

# Sugars from sago frond as prebiotic substrate to enhance the growth of *Lactococcus lactis* IO-1 and production of L-lactic acid.

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## Abstract

Sago palm is often discredited for exhibiting long maturity period and barrenness of pre-harvest products, which restrain its potentials as an alternative and eternal starch provider. The use of sago fronds to produce prebiotic and fermentable sugars from pruned palms and fronds discarded upon harvesting is a possible enterprise to provide income for the cash-strapped sago farmers while waiting for the sago trunks to be harvestable. Dried sago frond powder coupled with the cellulolytic enzyme and incubated for 48 hours, producing a maximum recovery of cellobiose at 25%. This is of great advantage in reducing the cost of large-scale processes since the yield and productivity from SFS is comparable to the Standard Medium and SFS amended with yeast extract at 0.85g/g and 85%, respectively. Meanwhile, the composition of cellobiose as main sugar component increase the viability of the *Lactococcus lactis* IO-1 by prolong the lifespan of the cell by perform as slow release carbon source, in fact, cellobiose was protected by  $\beta$  (1-4) glycosidic bond made it consumable to specific probiotic in human digestive system conceive that cellobiose as potential prebiotic component for human. Clearly, the use of sago frond is highly economical and sustainable as the raw material for the manufacturing of fermentable sugars and subsequently as the sustainable substrate for large-scale production of L-lactic acid.



## 1.0 INTRODUCTION

Lactic acid is widely used in the food industry (as a preservative), in the cosmetic industries (skin and hair care products), and in the pharmaceutical and textile industries. Lactic acid can be produced both by microbial fermentation and synthetically. Microbial fermentation using lactic acid bacteria and biological wastes is highly regarded since it utilizes renewable resources and does not emit greenhouse gasses especially carbon dioxide [8]. *Lactococcus lactis* IO-1 is the homolactic bacteria widely used for the production of lactic acid due to ability of the strain produce L-(+) lactic acid as the main product [9]. Generally, lactic acid is produced using starch from food crops such as maize, sugar cane and tapioca as the main raw material. However, production of lactic acid from food crops for use other than as food preservatives is deemed unethical and hence must be avoided.

Consequently, alternative raw materials for the production of lactic acid such as agricultural wastes should be encouraged to ensure sustainability which guarantees production and concomitantly minimise pollution. Therefore, in this research the use of sago frond as the alternative raw material for the production of lactic acid is proposed due to the humongous amount of this waste generated upon harvesting of sago trunks for the production of sago flour. Utilization of sago frond for the production of lactic acid not only will reduce environmental problems due to the slow degradability of sago fronds but will also increase the sago farmers' income, while denying ethical issues of producing lactic acid from food crop commodity.

## 2.0 MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Sago Frond Sugar (SFS)

Sago fronds were obtained from locally cultivated sago palms in the District of Pusa, Sarawak. Sago Frond Sugar was produced through enzymatic hydrolysis of sago frond. Deskined sago frond was cut into small 2-3 cm cubes, oven dried and ground to produce powder, name as Sago Frond Powder (SFP). About 10% (v/w) of the enzyme Celluclast 1.5L (60 FPU/mL) was used to convert cellulose in SFP into SFS. The hydrolysis was conducted at 45°C for 48 hours. Detail procedure where treated sago frond fibre has been enzymatically hydrolysed using the cellulolytic enzyme producing Sago Frond Sugar (SFS), which mainly contains cellobiose and glucose at 15-17 g/L and 5-7 g/L, respectively [2].

#### 2.1.2 Microorganism and Standard Media

The strain of *Lactococcus lactis* IO-1 was obtained from the Japanese Collection of Microorganism (JCM) with an optimum growth temperature at 37°C [10]. Stock culture was revived in Nutrient Broth (NB) media for 18 hours at 37°C. Exactly 10% (v/v) of the culture was transferred to the inoculum medium.

#### 2.1.3 Inoculum

A 10% (v/v) inoculum was used as standard in this experiment. The inoculum medium consisted of 20 g/L commercial glucose and 5 g/L yeast extract. Once the medium was sterilized, the inoculum was incubated for 18 hours at 37°C with agitation. The incubated inoculum was divided into 3 sterile falcon tubes at 10% (v/v) of working volume, and centrifuged (8,000 rpm) at 4°C. The supernatant from the inoculum was discarded.

