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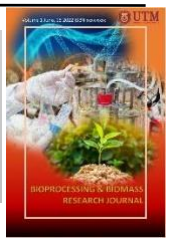

Research Article

Effects of Initial Rice Bran Concentration and Inoculum's Ratio on Microbial Growth of Co-culture Fermentation

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ABSTRACT

Co-culture fermentation is widely applied for its synergistic effects. The synergistic effects of lactic acid bacteria (LAB) and propionic acid bacteria (PAB) are reported to improve the ruminant feed efficiency through the supplementations of probiotics. However, although co-culture fermentation of LAB and PAB has been recently demonstrated, the effects of carbon source and inoculum's ratio on the microbial growth in co-fermentation are still not well-explored. Thus, this study was carried out to investigate the effect of carbon source concentration and inoculum's ratio on the growth of *Lactobacillus casei* and *Propionibacterium jensenii* in co-culture fermentation. Rice bran was used in this study, and the reducing sugar was extracted from rice bran through autoclave at 121 ℃ for 15 minutes. The co-culture fermentation was carried out in 2 stages: rice bran extract concentration's variation and inoculum's ratio variation. Co-culture in 20% w/v of RBE concentration showed the highest yield coefficient of $Y_{X/S}$ of 0.265 g biomass/g substrate and $Y_{P/S}$ of 0.715 g propionic acid/g substrate. Therefore, 20% w/v RBE concentration was used for the study of inoculum's ratio. The $Y_{X/S}$ (0.254 g biomass/g substrate) and $Y_{P/S}$ (0.653 g propionic acid/g substrate) of ratio 1:4 was slightly lower than ratio 1:8, but the viability of *L. casei* (8.934 $\log_{10}$ CFU/mL) and *P. jensenii* (9.420 $\log_{10}$ CFU/mL) was the highest in ratio 1:4. Although increase of PAB ratio can increase biomass produced, but ratio 1:4 can achieve higher microbes' viability which is important in the development of probiotics products.

INTRODUCTION

Co-culture fermentation is described as cultivation setup of two species microbes grown simultaneously in the same culture with some degree of contact among them (Bader et al., 2010). In co-culture fermentation, there are synergistic activities between the microbes in the culture, such as the fermentation product served as the carbon source for the coupled species for growth and synthesis. In the last 10 years, studies of co-culture fermentation have been on the rise. This approach has been utilised to produce organic acid (Klongklaew et al., 2021), biosurfactant (Hamza et al., 2018), bacterial cellulose (Hu et al., 2021), biofuel (Das et al., 2021; Chen, 2011), natural folate and vitamin B12 (Hugenschmidt et al., 2011), microalgal biomass (Kim et al., 2020) and probiotics (Mohamed Esivan et al., 2021; Ranadheera et al., 2016). It can be said that co-culture fermentation is employed for two purposes; to enable substrate conversion and to improve process performance (Canon et al., 2020).

Lactobacillus sp. is one of the lactic acid bacteria (LAB) commonly used as probiotics for animal

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consumptions. Due to its properties such as environmentally friendly and its ability to alter the environment through several mechanisms, making it is more effective than other type of probiotics for animal consumption. On a related note, a group of propionic acid bacteria such as dairy propionibacterium has been reported to demonstrate optimal properties as probiotics (Alazzeh et al., 2014). Interestingly, in an environment with two types of carbon source existed, reducing sugars and lactic acid, dairy propionibacterium is reported has lactic acid preferences over reducing sugars as carbon source (Xie et al., 2019).

A co-culture of an LAB bacteria and propionic acid bacteria (PAB) has been discovered to have positive effects to the ruminant's growth (Seo et al., 2010). A combination of LAB and PAB as probiotics for animal consumption has been reported to improve the digestive process and enhance adsorption of nutrients (Seo et al., 2010). It is also reported to further prevent the drastic pH drops in rumen due to the accumulation of lactic acid, thus preventing acidosis in ruminants (Elghandour et al., 2015; Derev et al., 2007).

