.676 g/g × 1 steps × 48 h) ÷ (0.676 g/g × 1 steps × 48 h)] × 100 = 100.

– All scores for sugars productivity in bracket were calculated as percentages based on the maximum productivity with standard errors of less than 0.1%.

in glucose as the final product. Thus, the suitable digestion sequence of for enzyme mixture containing glucoamylase, namely, EzM 4 could be the last one in order to enhance the yield of monosaccharides being released. In contrast, the release of monosaccharides was lower when glucoamylase was placed in the first position of the sequence as indicated in Table 5. For instance, glucose yields from the EzM (4→5→3) and EzM (4→3→5) sequences were only 0.204 ± 0.017 and 0.207 ± 0.005 g glucose / g WF–LSWP, respectively. These were significantly lower ($p \leq 0.05$) than the other three conditions, namely, EzM (3→5→4), (5→3→4), and (5→4→3) which were in range of 0.315 ± 0.026 – 0.346 ± 0.039 g glucose / g WF–LSWP.

In the situation of double / single combination, yields and the total sugars concentration levels were in the range of 0.568 – 0.679 g / g WF–LSWP and 39.9 – 47.6 g / l hydrolysate for all sequences. Comparison of two cases showed that both of yields and the total sugars concentration levels from double / single combination were significantly higher ($p \leq 0.05$) than the single / double combination. For example, the EzM (4+5)→ 3 sequence obtained 0.679 ± 0.029 g total sugars /g WF–LSWP and 47.6 g/l, respectively, whereas EzM 3→ (4+5) were 0.600 ± 0.004 g total sugars /g WF–LSWP and 42.0 g/l, respectively. These results demonstrated the synergistic action of enzyme mixtures and the importance of digestion sequence which was supported by the study of Jadhav and Singhal [35]. The co – conjugation effect of both α – amylase and glucoamylase could significantly enhance the release of glucose from raw materials to hydrolysate.

Lastly, the triple combination EzM (3 + 4 + 5) could produce the comparable quantity of glucose yield (0.402 ± 0.003 g/g WF–LSWP) and total sugars yield (0.676 ± 0.007 g/g WF–LSWP) with the maximum sugar productivity score of 100 ((141 ± 1.4) × 10^{-4} g total sugars / g WF–LSWP / h / step) when the overall digestion time and number of step(s) involving in the digestion sequence. This result might also be used as illustration for the synergistic action of multiple enzyme mixtures. Evidently, the morphological changes of untreated WF–LSWP and digested WF–LSWP by triple combination were compared under SEM as shown in Figure 1. The surface of untreated WF–LSWP was relatively smooth with characteristics of homogeneous sheetlike appearance without trace of erosion as shown in Figure 1(a) whereas the hydrolyzed WF–LSWP using the triple combination EzM (3 + 4 + 5) was relatively rough and appeared to have numerous spongelike surfaces as shown in Figure 1(b). These results thus indicated the impact of enzymatic digestion on an overall physical structure of WF–LSWP. As WF–LSWP contained the relatively high content of starch, pectin, and other carbohydrates, Presecki *et al.* [38] proposed that complete starch hydrolysis in raw materials could be yielded from the synergistic action between glucoamylase as well as amylase. Furthermore, Lesiecki *et al.* [39] reported that simultaneous application of amylolytic, cellulolytic and pectinolytic enzymes in the raw material digestion was the most effective way of carrying out the process with recorded efficiency reaching 90%. The total sugars concentration levels and respective sugar yields from the double / single combination such as EzM (4+5)→3 were similar to triple combination. This was possible as the synergistic action of enzyme mixture in this specific sequence could also digest WF–LSWP in the similar manner as the triple combination case.