### 2.2 Fermentation

The experiment was conducted using batch fermentation system in 2L shake flasks at 1L working volume. Three types of fermentation media were used in this experiment, these are Standard Medium (SM) made of commercial glucose and yeast extract at 20 g/L and 5 g/L respectively, SFS supplemented

with yeast extract (SFS+YE) and SFS, without the addition of yeast extract. The fermentation media were incubated for 48 hours at 37°C with the agitation speed of 150 rpm. Exactly 10ml samples were collected into pre-weighed centrifuge tubes for every 6 hours to determine the cell growth profile, sugar consumption and production of lactic acid.

### 2.3 Growth Profile

The growth profile of *L. lactis* IO-1 was determined using the dry cell weight (DCW) method. Samples collected from the fermentation process were centrifuged (10,000 rpm) at 4°C. The supernatant was placed into new falcon tubes to determine the sugar consumption and lactic acid production. The cell pellets were dried in oven at 60°C until the weight is constant.

### 2.4 Sugar Consumption and Lactic Acid Production

The supernatant obtained from the fermentation process was analysed for sugar and L-lactic acid using High Performance Liquid Chromatography (Shimadzu, Japan) with BioRad Fermentation Monitoring Column at 1.0 ml/min flowrate using 0.005M H<sub>2</sub>SO<sub>4</sub> as mobile phase at 65°C column temperature.

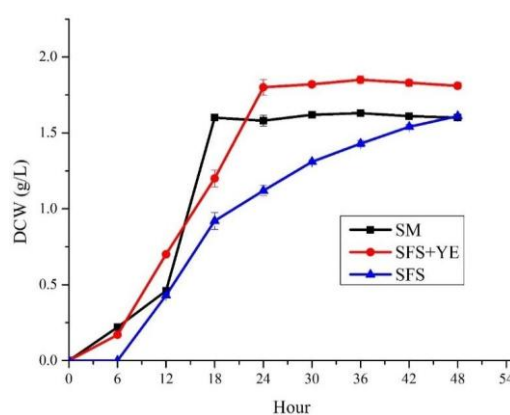
### 2.5 Statistical Analysis

All data obtained from all of experiments were compared and analysed using a One Way and Two-Way ANOVA. LEAD Technologies, MINITAB Version 17.1.0 was used to perform the statistical analyses and all graphs were generated using OriginPRO Version 9.0.0 software by OriginLab.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Growth Profile of *L. lactis* IO-1 in SM, SFS+YE and SFS

The growth of *Lactococcus lactis* IO-1 in SM showed that the lag phase commenced in less than 6 hours upon incubation, followed by the exponential phase which remains up to 12 hours later (figure 1). The growth reached stationary phase after 18 hours with a biomass (DCW) of 1.60 g/L.



**Figure 1:** Growth profile of *L. lactis* IO-1 in different types of media; standard medium (SM), sago frond sugar amended with yeast extract (SFS+YE) and only sago frond sugar (SFS), based on dried cell weight (DCW, g/L).

In the medium where SFS was amended with yeast extract (SFS+YE), the growth of *Lactococcus lactis* IO-1 showed the lag phase started in less than 6 hours, similar to the SM, following which the cell grew exponentially for the remaining 18 hours in the lag phase. Stationary phase was reached after 24 hours of incubation with a biomass (DCW) of 1.80 g/L.

Comparatively, in the medium where SFS was used as the sole substrate (SFS medium), *Lactococcus lactis* IO-1 remained in the lag phase for 6 hours. But after this, the biomass promptly increased and remains in the lag phase for 12 hours of exponential growth. Based on this observation, the cells cultured in SFS continued to proliferate even after 48 hours of incubation, where the amount of biomass (DCW) was 1.61 g/L. Evidently, incubation of *Lactococcus lactis* IO-1 in SFS+YE produced the highest DCW (1.80 g/L) compared with SM and SFS, which was both at about 1.60 g/L. Utilization of cellobiose as the main carbon source enhanced the growth of *Lactococcus lactis* IO-1 but it needs longer incubation time.