However, in co-culture fermentation, a careful selection of inoculum's ratio of the two microbes must be conducted. For example, reducing sugar is utilized by LAB to convert to lactic acid, which then consumed by PAB to synthesis propionic acid and acetic acid. PAB has been reported to grow significantly at a slower rate compared to LAB (Wu et al., 2012). Therefore, incompatible in combination ratio can lead to the accumulation of lactate that significantly decrease the pH of fermentation medium and affect the synergistic effect (Farhadi et al., 2013). Moreover, medium's pH lower than 4.5 can lead to self-inhibiting effect (Campaniello et al., 2015). Although co-culture fermentation studies for LAB and PAB have been reported, however, studies of the effect of carbon source concentration and the inoculum's ratio of LAB and PAB to the microbial growth in co-culture fermentation are not well explored.

Thus, in this study, reducing sugars (TRS) were extracted from solid rice bran to be used as the sole carbon source for the co-culture fermentation of *Lactobacillus casei* and *Propionibacterium jensenii*. The effects of rice bran extract (RBE) concentration and the inoculum's ratio of LAB and PAB on microbial growth were observed and reported. The suitable value for the two factors were evaluated to improve this co-culture fermentation for better microbes' viability and biomass yield for probiotic production purposes.

MATERIALS AND METHOD

Materials

Microorganisms used in this study were *Lactobacillus casei* ATCC 393 and *Propionibacterium jensenii* ATCC 4871. Both microorganisms were bought from American Type Culture Collection (ATCC) (Virginia, USA). Strain of *L. casei* was activated in de Man Rogose & Sharpe (MRS) broth (Merck) at aliquot of 1 %v/v (1 mL to 100 mL of broth) and incubated at 37 °C for 24 hours without stirring. Strain of *P. jensenii* was activated in Reinforced Clostridial Media broth (Oxoid) at aliquot of 4% v/v (4 mL to 100 mL of broth) and incubated at 30 °C for 48 hours under static condition. These cultures were used as the inoculum for the co-culture fermentations.

Preparation of Fermentation Medium

The rice bran was used as the carbon source in the co-culture fermentation. The extraction of reducing sugar from solid rice bran was conducted by heat treatment at 121 °C for 15 minutes to break the cellulose of cell wall. For RBE concentration experiment, different weight of rice bran solid to distilled water volume was used to prepare the extracted medium. The working volume was set to 300 mL. Solid residues were removed by centrifugation at 5,000 x g for 15 minutes in room temperature. Initial pH of extracted medium was adjusted to 6.5 by adding 1.0 M of NaOH. The medium was then left overnight to remove suspending particles through sedimentation. Afterwards, the medium was filtered and sterilised prior to the fermentation.

Co-culture Fermentation

The co-culture fermentations were conducted in a 500 mL conical flask with 300 mL of RBE. The co-culture was carried out statically to create a microaerophilic environment. Total fermentation time was 168 hours, and the incubation temperature was set at 30 ℃ with initial pH of 6.5. The effect of RBE concentration was studied for 10, 20 and 25% w/v of RBE concentration. For the study of inoculum's ratio of *L. casei* to *P. jensenii*, the inoculum concentration of *L. casei* was maintained at 1% v/v while the concentration of *P. jensenii* was varied into 1, 4, and 8% v/v. Thus, there were three inoculum's ratios studied, 1:1, 1:4 and 1:8.

Determination of Cell Growth

Cell biomass produced through the co-culture fermentation was determined through cell dry weight analysis. Exact 1 mL of sample collected was micro-centrifuged at 10,000 x g for 5 minutes. The pellet obtained was dried up in oven at 80 °C for 24 hours and weighted. Cell density was determined through measurement of optical density (OD) values at wavelength of 600nm. The OD values were plotted into semi-log graph against fermentation time to obtain growth curve. Viability of *L. casei* and *P. jensenii* was done on MRS and sodium lactate agar, respectively using spread plate method. Serial dilution was performed up to 10^6 times to obtain higher resolution of viability in colony forming unit (CFU) per unit volume by using saline water at 0.85% w/v to reduce osmotic stress caused to microbes. All the analysis were done in triplicate.

