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# Growth of probiotic *Lactobacillus rhamnosus* GR-1 using saccharified oil palm mesocarp fibre as affected by saccharification time and fermentation time

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**Abstract**

Abundant low-cost oil palm mesocarp fibre could serve as a potential feedstock for the production of oligosaccharides which may have prebiotic properties. Thus, a study was carried out to determine the ability of saccharified oil palm mesocarp fibre (OPMF) to support the growth of a probiotic microorganism (*Lactobacillus rhamnosus* GR-1*)*. The saccharified OPMF was produced using different saccharification times (1, 3 and 6 hrs) while fermentation of the saccharified OPMF with *L. rhamnosus* GR-1 was carried out for 0, 12 and 24 hrs. Growth of *L. rhamnosus* GR-1 was measured based on $OD_{600}$ using a UV-spectrophotometer along with the pH of the growth media. The results showed a significantly higher ($p<0.05$) growth of *L. rhamnosus* GR-1 with increasing saccharification time and fermentation time. Increased saccharification time of up to 6 hrs also resulted in a higher variety of oligosaccharides compared to 1 hour and significantly reduced ($p<0.05$) pH of the growth media. The use of saccharified OPMF produced comparable growth of *L. rhamnosus* GR-1 with significantly lower ($p<0.05$) pH compared to using fructooligosaccharide in the growth media. The results showed the ability of saccharified OPMF to support the growth of probiotic *L. rhamnosus* GR-1.

## 1. Introduction

The World Health Organization defines a probiotic as ‘‘live microorganisms that when administered in adequate amounts confer a health benefit on the host when ingested” (Williams and Hekmat, 2017; White and Hekmat, 2018). Examples of probiotic microorganisms include *Lactobacillus rhamnosus*, *Lactobacillus plantarum* (Asmariah *et al*., 2018), *Lactobacillus salivarius*, *Lactobacillus reuteri* (Shamsudin *et al*., 2019) as well as *Bifidobacterium* species (Williams and Hekmat, 2017). The growth and metabolic activity of probiotic bacteria such as lactobacilli can be selectively accelerated by various dietary carbohydrates that were not digested by the host (Leblanc *et al*., 2017). Prebiotics are defined as a selectively fermented ingredient that concedes specific changes, both in condition and/or activity in the gastrointestinal microflora, that bestow health benefits (White and Hekmat, 2018).

Nowadays, it is a great attraction to produce novel forms of prebiotic from readily available and renewable carbohydrate resources (Shi *et al*., 2018). Recently, there has been increased interest in the potential of oligosaccharides as prebiotics such as xylooligosaccharides, fructooligosaccharides (FOS), and several others (Dávila *et al*., 2019). An oligosaccharide is a molecule containing a low number of monosaccharide residues connected by glycosidic linkages (Nguyen and Haltrich, 2013). According to Patel and Goyal (2011), functional oligosaccharides are gaining outstanding popularity among the non-digestible carbohydrates, owing to their physiological benefits to the consumer. Moreover, they positively affect the composition and metabolic activity of the intestinal microflora and a daily moderate supplement of these non-digestible oligosaccharides stimulates mineral absorption especially Ca and Mg (Roberfroid *et al*., 2010; Kunova *et al*., 2012).

Biomass from plant material is the most abundant and widespread renewable raw material for sustainable development and can be employed as a source of

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polymeric and oligomeric carbohydrates (Gullon *et al*., 2009). Cellulose is a high molecular weight linear polymer composed of β-glucose (5000–10,000 units) and linked by β-1,4-glycosidic bonds (Srivastava *et al*., 2019). Enzymatic hydrolysis using cellulase from *Trichoderma reesei*, a mixture of endoglucanases, exoglucanases, and β-glucosidase, is widely used for the degradation of cellulose into soluble oligo glucan/ oligomers, cellobiose and glucose (Pasma *et al*., 2013). Cellulose from oil palm mesocarp fibre (OPMF) possibly can be depolymerised using enzymes to produce oligosaccharides through saccharification. About 80 million tonnes of oil palm biomass was produced in Malaysia in 2017. In addition, it was forecasted that the quantity of solid biomass from oil palm will reach 111 million tonnes a year by 2020 (MGCC, 2018). Moreover, approximately 11% of oil palm mesocarp fibre (OPMF) is generated from palm fruits after oil extraction. OPMF, also known as palm pressed fibre (PPF), is the biomass residue obtained after pressing the palm fruits for palm oil extraction (Nordin *et al*., 2013). Thus, it is a potential source of biomass that can be utilised for the production of valuable compounds (Pasma, Rusli and Maskat, 2019). However, in order for the saccharified OPMF to function as a prebiotic, it has to be shown to be able to support the growth of probiotic microorganisms.