Analyses of the kinetic parameters of the bacterium cultured in SM, SFS+YE and SFS (table 1) shows that the productivity of *L. lactis* IO-1 in SFS+YE medium (0.04<sup>b</sup>) was only slightly lower compared to SM (0.05<sup>a</sup>). This also confirmed that cellobiose in SFS requires a little more time for the bacterium to be used as substrate.

**Table 1:** Kinetic parameters of *Lactococcus lactis* IO-1 grown in SFS+YE and SFS media.

Parameters	Types of Medium		
	SM	SFS+YE	SFS
Maximum DCW Concentration (g/L)	1.6 <sup>b</sup>	1.8 <sup>a</sup>	1.61 <sup>b</sup>
Specific Growth Rate/ $\mu$ (h <sup>-1</sup> )	0.03 <sup>b</sup>	0.04 <sup>a</sup>	0.03 <sup>b</sup>
Yield (g/g)	0.07 <sup>b</sup>	0.08 <sup>a</sup>	0.08 <sup>a</sup>
Productivity (g/L/hour)	0.05 <sup>a</sup>	0.04 <sup>b</sup>	0.03 <sup>c</sup>

Statistical analysis was determined using One-way ANOVA and Tukey tests. Each value is the mean  $\pm$  SE of 3 replicates. Means with the same letter are not significant at  $p < 0.05$

*Lactococcus lactis* ssp. has a phosphoenolpyruvate dependent transport system (PTS) for fructose, mannose, sucrose or trehalose, mannitol and cellobiose [4]. This characteristic consents *L. lactis* IO-1 to utilize cellobiose as the carbon source. However, excessive cellobiose extremely inhibits glucose uptake up to 81% [11]. Hence, *L. lactis* IO-1 depend mainly on cellobiose as the carbon source. In order to utilize cellobiose, *L. lactis* IO-1 needs to hydrolyse cellobiose into glucose before it can be consumed for energy. Therefore, the productivity of *L. lactis* IO-1 in medium supplemented with cellobiose as the carbon source is slightly lower with longer lag phase compared to growth in medium using glucose as the carbon source. Consequently, cellobiose supplementation enhances the growth of *L. lactis* IO-1 as proven in prebiotic analysis. The specific growth rate (0.04 h<sup>-1</sup>) and the yield (0.08 g/g) of cell biomass of *L. lactis* IO-1 in SFS+YE media slightly higher compared to SM (0.03 h<sup>-1</sup> specific growth rate, 0.07 g/g yield). Slow degradation of cellobiose as carbon source by *L. lactis* IO-1 allowed the strain to undergo longer exponential period and multiply more at higher growth rate lead to higher yield of biomass.

Based on these results, the lag phase in SFS+YE medium was shorter compared to SFS medium. This shows that supplementation with YE have boosted the growth of *L. Lactis* IO-1, similar to the finding of [15]. Likewise, the lag phase of *L. lactis* IO-1 in SFS medium was much longer compared to

SFS+YE medium. This is because of the productivity and growth rate of cells in SFS+YE are higher compared to in SM. Higher productivity and growth rate mean increased growth of *L. lactis* IO-1. Hence the amount of biomass of *L. lactis* IO-1 in SFS+YE was higher compared to SFS.

Supplementation of yeast extract was important for fast growth of *L. lactis* IO-1 and subsequently the concentration of biomass, potentially reducing the duration of fermentation [15]. In the absence of yeast extract, the growth of *L. lactis* IO-1 in SFS media was comparable to SM. There was no significant different in maximum DCW and growth rate of *L. lactis* IO-1 in these two media, in fact the yield of biomass in SFS was higher compared to SM. This mirrors the potential of cultivation *L. lactis* IO-1 in SFS without the amendment with yeast extract.

Sago frond contains natural nitrogen source, at approximately 2.26% DM. In our project, 6% w/v of dry matter was used for the enzymatic hydrolysis process (60 g sago frond powder in 1L mixture), containing about 1.35 g of nitrogen which naturally exists in the fermentation medium [6]. Hence, SFS have high potentials to be used as an inoculum or a fermentation medium to grow *L. lactis* IO-1.

Aerobic fermentation of *Lactococcus lactis* spp. require superoxide dismutase (SOD) to utilize oxygen free radical ( $O_2^{\cdot -}$ ) into hydrogen peroxide ( $H_2O_2$ ) by via dismutation process [17]. The present of hydrogen peroxide as free radical compound inhibits the growth of *L. lactis*. Hence, catalase enzyme will be produced to convert hydrogen peroxide into water and stable oxygen ( $O_2$ ) molecule.