The selection of the optimal enzymatic digestion sequence of WF–LSWP to produce total sugars at the highest concentration level was considered by the sugars productivity and weight scoring of sugars productivity. Although

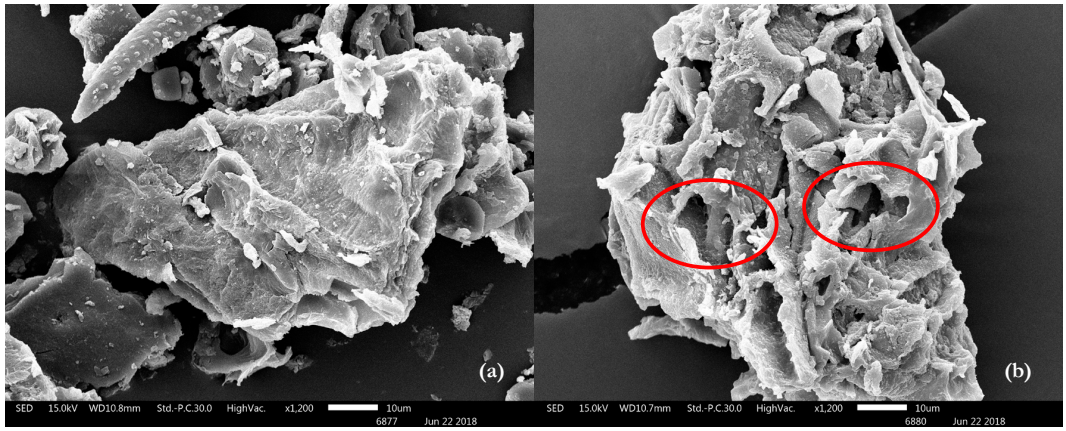

Figure 1. JSM – IT300 SEM photomicrographs of (a) WF–LSWP before enzymatic digestion and (b) WF–LSWP after enzymatic digestion (1,200 × magnification).

EzM (4+5)→3 could produce total sugars with the highest mean of concentration level, the procedure during this process was more complicate than EzM (3 + 4 + 5) which was completed in a single step and could produce total sugars at the statistically similar level ($p > 0.05$). Hence, the triple enzymes combination or EzM (3 + 4 + 5) was selected due to the highest total sugars productivity and score.

3.3 Kinetic of *C. tropicalis* TISTR 5306 Cultivation in Erlenmeyer Flask

In this section, WF–LSWP extract among three initial concentration levels, namely, 16, 45, and 90 g/l were selected as the carbon sources for the cultivation of *C. tropicalis* TISTR 5306 in 250 ml Erlenmeyer flask under shaking condition. Kinetic parameters (μ, t_d, q_S, q_P, $Y_{P/X}$, $Y_{P/S}$, and $Y_{X/S}$) from the yeast cultivation of *C. tropicalis* TISTR 5306 were determined at different time intervals as shown in Table 6 with bar charts illustrating the decreasing and increasing trends of substrates (glucose, xylose, fructose, and total sugars) concentration, products (ethanol and dried biomass) concentration, as well as by–product (acetic acid) concentration for each condition in Figure 2. Lag phase (if any) can be elaborated in the further study with detail time course during 0 – 12 h. The reason for choosing time 0 h as the basis of calculation in this study was based on the assumption of lag phase absence which could be compared to the later detail kinetic study in the same or other conditions.

Specific growth rate (μ) from the cultivation of *C. tropicalis* TISTR 5306 using concentrated WF–LSWP extract at 90 g/l was at the highest ($p \leq 0.05$) level of 0.097 ± 0.001 h^{-1} which corresponded to the shortest doubling time (t_d) of 7.17 ± 0.11 h during 0 – 12 h. These results could be compared to the range of lower specific growth rates and longer doubling times of this microbe between 0.050 ± 0.003 h^{-1} and 13.8 ± 0.7 h in 16 g/l WF–LSWP extract to 0.072 ± 0.005 h^{-1} and 9.59 ± 0.65 h in 45 g/l WF–LSWP extract. The specific growth rate from this cultivation in 90 g/l WF–LSWP extract was also statistical significantly higher ($p \leq 0.05$) than in assorted grade fresh longan juice of 100 ml scale at which μ was only 0.028 ± 0.004 h^{-1} during 24 – 48 h cultivation period from our previous study [22]. This could imply that evaporation effect of WF–LSWP predigested with EzM (3 + 4 + 5) by two times did not cause detrimental effect to specific growth rate of *C. tropicalis* TISTR 5306 during the first 12 h. In addition, further investigation of detailed growth kinetics to elucidate the

Table 6. Kinetic parameters (m, t_d, q_S, q_P, $Y_{P/X}$, $Y_{P/S}$, and $Y_{X/S}$) for four periods (0–12 h, 0–24 h, 0–36 h, and average values) with different initial total sugars concentration levels.