Determination of pH and Titratable Acidity

The pH values of samples were measured by using pH meter at room condition. Another 5 mL of samples were used to determine organic acid concentration present through titratable acidity method. Exact 3 drops of phenolphthalein were used as indicator. The samples were titrated using 0.1 M of NaOH until the samples turn pink colour. The acid concentration was calculated by using the **Equation (1)**.

$$TA = \frac{Vol_{0.1M\ NaOH\ used}}{Vol_{sample}} \times \frac{0.1\ mol}{L} \times \frac{74g}{mol} \qquad (1)$$

Where $Vol_{0.1M\ NaOH\ used}$ is the volume of base used to neutralise the sample and the Vol_{sample} used is the volume of sample. The concentration of acid measured was assumed to be propionic acid only due to the consumption of lactic

acid by *P. jensenii* to synthesis propionic acid. All the analysis were done in triplicate.

Determination of Total Reducing Sugar (TRS)

A 1 mL sample was collected and centrifuged 10,000 x g for 5 minutes. The supernatant was taken for the determination of total reducing sugar via the dinitrosalicylic acid (DNS) method. The supernatant sample was diluted and reacted with DNS reagent in hot water bath for 5 minutes. The colour intensity of samples was detected with spectrophotometer at 540 nm wavelength and compared to standard glucose curve to obtain reducing sugar concentration present. The determination of TRS was done in triplicate for each sample.

Determination of Growth Rate and Yield Coefficient

From the growth curve obtained from the semi-log graph from OD readings, the microbial growth rate was calculated from the gradient of linear part found in exponential phase of growth curve. The yield of coefficient was calculated based on amount of biomass (from cell dry weight) and propionic acid (from titratable acidity) per amount of reducing sugar consumed.

RESULTS AND DISCUSSION

The yield of co-culture fermentation is strongly depended on the growing condition of microbes. In this study, the growth rate of *L. casei* and *P. jensenii* was included as one of our analyses to understand the growing condition inside the culturing medium. If the condition satisfies for their co-culturing, the cells will grow and replicate with the incubation time (Maier, 2015). In this study, the suitable values of rice bran extract concentration and microbes' combination ratio were determined through two separated experiments. The optimum RBE concentration was determined first, and afterwards, the optimum microbe's combination ratio was determined. The optimum condition was based on the viability and growth rate of both microbes. However, the yield of biomass and product over substrate were also compared to reflect on the commensalistic relationship between *L. casei* and *P. jensenii*.

Effects of RBE Concentration

The total reducing sugar (TRS) in 10%, 20% and 25% w/v of RBE concentration were 4.74 g/L, 10.09 g/L and 12.72 g/L, respectively. The TRS present in RBE served as the primary carbon source for the growth of *L. casei*, and lactic acid produced was consumed by *P. jensenii* to produce propionic acid. *L. casei* and *P. jensenii* was inoculated at ratio 1:4% v/v in each RBE concentration. The time profile for the accumulation of dry cell and the pH changes in three different RBE concentration are represented in **Figure 1** and **Figure 2**, respectively.

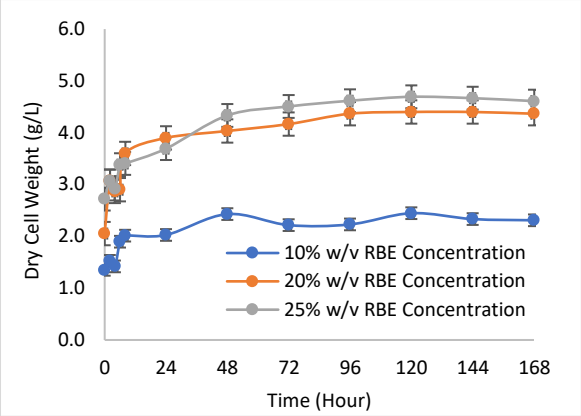

Figure 1 Dry cell weight produced from different RBE concentration

From **Figure 1**, it was observed that after 168 hours of fermentation, co-culture in 25% w/v of RBE concentration produced the highest cell biomass, 4.61 g/L. Co-culture in 20% w/v of RBE concentration produced 4.37 g/L and co-culture in 10% w/v of RBE concentration gave the lowest concentration of cell biomass, 2.31 g/L. Prior to the fermentation, the RBE pH was adjusted to 6.5, and the fermentation was conducted without pH control.