*Lactobacillus rhamnosus* GR-1 was chosen for this study since it was known as the second most scientifically documented *L. rhamnosus* probiotic. It was discovered in 1981 in Kingston, Ontario (Westerik *et al*., 2018). *L. rhamnosus* GR-1 is a probiotic strain that is bile and acid resistant and also can survive the passage through the gastrointestinal tract. These properties made *L. rhamnosus* GR-1 exert a number of human health benefits such as the prevention of urinary tract infections and oral intake of GR-1 also provide benefits to the urogenital tract, the gut as well as respiratory tract (Westerik *et al*., 2018; White and Hekmat 2018; Figueroa-Gonzalez *et al*., 2019). Due to its properties, *L. rhamnosus* GR-1 has been attributed as one of the main probiotic strains that are suitable for applications in the production of yoghurt and fermented foods in Africa (Wekstrik *et al*., 2018). Meanwhile, Zare *et al*. (2012) reported that *L. rhamnosus* has been the main species used as a probiotic over the years.

Williams and Hekmat (2017) have described the addition of short-chain inulin, long-chain Inulin and oat have no adverse supplementation effects in the viability of *L. rhamnosus* GR-1, with 4% of short-chain inulin showing the highest viability. While, White and Hekmat (2018) were found that the fermentation of apple cider, grape and orange juice fortified with inulin fibre with *L. rhamnosus* GR-1 improved the viable bacterial counts that were greater than the minimum requirement of $10^6$ CFU/mL, to be classified as prebiotics. Other than that, results from the sensory evaluation indicated may be potential for probiotic fruit juices in the commercial market. A study conducted by Dunkley and Hekmat (2019) has studied the growth and viability of *L. rhamnosus* GR-1 in carrot juice (CJ), carrot apple juice (CAJ), carrot orange juice (COJ) and carrot apple juice (CBJ) over 72 h of fermentation and 30 days storage at 4°C. The result showed a significant microbial growth only juices CJ and COJ with a significant decline in pH.

Due to its abundance, OPMF fibre has the potential to serve as a lower-cost cellulosic feedstock to produce oligosaccharides as an alternative source of prebiotic. As there is limited literature on the use of saccharified OPMF as feedstock for probiotics, this study was carried out to determine the ability of saccharified OPMF to support the growth of *L. rhamnosus* GR-1 which is a known probiotic as affected by saccharification time and also fermentation time.

## 2. Materials and methods

### *2.1 Materials*

The oil palm mesocarp fibre (OPMF) was sourced from a local oil palm mill in Hulu Langat, Selangor, Malaysia. Acetonitrile, manganese (II) sulfate tetrahydrate and Tween-80 were purchased from Merck and Co. (USA), while acetate buffer solution (pH 4.0) and di-potassium-hydrogen phosphate were obtained from R&M Chemicals. Di-ammonium-hydrogen citrate, sodium acetate and magnesium sulphate were purchased from Chemiz, Malaysia. Other chemicals, sodium hydroxide (NaOH), sodium acetate anhydride, ethanol (95% v/v), ethyl acetate and meat extract were purchased from Systerm, Malaysia. Meanwhile, dextran molecular weight ladder (Mw 5000), ammonium acetate, 2-aminobenzamide (2-AB), sodium cyanoborohydride, fructooligosaccharide (FOS), phosphate buffered saline (PBS), cysteine hydrochloride and enzyme cocktail Celluclast (1.5 L) from *Trichoderma reesei* (>700 units/ g) were obtained from Sigma Aldrich Co. (St. Louis MO, USA). Chemicals for probiotic medium preparation, Man -Rogosa-Sharpe (MRS) broth for microbiology with carbohydrate source was obtained from Fluka Analytical and MRS agar, bacteriological peptone, and yeast extract were purchased from Oxoid (Hampshire, UK). Man-Rogosa-Sharpe (MRS) liquid without carbohydrate source was prepared by dissolving 10 g of bacteriological peptone, 8 g of meat extract, 4 g of yeast extract, 1 g of di-potassium-hydrogen phosphate, 2 g of Tween-80, 2 g of di-ammonium-hydrogen citrate, 5 g of sodium acetate, 0.2 g of magnesium sulphate and 0.04 g of manganese (II) sulphate tetrahydrate per litre. The

 

medium was autoclaved and stored at 4°C until further use.