[Total sugars]	Time (h)	μ (h^{-1})		t_d (h)		q_S (gS/gX/h)		q_P (gP/gX/h)		$Y_{P/X}$ (gP/gX)		$Y_{P/S}$ (gP/gS)		$Y_{X/S}$ (gX/gS)	
Fig. 2 16 g/l	0 – 12	0.050 ± 0.003	A(c)	13.8 ± 0.7	B(a)	0.553 ± 0.012	A(b)	0.123 ± 0.004	A(c)	2.44 ± 0.14	A(b)	0.222 ± 0.006	A(c)	0.091 ± 0.005	B(b)
	0 – 24	0.029 ± 0.007	B(b)	**24.1 ± 5.9**	**A(a)**	0.261 ± 0.021	C(b)	0.051 ± 0.004	C(b)	1.76 ± 0.41	B(a)	0.195 ± 0.004	C(b)	0.111 ± 0.026	B(c)
	0 – 36	0.028 ± 0.007	B(b)	**25.1 ± 6.4**	**A(a)**	0.135 ± 0.015	D(c)	0.027 ± 0.003	D(b)	0.976 ± 0.223	C(b)	0.200 ± 0.005	BC(b)	**0.205 ± 0.047**	**A(a)**
	Average	0.036 ± 0.003	B(c)	21.0 ± 2.9	AB(a)	0.316 ± 0.010	B(b)	0.067 ± 0.002	B(c)	1.73 ± 0.16	B(a)	0.206 ± 0.003	B(b)	0.135 ± 0.018	B(c)
Fig. 3 45 g/l	0 – 12	0.072 ± 0.005	A(b)	9.59 ± 0.65	C(b)	**0.642 ± 0.039**	**A(a)**	0.203 ± 0.010	A(b)	**2.81 ± 0.21**	**A(a)**	0.317 ± 0.022	A(b)	0.113 ± 0.009	D(b)
	0 – 24	0.055 ± 0.001	B(a)	12.5 ± 0.22	B(b)	0.270 ± 0.010	C(b)	0.052 ± 0.002	C(b)	0.94 ± 0.04	C(b)	0.193 ± 0.011	B(b)	0.206 ± 0.008	B(a)
	0 – 36	0.041 ± 0.001	C(a)	16.9 ± 0.1	A(b)	0.164 ± 0.005	D(b)	0.025 ± 0.005	D(b)	0.60 ± 0.12	D(c)	0.150 ± 0.029	C(c)	**0.250 ± 0.008**	**A(a)**
	Average	0.056 ± 0.002	B(b)	13.0 ± 0.2	B(b)	0.358 ± 0.014	B(a)	0.093 ± 0.004	B(b)	1.45 ± 0.08	B(b)	0.220 ± 0.013	B(b)	0.189 ± 0.005	C(a)
Fig. 4 90 g/l	0 – 12	**0.097 ± 0.001**	**A(a)**	7.17 ± 0.11	C(c)	0.538 ± 0.054	A(b)	**0.221 ± 0.010**	**A(a)**	2.28 ± 0.10	A(b)	**0.411 ± 0.044**	**A(a)**	**0.180 ± 0.018**	**A(a)**
	0 – 24	0.054 ± 0.002	C(a)	12.9 ± 0.42	B(b)	0.329 ± 0.013	B(a)	0.088 ± 0.005	C(a)	1.64 ± 0.10	C(a)	0.268 ± 0.018	BC(a)	0.163 ± 0.007	A(b)
	0 – 36	0.035 ± 0.001	D(ab)	**19.5 ± 0.32**	**A(ab)**	0.260 ± 0.011	C(a)	0.065 ± 0.004	D(a)	1.83 ± 0.11	B(a)	0.249 ± 0.018	C(a)	0.136 ± 0.006	B(b)
	Average	0.062 ± 0.001	B(a)	13.2 ± 0.18	B(b)	0.375 ± 0.19	B(a)	0.125 ± 0.004	B(a)	1.92 ± 0.06	B(a)	0.309 ± 0.017	B(a)	0.160 ± 0.007	A(b)

Note:

– Values with different capital alphabets (A – D) in the same column indicated significant difference ($p \leq 0.05$) between time intervals of the same individual sugar source.