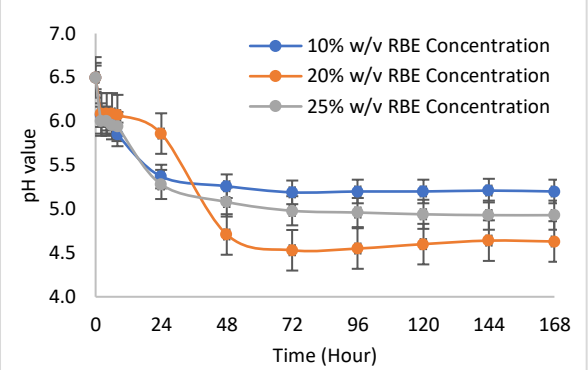

Figure 2 The pH changes in different RBE concentration

In **Figure 2**, all the three concentrations were observed to have sharp pH drop at first 2 hours and slowed down at time interval of 4 to 8 hours. This sharp drop suggested that the rapid growth of *L. casei* had caused the lactic acid concentration to increase sharply while the slow drop of pH from 4 to 8 hours was due to the active consumption of lactic acid by *P. jensenii* to synthesis propionic acid, which then caused the pH to continue drop. According to Guyot et al., (2001), pH inhibitory effect was observed in the cultivation of LAB when pH dropped to 4.5. The final pH value of variation of 20% w/v was observed to be lower compared to 10% w/v and 25% w/v after 168 hours of fermentation, which was suggested to have higher production of propionic acid and more suitable in concentration. The carbon consumption against time is shown in **Figure 3**.

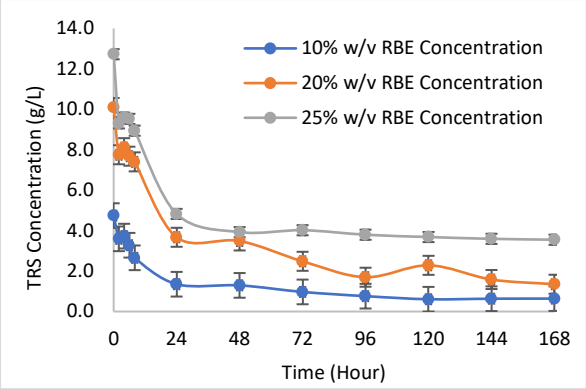

Figure 3 TRS concentration (g/L) for different RBE concentration over time.

From **Figure 3**, TRS was seen gradually decreasing over time, suggesting that TRS was consumed by the microbes. A slight increase in TRS concentration at t = 4 hours was observed in all the three RBE concentrations. It was suggested that the starch present in RBE was hydrolysed to glucose by *L. casei* during two-step fermentation process (Akoetey, 2015). Lactate produced by *L. casei* was consumed by *P. jensenii* as preferred carbon source to synthesis propionic acid as metabolic product (Wu et al., 2012). The greater amount of product produced with lower amount of substrate consumed, the higher the yield coefficient, which indicated the higher efficiency in fermentation process. **Figure 4** shows the co-culture cell density in OD against time.

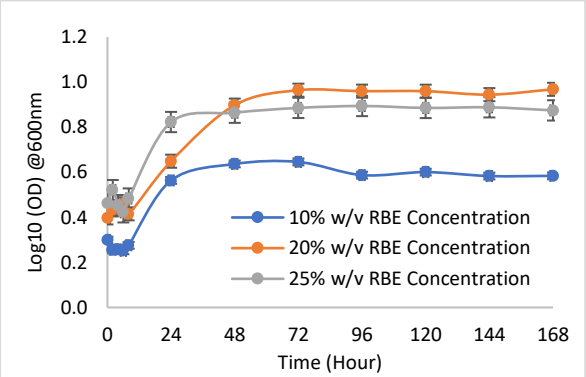

Figure 4 Log_{10} (OD) value against time (hour) for three different RBE concentration