Some of the nutrients are important in enzymatic reaction to enhance the activity of the enzyme that responsible as cofactor mineral. Superoxide dismutase form can be influence by the present of zinc, copper and manganese [14]. These minerals found in raw SFS which can stimulate the activity of SOD produced by *L. lactis* IO-1.

*Lactococcus lactis* require at least 0.06 mg/L of manganese in the fermentation media to stimulate the activity of superoxide dismutase enzyme in aerobic fermentation [7]. For addition, the concentration of magnesium lower than 0.36mg/L affect the growth rate of the *L. lactis*, nevertheless the final biomass produced were reliant on the initial Mg concentration of the fermentation media that contain lower than 2.4 mg/L [12]. Both nutrients (Appendix C) exists naturally in SFS and the amount was adequate (Mn = 18.37 mg/L, Mg = 72.66 mg/L) for the growth of *L. lactis* IO-1 in the production of lactic acid.

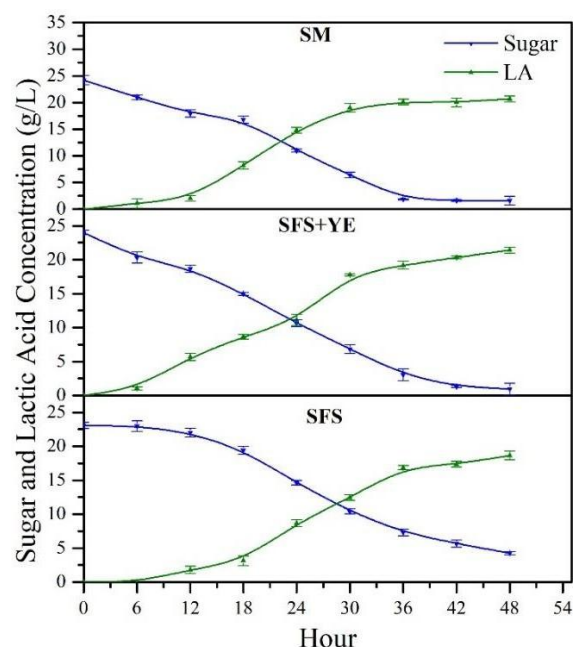
Due to the presence of these nutrients, production cost of lactic acid via fermentation of SFS using *L. lactis* IO-1 can be reduced due to unnecessarily supplementation of nitrogen sources such as yeast extract which cost up to RM 4,775/tonne for fermentation of SFS.

SOD is a very important antioxidant in neutralizing thousands of toxic free radical and for the production of anti-radical medicine and prevent free radical skin damage in cosmetic products [16]. Therefore, SOD produced from the fermentation of *L. lactis* IO-1 in SFS can be extracted and purified or combined with lactic acid for the production of high-value product which will enhance development of the sago industry.

### 3.2 Consumption of Sugar and Production of Lactic Acid

It was observed that maximum concentration of lactic acid produced from the fermentation of *L. lactis* IO-1 in SM was  $22.81 \text{ g/L} \pm 0.06$  after 42 hours (figure 2). The amount of residual sugar was  $0.85 \text{ g/L} \pm 0.02$ . Hence, the amount of sugar consumed was 22.51 g (93.79%) with a lactic acid yield of 94.21%. The highest production of lactic acid in SFS+YE medium was 21.40 g/L (89.16% yield) with a residual sugar of 0.91 g/L (96.21% sugar consumed) after 48 hours. Within the same duration (48

hours of incubation) the highest amount of lactic acid in SFS medium was  $18.64 \text{ g/L} \pm 0.06$  (80.8% yield) and final sugar concentration of  $4.23 \text{ g/L} \pm 0.23$  (81.66% sugar consumed).



**Figure 2:** Comparison in production of Lactic acid by *L. lactis* IO-1 in different media.

Evidently in this project, all parameters are superior in SM medium compared to the other two media. The yield of lactic acid from SM medium was higher compared to SFS+YE (table 2), since the fermentation of glucose has higher conversion rate to lactic acid than cellobiose (as in SFS+YE and SFS). The efficiency of lactic acid production in SM was higher compared to SFS+YE and SFS media together with shorter duration of fermentation (36 hours in SM), faster by 12 hours than SFS+YE (48 hours).