– Values with different small alphabets (a – c) in the same column indicated significant difference ($p \leq 0.05$) between different sugar sources across the same time interval.

– Kinetic parameters between 0 – 48 h were not shown as the microbial cultures had already reached stationary phase at 48 h.

– Experimental results with the highest statistical values ($p \leq 0.05$) for both comparison of time intervals and sugar sources (A(a)) were bolded and underlined.

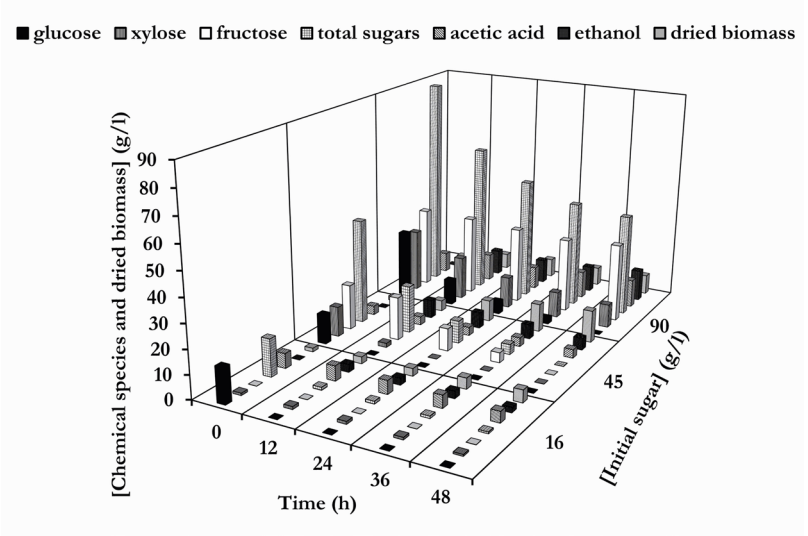

Figure 2. Concentration levels of individual and total sugar(s), acetic acid, ethanol, and dried biomass during the cultivation of yeast *C. tropicalis* TISTR 5306 using WF–LSWP extract (EzM 1) with initial sugar concentration level of 16 g/l, WF–LSWP extract (EzM 3 + 4 + 5) with initial sugar concentration level of 45 g/l, as well as evaporated WF–LSWP extract (EzM 3 + 4 + 5) with an initial sugar concentration level of 90 g/l. All media were supplemented with nitrogen source and the cultivation was carried out in batch mode. Standard error of each data set in this chart was less than 3%.

evaporation effect of WF–LSWP digestive extract on microbial growth, probably by a mathematical model, might be necessary in a future study. In another study, cultivation of *C. tropicalis* on barley malt extract resulted in doubling time of 3 h [40]. The relatively higher specific growth rate from our study might also suggest the advantage of the proposed enzymatic digestion sequence and fermentation condition to promote growth of *C. tropicalis* TISTR 5306 using concentrated WF–LSWP extract at 90 g/l. In addition, the corresponding specific rate of sugars consumption (q_s) and specific rate of ethanol production (q_p) were in ranges of 0.260 ± 0.011 – 0.538 ± 0.054 g utilized sugars (S) /g formed biomass (X) /h and 0.088 ± 0.005 – 0.221± 0.010 g produced ethanol (P)/gX/h, respectively. Q_p value (0.221 ± 0.010 gP / gX /h) was statistical significantly higher ($p \leq 0.05$) than that of 45 g/l WF–LSWP extract (0.203 ± 0.010 gP /gX / h) during 0 – 12 h. This was in contrast to q_S whose value (0.538 ± 0.054 gS /gX / h) was statistical significantly lower ($p \leq 0.05$) than it's counterpart (0.642 ± 0.039 gS/gX/h) during 0 – 12 h. Evidently, Nunta *et al.* [22] reported that q_S and q_p from the cultivation of *C. tropicalis* TISTR 5306 using assorted grade fresh longan juice during 24 – 48 h were 1.31 ± 0.03 gS / gX / h and 0.508 ± 0.014 gP / gX / h, respectively, which were significantly higher ($p \leq 0.05$) than 90 g/l WF–LSWP extract. These results indicated that the different initial concentration level of total sugars could have impact on q_S and q_p with the highest rates occurring at 45 and 90 g/l initial total sugar. This was supported by Azhar *et al.*, [41] who mentioned that ethanol productivity and yield in batch fermentation depended on the initial sugar concentration with enhancing effect when higher initial sugar concentration was implemented. Furthermore, the cultivation of *C. tropicalis* TISTR 5306 in either of fresh