In **Figure 4**, all the three RBE concentrations were observed to enter exponential phase after 8 hours of fermentation. Diauxic-like growth pattern was observed in the 20% and 25% w/v concentrations during fermentation time of 4 to 6 hours instead of noticeable lag phase. According to Lee et al., (1974), the observation was explained with the inhibiting effect due to low pH of fermentation medium. However, the pH measured at that time interval was about 6.0. Therefore, it is suggested that the secondary growth pattern was possibly caused by the late lactate utilisation of *P. jensenii* before switching from reducing sugar as main carbon source.

The propionic acid produced in the culture was estimated through titratable acidity method (**Figure 5**). All the three concentrations showed a slight decrease in acid concentration during the time interval from 4 to 6 hours. It was suggested that the late lactate utilization of *P. jensenii* had caused the consumption of lactic acid more than production of propionic acid. According to Farhadi et al. (2013), highest propionic acid concentration was achieved at 7.7g/L after co-fermenting for 69 hours using lactose as main substrate. In this study, RBE concentration of 20% w/v achieved highest propionic acid concentration of 6.25 g/L after 168 hours of fermentation. Next, the growth rate and yield coefficient in different RBE concentrations were calculated and tabulated in **Table 1**.

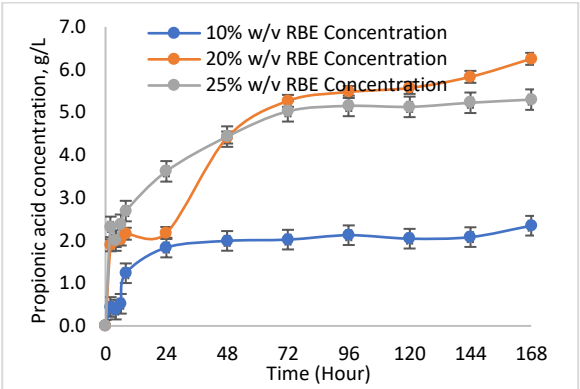

Figure 5 Propionic acid concentration (g/L) in different RBE concentrations

Table 1 Comparative growth rate and yield coefficient for different RBE concentration

RBE concentration	Growth rate, μ (h^{-1})	$Y_{X/S}$ (g biomass/g substrate)	$Y_{P/S}$ (g propionic acid/g substrate)
10% w/v	0.0174±0.005	0.233±0.015	0.572±0.018
20% w/v	0.0121±0.013	0.265±0.021	0.715±0.032
25% w/v	0.0223±0.006	0.206±0.061	0.578±0.041

From the **Table 1**, it was observed that the highest growth rate was obtained at 25% w/v RBE concentration, followed by 10% w/v and the lowest growth rate was obtained in 20% w/v RBE concentration. Thus, in regards of microbial growth rate, 25% w/v was suggested to be more suitable although the overall increase in cell density was slightly lower than 20% w/v. However, RBE concentration of 20% w/v RBE concentration achieved higher yield coefficient of biomass and propionic acid over substrate than 25% w/v RBE concentration. Therefore, it is suggested that further increase in RBE concentration from 20% w/v to 25% w/v does not significantly increase biomass and product produced. Hence, 20% w/v of RBE concentration was chosen to study the effect of inoculation ratio on the microbial growth of co-culture fermentation.

Effects of Inoculation Ratios

The inoculum concentration of *P. jensenii* was varied into three different concentrations, 1%, 4% and 8% v/v, and the inoculum concentrations of *L. casei* was maintained at 1% v/v. **Figure 6** shows the accumulation of dry cell over time in different inoculation ratios. Variation of 1:4 showed a late increase in cell dry weight started from 72 hours of fermentation and surpassed the dry cell weight of ratio 1:8 at 144 hours. According to Liu & Moon (1982), a significant shift in metabolism was observed after lactate had been reduced to very low concentration and glucose consumption was continued. Therefore, it was suggested that the reutilization of TRS by *P. jensenii* due to low concentration of lactate had caused late increase in cell dry weight of ratio 1:4.