**Table 2:** Kinetic parameter of L-lactic acid production of *L. lactis* IO-1 in SM, SFS+YE and SFS media.

Parameters	Types of Medium		
	SM	SFS+YE	SFS
Yield (g/g)	$0.91^a \pm 0.008$	$0.85^b \pm 0.006$	$0.85^b \pm 0.001$
Productivity (g/L/hour)	$0.64^a \pm 0.004$	$0.45^b \pm 0.009$	$0.39^c \pm 0.001$
Efficiency (%)	$92.40^a \pm 0.77$	$85.03^b \pm 0.63$	$84.76^b \pm 0.85$

All kinetic parameters showed that glucose is the better substrate for the production of lactic acid. This is because of the molecular structure of cellobiose is more complex than glucose. Glucose can be directly utilized into lactic acid by lactic acid bacteria such as *L. lactis* IO-1 while cellobiose was protected by the  $\beta$  (1-4) glycosidic bond that must be cleaved into glucose molecules using the enzyme  $\beta$ -glucosidase before it can be converted to lactic acid. This needs more time and reduced the convertibility of cellobiose into lactic acid by *L. lactis* IO-1. *L. lactis* strains that encode pBL1 and celB genes produce a phospho- $\beta$ -glucosidase for the hydrolysis of cellobiose indicate the growth profile of

strain grown in chemically defined medium containing cellobiose (CDM-Cel) is slower compared glucose as carbon source due to existence of those gene that shifted the fermentation products toward a mixed acid profile and promoted significant changes in intracellular pool size for glycolytic intermediates in cells growing on cellobiose consumption [5]. Moreover, cultivation of *L. lactis* strain in CDM-Cel for 24 hours shows a very long lag phase that resulted in the increase the culture optical density [13].

However, utilization of SFS as the alternative medium for the production of lactic acid is very much akin to SM. It is possible to have incomplete conversion of cellobiose to glucose via enzymatic hydrolysis, but addition of more  $\beta$ -glucosidase enzyme was unnecessary due to convertibility of cellobiose to lactic acid by *L. lactis* IO-1. The results obtained was similar to [1] where the lactic acid recovery from the utilization of pure cellobiose was 90% using *Lactobacillus delbrueckii* mutant Uc-3 strain after 40 hours of incubation. This shows that utilization of cellobiose as the main and cheaper source of carbon in SFS is capable for large-scale production of lactic acid.

The productivity of lactic acid from SFS+YE medium was higher compared to SFS, which supports that addition of yeast extract speed up the production of lactic acid by enhance the growth of *L. lactis* IO-1. However, rapid growth of *L. lactis* IO-1 in SFS+YE medium creates a stress environment to the cells due to increased biomass concentration which leads to competition for the carbon source. This competition forced the bacteria to produce lactic acid to exterminate other bacteria [3]. As such, productivity of lactic acid in SFS+YE medium is higher compared to SFS. Low productivity will lead to slower production of lactic acid. Hence, production of lactic acid and consumption of sugar in SFS medium was incomplete after 48 hours of incubation.

However, the yield and efficiency of lactic acid in SFS and SFS+YE medium was not significantly different. This proves that addition of yeast extract does not influence the conversion of cellobiose to lactic acid and it does not influence the conversion of cellobiose to glucose. This is because nitrogen source naturally exist in SFS. Thus, supplementation of yeast extract only speeds up the conversion of sugar to lactic acid. Therefore, production of lactic acid from the utilization of SFS can be conducted efficiently without the need to add yeast extract, it only requires slightly longer incubation time.

#### 4.0 CONCLUSION

In conclusion, sago frond has high potential to be utilized as alternative raw material as viable prebiotic media to enhance growth of lactic acid bacteria (LAB) and excellent substrate for production of lactic acid. Large-scale production of lactic acid is then possible, without the need to interfere with production of starch food products and crops such as maize, sugar cane and sago starch. Lactic acid produced from the fermentation of SFS by using *L. lactis* IO-1 strain is purely of the L-lactate type, which has higher values for use as food preservatives and cosmetics.

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