longan juice [22] or WF–LSWP extract from the enzymatic digestion also produce ethanol at different rates, partly from different initial total sugars concentration.

Three principle composition of carbon sources, namely, glucose, xylose, and fructose in various types of WF–LSWP with initial concentration of total sugars were analyzed as indicated in Figure 2. The absence of fructose in case of 16 g/l WF–LSWP when EzM 1 was used as a sole digestive enzyme was evident and compared with 45 and 90 g/l WF–LSWP when EzM (3 + 4 + 5) was employed in which fructose was present in significant amount between 12 – 28 g/l. In our previous report, fresh longan juice had a nearly identical amount of glucose and fructose [22] which might be carried over to WF–LSWP. The significant quantity of fructose might also be released from pulp portion when WF–LSWP was subjected to the more efficient EzM (3 + 4 + 5) digestive enzyme in the situation of concentrated WF–LSWP in comparison to EzM 1 alone with a relatively lower level of WF–LSWP extract. The presence of fructose in EzM (3 + 4 + 5) stock solution was also negligible (unpublished data). The release of glucose and xylose to the digestive extract were as expected since all of the implemented EzMs were capable of cleaving existed polysaccharides in WF–LSWP which would eventually yield either glucose or xylose as end products [42]. Evidently, *C. tropicalis* TISTR 5306 was able to utilize all types of sugars in WF–LSWP extract as indicated in the case of 16 and 90 g/l initial sugar with the following order of preference (1) glucose, (2) xylose, and (3) fructose. Cason *et al.* [43] described the effect of fructose concentration ($\geq$ 20 g/l) at which fructose utilization was usually slower than glucose in all brewing strains and fructose would not be completely consumed by the end of fermentation. In addition, the preferential utilization of glucose over fructose was probably due to competitively inhibition effect of glucose towards fructose uptake by the membrane carrier. This was in contrary to the cultivation of *C. tropicalis* TISTR 5306 using longan juice as substrate during which both glucose and fructose were simultaneously consumed and depleted [22].

In the case of acetic acid profile, all three media had the initial concentration levels of acetic acid between 4 – 8 g/l due to the utilization of acetate buffer for maintaining pH level during enzymatic digestion step for the production of WF–LSWP extract. Moreover, acetic acid was one of the possible by–products from ethanol production by yeast [44]. The production of acetic acid by *C. tropicalis* TISTR 5306 during cultivation in this study (Figure 2) was significantly higher ($p \leq 0.05$) than the situation where fresh longan juice was used as a cultivation medium (< 2 g/l) [22]. Sousa *et al.* [45] mentioned that yeast cells could react to adverse conditions by triggering a stress response thereby enabling them to adapt to the new environment. Yeast was able to degrade acetic acid when the cultivation was carried out under limited–aerobic condition in a medium containing both glucose and acetic acid [46]. This phenomenon was also observed in case of 16 g/l initial sugar with slight decrease of acetic acid in the presence of both glucose and xylose.

The comparison of yields such as product yield based on dried biomass produced ($Y_{P/X}$), product yield based on sugars utilized or ethanol yield ($Y_{P/S}$), and dried biomass yield based on sugars utilized ($Y_{X/S}$) were made between the same time interval across different sugar sources as shown in Table 6. $Y_{P/S}$ was at the statistical significantly highest ($p \leq 0.05$) level of 0.411 $\pm$ 0.044 gP/gS or at 80.4 $\pm$ 8.6% of the theoretical yield value when *C. tropicalis* TISTR 5306 was cultivated in 90 g/l WF–LSWP during 0 – 12 h with the corresponding $Y_{X/S}$ value of 0.180 $\pm$ 0.018 gX/gS. The latter did not differ statistically significant ($p > 0.05$) from