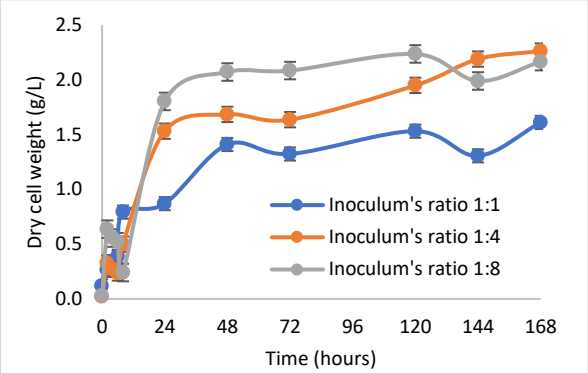

Figure 6 Dry cell weight from different inoculation ratios

Figure 7 shows the trend of pH culture recorded from three inoculation ratios. From the figure, the pH culture drops immediately during the two hours of fermentation and then remained at the same value until from 2 hours to 8 hours of fermentation. Afterwards, the pH culture continues to drop until it reached the lowest value after 24 hours of fermentation. According to Ahmadi et al. (2016), the mid-stationary phase was suggested to be caused by the faster growth rate of lactic acid bacteria (LAB) than propionic acid bacteria (PAB). Thus, a high concentration of lactic acid produced.

During this time, lactic acid produced was consumed by PAB to produce propionic acid, which caused the pH to remain unchanged temporary. In general, pH values decreased as the fermentation time increased. However, rapid decreasing of pH value was observed for inoculum's ratio at 1:1 compared with other ratios as depicted in **Figure 7**. As propionibacteria grow slower than lactic acid bacteria (Wu et al., 2012), the rapid decrease of pH could be attributes to the rapid production of lactic acid. According to Ahmadi et al., (2016), a rapid drop of pH culture affects the growth of *Propionibacterium* sp. negatively. Therefore, based on pH trend, it is suggested that the microbes are not suitable with inoculum's combination ratio at 1:1.

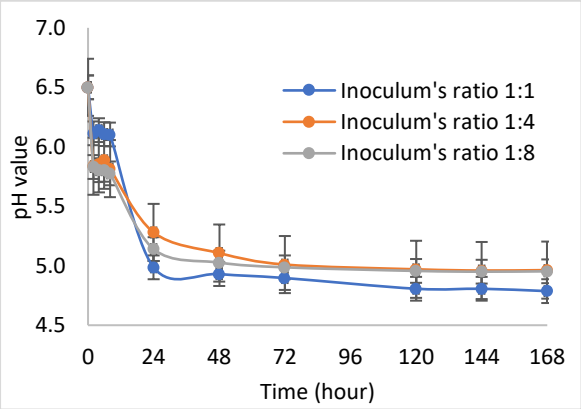

Figure 7 The pH changes in different inoculation ratios

The consumption of TRS is shown in **Figure 8**. The same consumption trend with slight increase in TRS concentration after 4 hours of fermentation was also observed. The highest TRS consumption was recorded from 0 until 6 hours of fermentation and became stagnant after 24 hours of fermentation. All the three inoculation ratios of were found to have similar TRS concentration left at the end of co-fermentation, in between 4.15 g/L and 4.50 g/L. The cell density of co-culture against time is shown in **Figure 9**.