*2.2 Preparation of carbohydrate substrates*

The mesocarp fibre was washed to remove foreign particles and dried in an oven at 80°C overnight (Pasma *et al*., 2019). Subsequently, the dried fibre was ground into approximately 0.25 mm particles, transferred into sealed plastic bags and stored at 4°C until used. The ground OPMF was deconstructed as according to Hassan *et al*. (2013) with slight modifications which involved pre-treatment using steam and 15% NaOH solution.

Subsequently, the OPMF was subjected to enzymatic saccharification using a commercial enzyme cocktail (Celluclast 1.5 L) in an incubator shaker (Wisecube wis 20) at 50°C, 120 rpm (Tang *et al*., 2018). The saccharification time was varied at 1, 3 and 6 hrs to produce samples at different degrees of polymerisation (DP) ranging between 2 and 4. The saccharified OPMF was extracted by centrifugation 2147×*g*, 15 mins (Thermo Scientific™ Sorvall™ model) and then dried using a rotary evaporator. The glucose units (GU) of saccharified OPMF was determined using high-performance liquid chromatography with fluorescence detection (HPLC-FD). Fluorescent labelling via chemical derivatisation with 2-aminobenzamide (2-AB) and the subsequent HPLC-FD analysis of saccharified OPMF samples were carried out as described previously (Woon *et al*., 2016; Jalaludin *et al*., 2017). The 2AB-labelled samples (10 µL of hydrolysate) were run on an HPLC instrument using an XBridge amide (4.6×250 mm, particle size 3.5 µm) column with a gradient elution system of ammonium acetate (pH 3.5) and acetonitrile as reported by Woon *et al*. (2016). The chromatography system consists of a Waters 600 pump, autosampler Waters 2707 with a Waters 2745 model fluorescent detector set at the wavelengths of 360 nm wavelength (excitation) and 426 nm (emission).

*2.3 In vitro fermentation of saccharified OPMF using Lactobacillus rhamnosus*

The fermentation of saccharified OPMF using *L. rhamnosus* GR-1 was carried out according to the methods by Shi *et al*. (2018) and Mueller *et al*. (2016) with slight modifications. A fresh overnight culture of *L. rhamnosus* GR-1 was prepared in MRS broth with carbohydrate source (*Fluka Analytical*) at 37°C for 16 hrs at 180 rpm using an incubator shaker (Wisecube wis 20) in aerobic condition and cell density was determined based on turbidity measurement as indicated by an increased $OD_{600}$ value using UV-spectrophotometer (Spectronic 200, Thermo Scientific™). Subsequently, the cultured bacteria were collected by centrifugation at 6708×*g* for 5 mins (Gryozen Mini, Korea) and the bacteria were washed two times in phosphate buffered saline (PBS) supplemented with cysteine hydrochloride to remove MRS broth residue. The cell amount for a final starting $OD_{600}$ of 0.1 was determined. The bacteria (1% v/v) were resuspended in the MRS broth medium in the absence of any carbohydrate source. Subsequently, the fermentation was conducted in the presence of 1% (v/v) saccharide OPMF samples (1, 3 and 6 hrs) in a 100 mL Erlenmeyer flask with a final volume of 30 mL. Aerobic fermentation of the saccharified OPMF was carried out for 0, 12 and 24 hrs at 37°C at 180 rpm incubator shaker (Wisecube wis 20). The growth of *L. rhamnosus* GR-1 was measured based on $OD_{600}$ using a UV-spectrophotometer. The negative control was incubated without sugar and FOS was chosen as a positive control. All experiments were conducted in triplicates.

*2.4 pH analysis*

The pH of all saccharified OPMF samples (1, 3 and 6 hrs) was measured using Sartorius Basic PB-10 pH meter (*Germany*) after 0, 12 and 24 hrs of fermentation. An average of three replicates of each sample at each fermentation time was taken.

*2.5 Statistical analysis*

All data reported in this study was indicated as means ± SD. To determine if there were significant differences in the growth of *L. rhamnosus* GR-1 a two-way ANOVA and Tukey test were performed using the statistical software JASP. Comparison of pH data was carried out using one-way ANOVA and Tukey test. The confidence interval of 95% was used.

## 3. Results and discussion

The effects of saccharification and fermentation time of OPMF on the growth of probiotic *L. rhamnosus* GR-1 are as shown in Table 1. Based on the results, the individual effect of saccharification time on *L. rhamnosus* GR-1 growth showed a significant ($p<0.05$) increase in $OD_{600}$ from saccharification time of 1 to 3 hrs. However, further increase of saccharification time from 3 to 6 hrs did not show any significant change in the growth of *L. rhamnosus* GR-1. The significant increase in growth of *L. rhamnosus* GR-1 ($p<0.05$) showed that saccharified OPMF was able to support the growth of probiotic bacteria.