the cultivation in 16 g/l and 45 g/l WF–LSWP during 0 – 36 h. $Y_{P/S}$ from the cultivation of non–*Saccharomyces* yeasts such as *Candida* spp. (i.e. *C. tropicalis*, *C. sake*, *C. stellate*, *C. zemplinina*, and *C. shehatae*), *Lachancea* spp., and *Metschnikowia* spp. generally exhibited the lower ethanol yield and corresponding concentration level in comparison with *Saccharomyces* yeasts due to the production of other products such as organic acids (acetic acid and succinic acid), rather than only ethanol, during the cultivation of these strains [47]. In addition, $Y_{P/S}$ from the cultivation of *Candida* species was usually in the range of 0.360 – 0.442 gP/gS or approximately 70.5 – 86.5% of the theoretical yield (0.511 g ethanol / g glucose consumed) [22, 48]. $Y_{P/X}$ was at statistical significantly highest ($p \leq 0.05$) level of 2.81 ± 0.21 gP/dX when *C. tropicalis* TISTR 5306 was cultivated in 45 g/l WF–LSWP during 0 – 12 h. These results demonstrated the effect of initial total sugars concentration in WF–LSWP extract on kinetic parameters of *C. tropicalis* TISTR 5306 cultivation which were statistical significantly higher ($p \leq 0.05$) than the cultivation in assorted grade fresh longan juice during 24 – 48 h ($Y_{P/S}$ of 0.388 ± 0.014 gP/gS, $Y_{X/S}$ of 0.157 ± 0.023 gX/gS, and $Y_{P/X}$ of 0.157 ± 0.023 gP/gX) [22].

4. Conclusions

In conclusion, the pretreatments of WF–LSWP by alkali or saturated steam before enzymatic digestion were unnecessary as WF–LSWP contained the relatively high starch content of 27.9 ± 0.9 % (w/w) and low lignin content of only 5.79 ± 0.43 % (w/w). The most suitable digestion sequence of WF–LSWP was by the triple combination sequence of three commercial enzymes (EzM 3 + 4 + 5) containing amylase, glucoamylase, cellulase, and xylanase in the one step enzymatic digestion at 50°C for 48 h which resulted in the statistical significantly highest ($p \leq 0.05$) specific overall sugars productivity of (141 ± 2) × 10^{-4} g total sugars / g WF–LSWP / digestion step / h. The yeast cultivation in 90 g/l WF–LSWP extract resulted in the statistical significantly highest ($p \leq 0.05$) values of $Y_{X/S}$ and $Y_{P/S}$ during 0 – 12 h. These trends of statistical significantly highest ($p \leq 0.05$) values were also observed for the other kinetic parameters such as (q_P, $Y_{P/X}$) and (m, q_S) during 0 – 12 h in 45 and 90 g/l WF–LSWP extracts, respectively. The concentrated effects of WF–LSWP digestate due to evaporation at different levels on growth and fermentation kinetics should be investigated further to elucidate the degree of evaporation that would result in the optimal growth kinetics for ethanol and biomass production from *C. tropicalis* TISTR 5306 with WF–LSWP as substrate.

Nomenclature

μ	specific growth rate (h^{-1})
AOAC	Association of Official Analytical Chemists
AR	analytical reagent
EC	enzyme classification
EzM	enzyme mixtures
HPLC	high pressure liquid chromatography
LSWP	longan solid waste powder
PAC	phenylacetylcarbinol
PDC	pyruvate decarboxylase
PO	peel–only
q_P	specific ethanol production rate (g ethanol / g dried biomass / h)
q_S	specific total sugars consumption rate (g total sugars consumed / g dried biomass / h)
SE	standard error
SEM	scanning electron microscope
SO	seed–only
t_d	doubling time (h)
TISTR	Thailand Institute of Scientific and Technological Research
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
WF	whole fruit

YM yeast – malt
$Y_{P/S}$ yield of ethanol produced over total sugars consumed (g ethanol / g total sugars)
$Y_{P/X}$ yield of ethanol produced over dried biomass consumed (g ethanol / g dried biomass)
$Y_{X/S}$ yield of dried biomass produced over total sugars consumed (g dried biomass / g total sugars)

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