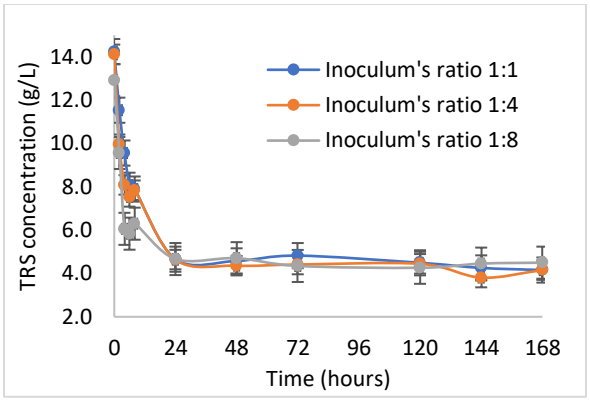

Figure 8 TRS concentration (g/L) for different inoculation ratios over time

In **Figure 9**, all the three ratio variations of inoculum's ratio had achieved final $\log_{10}$ OD values in the range between 0.50 to 0.70. According to Farhadi et al. (2013), the short lag phase could be due to the faster growth rate of LAB at suitable condition. The same secondary growth pattern was also observed in the ratio variations of 1:1, 1:4 and 1:8, which was suggested to be caused by switching to lactate utilization of *P. jensenii* from reducing sugar as mentioned in previous section.

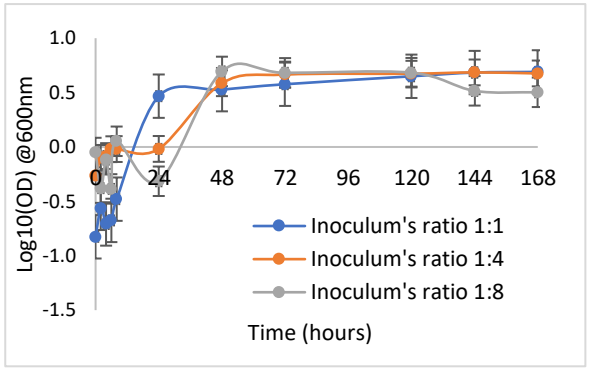

Figure 9 Log_{10} (OD) value against time (hour) for different inoculation ratios

The production of propionic acid against time is shown in **Figure 10**. From the figure, the acid production was found to increase after 24 hours of fermentation. Meanwhile, the TRS consumption reached the lowest concentration after 24 hours of concentration as shown in **Figure 9**. Therefore, it was suggested that the lactic acid produced was being utilized instead of TRS by *P. jensenii* to synthesis propionic acid during the secondary exponential growth phase. All the three inoculum's ratios had achieved final propionic acid concentration at about 5.5 g/L after 168 hours of co-fermentation. Next, the growth rate and yield coefficient in different inoculation ratios were calculated and tabulated in **Table 2**.

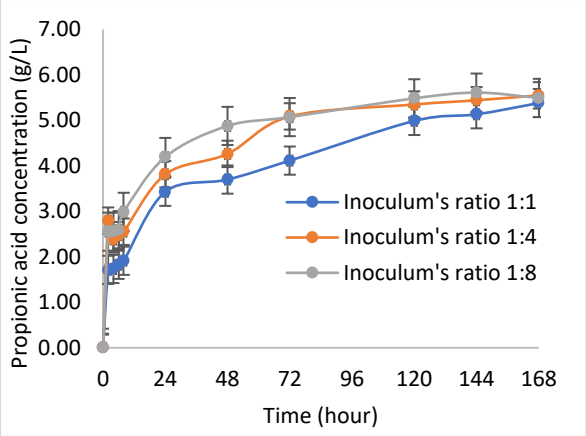

Figure 10 Propionic acid concentration (g/L) in different RBE concentrations

Table 2 Comparative growth rate and yield coefficient for different inoculation ratios

Inoculum's ratio	Growth rate, μ (h^{-1})	$Y_{X/S}$ (g biomass/g substrate)	$Y_{P/S}$ (g propionic acid/g substrate)
1:1	0.0587±0.001	0.148±0.05	0.534±0.005
1:4	0.0252±0.007	0.225±0.004	0.557±0.006
1:8	0.0421±0.003	0.254±0.001	0.653±0.012

From **Table 2**, inoculum's ratio at 1:1 had achieved growth rate at 0.0587 (h^{-1}) as shown in **Table 2**. It is suggested that the lower PAB ratio had led to lower substrate competition during the exponential phase of LAB. Therefore, it is suggested that ratio 1:1 was more suitable in terms of microbial growth rate, which indicated a compatible combination for this co-culture fermentation.