The increase in growth from 1 to 3 hrs of saccharification time may be due to the ease in using the substrate as the longer duration of saccharification will result in a lower degree of GU of the cellulose in OPMF (Meng *et al*., 2016). However, saccharification beyond 3

 

hrs may not have produced any further decrease in the GU of cellulose thus resulting in the insignificant growth measured. Results from Table 2 supported the mentioned possibility as it can be seen that the type of oligosaccharide after 3 and 6 hrs of saccharification (1, 2, 3 and 4 glucose units) were higher compared to 1 hr (1 and 2 glucose units). In addition, no increase in the type of oligosaccharide was observed between 3 and 6 hrs of saccharification.

Table 1. Individual and interactive effects of saccharification time and fermentation time based on two-way ANOVA for the growth of *Lactobacillus rhamnosus* GR-1 using oil palm mesocarp fibre as carbon source.

| Effects | Mean of $OD_{600}$ | p |
|---|---|---|
| Saccharification time (hrs) | | |
| 1 | $0.315^{b}$ | 0.008 |
| 3 | $0.562^{a}$ | |
| 6 | $0.515^{a}$ | |
| Fermentation time (hrs) | | |
| 0 | $0.038^{c}$ | <0.001 |
| 12 | $0.832^{a}$ | |
| 24 | $0.522^{b}$ | |
| Interaction | | 0.028 |

Values are presented as mean. Values with different superscript within the same saccharification time or fermentation time are significantly different ($p<0.05$).

Table 2. The number of glucose units for OPMF cellulose oligosaccharide at different saccharification time and FOS as carbon source.

| Samples | Number of glucose units (GU) |
|---|---|
| OPMF (1hr) | 1, 2 |
| OPMF (3hrs) | 1, 2, 3, 4 |
| OPMF (6hrs) | 1, 2, 3, 4 |
| FOS | 3-Sep |

A previous study reported that *L. rhamnosus* GR-1 grew on β-glucan oligosaccharides where it consistently consumed mono- and disaccharides and preferentially consumed tri-saccharides (degree of polymerisation (DP) 3) (Sims *et al*., 2014). Several studies have described that *L. rhamnosus* consumed both oligosaccharides (tri- and disaccharides) as well as glucose as reported in a study on the consumption of Oligomate 55 (galactooligosaccharide 55%) in fermentation media (Figueroa-Gonzalez *et al*., 2019).

Table 1 also shows a significant individual effect ($p<0.05$) of fermentation time on the growth of *L. rhamnosus* GR-1. An increase in fermentation time from 0 to 12 hrs resulted in a significant increase ($p<0.05$) in the growth of *L. rhamnosus* GR-1. However, a further increase from 12 to 24 hrs showed a significant decrease ($p<0.05$) in the growth of the probiotic bacteria. In general, the longer the fermentation time, the higher the opportunity for the bacteria to consume the saccharified OPMF (Hawashi *et al*., 2017). Thus, this explained the significant increase ($p<0.05$) in growth when fermentation time was increased to 12 hrs. The decrease in growth when fermentation time was extended to 24 hrs may be due to the limitation of nutrients to support growth (Hawashi *et al*., 2017).

The significant interaction between saccharification time and fermentation time as shown in Table 1 is elaborated in Table 3. The effect of increased saccharification time was dependent on fermentation time where at fermentation time of 12 hrs, growth of *L. rhamnosus* GR-1 increased significantly ($p<0.05$) from 1 to 3 hrs but not significantly different when further increased to 3 hrs. Similarly, the interactive effect between saccharification time and fermentation time can be observed for saccharification time of 6 hrs where the increase in fermentation time from 12 to 24 hrs resulted in a significant decrease ($p<0.05$) in bacterial growth.

Table 3 also shows the combined effects of saccharification time and fermentation time compared to FOS. FOS samples showed a similar trend for growth to OPMF 1 and 3 hrs where there was a significant increase ($p<0.05$) when fermentation time was increased from 0 to 12 hrs. Increasing fermentation time from 12 to 24 hrs did not produce any significant difference in growth. Thus, OPMF samples showed similar growth compared to FOS suggesting the possible potential of OPMF as a prebiotic material. However, further studies need to be carried out to ascertain the potential.