The combination ratio at 1:8 showed the highest yield coefficient at 0.254 g biomass/g substrate, which was slightly higher than 1:4 with yield coefficient of 0.225 g biomass/g substrate as shown in **Table 2**. The ratio of 1:8 had also achieved higher yield coefficient than 1:1 and 1:4 of 0.653 g propionic acid/g substrate. It is suggested that inoculum's ratio at 1:8 had better performance in term of yield production per amount of substrate consumed compared to ratio 1:1 and 1:4.

Comparative of Cell Viability

The viability of *L. casei* and *P. jensenii* while entering the stationary phase, mid stationary phase and at the end of fermentations are tabulated in **Table 3** and **Table 4**. *L. casei* and *P. jensenii* reached stationary phase at different time of fermentation, with *L. casei* enter the stationary phase earlier than *P. jensenii*.

Table 3 Comparative viability of *L. casei* with different inoculum's ratio

Inoculum's ratio	Log_{10} (CFU/mL)		
	1:1	1:4	1:8
t=48 hours	8.241±0.24	8.687±0.96	7.556±0.24
t=120 hours	8.195±0.29	8.699±0.05	7.879±0.38
t=144 hours	8.023±0.31	8.934±0.047	8.092±0.37

L. casei enter the stationary phase after 48 hours of fermentation. The viability of 1:1 inoculation ratio was seen to decrease over time; however, the viability remains high after 144 hours of fermentation. The viability of *L. casei* continue to increase at inoculation ratio of 1:4 and 1:8. The highest viability cell of *L. casei* was obtained in 1:4 inoculation ratios. The finding in this study is slightly higher than the findings reported by Farhadi et al., (2013). In their study, the highest viability of *L. acidophilus* during co-culture with *P. freudenreichii* was 8.06 log_{10} CFU/mL. Therefore, it is suggested that *L. casei* and *P. jensenii* are more suitable with inoculum's ratio at 1:4, while excessive increase in inoculation ratio can lead to nutritional lack and reduce the viability of *L. casei*.

Table 4 Comparative viability of *P. jensenii* with different inoculum's ratio

Inoculum's ratio	Log_{10} (CFU/mL)		
	1:1	1:4	1:8
t=72 hours	8.428±0.52	9.420±0.27	8.045±0.54
t=120 hours	7.976±0.64	8.100±0.54	8.108±0.25
t=144 hours	8.372±0.13	8.190±0.48	8.033±0.21

P. jensenii enter its stationary phase later than *L. casei*, which was after 72 hours of fermentation. The highest viability of *P. jensenii,* 9.42 log_{10} CFU/mL was obtained with inoculation ratio of 1:4 after 72 hours of fermentation. The finding was much higher than the result reported by Farhadi et al., (2013). In the study by Farhadi et al., (2013), the highest viability of *P. freudenreichii* (8.60 log_{10} CFU/mL) was achieved at inoculation ratio of 1:4. Farhadi et al. (2013) suggested that the function of PAB did not have any interference with LAB. However, the increase in ratio of *P. jensenii* had led to higher concentration of propionic acid produced which is causing the inhibiting effect to both *L. casei* and *P. jensenii*. Therefore, the more suitable inoculum's combination ratio for *L. casei* and *P. jensenii* was suggested to be 1:4 through findings of this study.

CONCLUSION

In general, this study was carried out to investigate the effects of initial rice bran concentration and inoculum's ratio on microbial growth rate of *L. casei* and *P. jensenii* in co-culture fermentation. From the results the highest viability for *L. casei* (8.934 log_{10} CFU/mL) was obtained at fermentation time of 144 hours and the highest viability for *P. jensenii* (9.420 log_{10} CFU/mL) was obtained at fermentation time of 72 hours, with inoculum's ratio of 1:4 and RBE concentration of 20% w/v. In conclusion, initial substrate RBE concentration and inoculum's ratio significantly affect the growth of the investigated co-culture fermentation. However, more research is necessary to further optimize the co-culture fermentation with rice bran as main substrate.

Acknowledgement

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