Table 3. Combined effect of saccharification time and fermentation time on the growth of *Lactobacillus rhamnosus* GR-1 using oil palm mesocarp fibre as carbon source.

| Sample | Fermentation time (hrs) | | |
|---|---|---|---|
| | 0 | 12 | 24 |
| Negative control (No sugar) | $0.015^{i}$ | $0.625^{def}$ | $0.320^{fgh}$ |
| OPMF 1 hr | $0.055^{ghi}$ | $0.510^{def}$ | $0.380^{efg}$ |
| OPMF 3 hrs | $0.050^{ghi}$ | $0.945^{abc}$ | $0.690^{cde}$ |
| OPMF 6 hrs | $0.010^{j}$ | $1.040^{ab}$ | $0.495^{def}$ |
| Fructo oligo saccharide (FOS) | $0.015^{j}$ | $0.810^{bcd}$ | $0.530^{def}$ |

Values with different superscript within the same row are significantly different ($p<0.05$).

The combined effect of saccharification time and fermentation time on pH during the growth of *L. rhamnosus* GR-1 using oil palm mesocarp fibre as carbon source was analysed as shown in Table 4. Comparison made among the samples showed that for saccharification time of 1, 3 and 6 hrs, fermentation up to 12 hrs showed a significant ($p<0.05$) decrease in pH. However, all OPMF samples (1, 3, and 6 hrs) did not show any significant changes ($p>0.05$) in pH after a further increase in fermentation time from 12 to 24 hrs. The trend showed for pH (Table 4) corroborated the trend of $OD_{600}$ representing the growth of *L. rhamnosus*

 

GR-1 as shown in Table 3. According to Sims *et al*. (2014), an increase in $OD_{600}$ of the culture broth and a decrease in pH of the culture media is the result of short-chain fatty acid production and reflected the ability of the bacteria to grow on the substrates. It has also been reported that *L. rhamnosus* is able to increase the production of SCFAs such as acetate, propionate and butyrate (Leblanc *et al*., 2017). Thus, these observations further support the ability of the probiotic *L. rhamnosus* to grow on saccharified OPMF.

Table 4. Combined effect of saccharification time and fermentation time on pH during growth of *Lactobacillus rhamnosus* GR-1 using oil palm mesocarp fibre as carbon source.

| Sample | Fermentation time (hrs) | | |
|---|---|---|---|
| | 0 | 12 | 24 |
| Negative control (No sugar) | $6.703^{ab}$ | $6.830^{a}$ | $6.857^{a}$ |
| OPMF 1 hr | $6.410^{ab}$ | $5.480^{cd}$ | $5.537^{c}$ |
| OPMF 3 hrs | $6.390^{ab}$ | $5.257^{d}$ | $5.277^{d}$ |
| OPMF 6 hrs | $6.363^{ab}$ | $5.197^{d}$ | $5.137^{d}$ |
| Fructo oligo saccharide (FOS) | $6.187^{b}$ | $6.230^{b}$ | $6.533^{ab}$ |

Values with different superscript within the same row are significantly different ($p<0.05$).

The results obtained (Table 4) also revealed that during fermentation for 12 hrs, an increase in saccharification time from 1 to 3 hrs, and subsequently to 6 hrs did not result in any significant ($p>0.05$) changes in pH for the growth of *L. rhamnosus* GR-1. However, when fermentation was extended for 24 hrs, OPMF saccharified for 3 hrs resulted in a significantly ($p<0.05$) decreased culture medium pH compared to OPMF 1 hr. Further increase in saccharification time from 3 to 6 hrs did not show any significant ($p>0.05$) difference in pH. The results suggested that an extended fermentation time was needed to realise the effects of increased saccharification time on pH.

Compared to FOS samples, the pH of all OPMF samples (1, 3 and 6 hrs) showed significantly lower ($p<0.05$) pH after fermentation for 12 and 24 hrs. As the lowering of pH may have been attributed to the production of short-chain fatty acids (Sim *et al*., 2014), the results suggested that saccharified OPMF may be able to produce more short-chain fatty acid by *L. rhamnosus* compared to FOS.

## 4. Conclusion

In our study, both saccharification time and fermentation time with 3 hrs and 12 hrs, respectively, showed a significant ($p<0.05$) effect on the growth of *L. rhamnosus* GR-1 using saccharified oil palm mesocarp fibre (OPMF) as carbon sources. The growth of *L. rhamnosus* GR-1 using OPMF was comparable to its growth when using FOS. In addition, the growth of *L. rhamnosus* GR-1 using OPMF saccharified for 3 hrs resulted in a significantly ($p<0.05$) decreased culture medium pH compared to FOS. Thus, this work suggested that saccharifying OPMF to oligosaccharides has the potential to be developed as a prebiotic for *L. rhamnosus* GR-1 probiotic.

## Conflict of interest

The authors declare no conflict of interest.

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