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Optimization of the production of *Weissella confusa* MBF8-1 lysate in plant-based media by using response surface methodology

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ABSTRACT

Aims: Bacterial lysate has been reported to possess many health-care-related benefits. This study aimed to determine the optimum conditions for producing *Weissella confusa* MBF8-1 lysate in two plant-based modified De Man, Rogosa, and Sharpe (MRS) media using the response surface methodology (RSM). In this study, we applied several condition factors and compared them to standard MRS media.
Methodology and results: *Weissella confusa* MBF8-1 was grown in two modified MRS media, which are MRS Vegitone and soy peptone modified-MRS. The optimized fermentation condition factors such as nitrogen sources (i.e., soy peptone, proteose peptone), dextrose concentrations, and fermentation time were measured, and the responses, such as bacteriocin-like inhibitory substance (BLIS) activity and lysate pH were observed. RSM results showed the diameter of BLIS activity-inhibition zone and pH decreases of the lysate produced in MRS Vegitone containing 1.50% dextrose, 0.75% proteose peptone for 11.75 h fermentation and in soy peptone modified-MRS containing 2.05% dextrose, 1.05% soy peptone for 7.53 h fermentation, i.e., 7.41 mm at 7.36, and 7.80 mm at 7.30, respectively. Whereas, lysate produced in standard MRS medium containing 2% dextrose, 1% peptone for 8 h fermentation showed 7.85 mm diameter of BLIS activity-inhibition zone at pH 7.26. *W. confusa* MBF8-1 lysate showed slightly lower pH, but higher BLIS activity when grown in standard MRS media compared to those of the two modified MRS media.
Conclusion, significance and impact of study: The data obtained provide the optimum condition of *W. confusa* MBF8-1 lysate production in plant-based media. The pH and BLIS activity possessed by *W. confusa* MBF8-1 lysate produced in soy peptone modified-MRS showed a more similar result as the standard one than the other modified one. Thus, the soy peptone modified-MRS is recommended as a plant-based alternative medium replacing standard MRS.

Keywords: BLIS activity, halal, soy peptone, vegitone, *Weissella confusa* MBF8-1

INTRODUCTION

Lysates are products derived from bacterial cell lysis and have many health-care-related benefits. For example, *Streptococcus pyogenes* lysates are used as immunomodulators in chronic obstructive pulmonary disease patients (Cazzola *et al*., 2012), *Staphylococcus aureus* lysates are used as adjunctive therapy for preventing atopic dermatitis (Bodemer *et al*., 2017), and *Bifidobacterium longum* lysates possess anti-allergic and anti-aging properties (Guéniche *et al*., 2010).

Lactic acid bacteria (LAB), such as *Weissella confusa*, produce lactic acid as the primary end product of carbohydrate fermentation. LAB are widely used in many fields, especially microbiology, biotechnology, and food industries, because, in addition to lactic acid, they produce important, beneficial compounds such as ethanol, carbon dioxide, bacteriocin, exopolysaccharides (EPS), oligosaccharides, proteolytic enzymes, vitamins, and aromatic compounds (Fessard and Remize, 2017). *Weissella confusa* lysates contain more than one active compound, which is efficacious. Besides, the process for obtaining lysates is faster than getting a single compound, such as bacteriocin or EPS. However, the production and uses of *W. confusa* lysate have never been studied.

Fermentation of *W. confusa* MBF8-1 is generally carried-out in De Man, Rogosa, and Sharpe (MRS) medium. Two alternative modified plant-based media are

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available for growing such LAB, i.e., MRS Vegitone and soy peptone modified-MRS. In the standard MRS medium, the source of nitrogen is animal based-peptone. Whereas, soy peptone is a vegetable peptone with high carbohydrate concentration and contains 8.7% nitrogen and 0.4% NaCl (Atlas, 2010). In addition, soy peptone modified-MRS medium is cheaper than standard MRS medium. MRS Vegitone medium also contains no material from animal sources but, instead, contains proteose peptone (a vegetable peptone). These applications of plant-based peptones were carried-out in order to promote Muslim's restriction for non-halal materials, as well as to support other communities who have vegan or vegetarian preferences. MRS Vegitone medium can enhance the safety of lysate production because there is no contamination by disease-causing substances, such as prions (principal factor proteins that cause a number of neurological diseases in mammals, such as bovine spongiform encephalopathy) (Fraser, 2014). In addition, the total price of the materials used in plant based-MRS media is relatively cheaper compared to that in standard MRS medium. However, those media have not been used previously as a fermentation medium for producing *W. confusa* lysate. Therefore, preliminary study is required to determine optimum conditions for bacterial lysate production in plant based-MRS media.

In previous studies, bacteriocins produced by *W. confusa* Cys2-2 had proven in diminishing the viability of Gram-negative bacteria at the early exponential growth phase by disrupting the integrity of target cells (Tenea and Lara, 2019), while cell-free culture supernatant of *W. confusa* DD_A7 isolated from kimchi was reported to exhibit antibacterial effect by inhibiting the growth of multidrug-resistant (MDR) extended-spectrum B-lactamase (ESBL) positive *Escherichia coli* (Dey *et al*., 2019). The cell free extract of *W. confusa* KR780676 isolated from an Indian traditional fermented food (Idli batter) showed probiotic properties and antioxidant activity by exhibiting lipid peroxidation inhibition, significant DPPH (2,2-diphenyl-1-picrylhydrazyl), superoxide anion radical scavenging potential, and inhibition of biofilm formation by *Pseudomonas aeruginosa* KT266804 (Sharma *et al*., 2018). Another study by Xiong *et al*. (2019) also reported that *W. confusa* strain BSP201703 isolated from Giant Panda (*Ailuropoda melanoleuca*) had probiotic potential by inhibiting common intestinal pathogens, possess an *in vitro* antioxidant activity, a high auto-aggregation ability, and a high surface hydrophobicity. Shah *et al.* (2016) also reported that *W. confusa* A110 had an antimicrobial effect on two clinical pathogenic strains, i.e., *E. coli* NG 502121 and *S. aureus* AY 507047 in co-culture. Result of previous study were also revealed *W. confusa* strains MBF8-1 and MBF8-2 potentially synthesize both glucan and fructan using glucansucrase and fructansucrase enzymes (Malik *et al.*, 2009). Also, *W. confusa* MBF8-1 possess a bacteriocin-like inhibitory substance (BLIS) activity which has been tested by an agar-based deferred antagonism assay (DAA) against a range of species including strains of *W. confusa*, *Weissella cibaria*, *Leuconostoc mesenteroides*, *Micrococcus luteus*, *Lactococcus lactis*, *Listeria monocytogenes* and several species of streptococci (Malik *et al.*, 2016). The draft genome of *W. confusa* MBF8-1, which was isolated from a homemade fermented soybean also exhibits antibacterial (bacteriocin) activity (Heng *et al.*, 2017). The bacteriocin-like peptides of *W. confusa* MBF8-1 also possess the potential for the development of narrow-spectrum antimicrobial agent and a novel spermicidal agent (Sartono *et al.*, 2019).

In this study, we produced *W. confusa* MBF8-1 lysate using standard MRS, soy peptone modified-MRS, and MRS Vegitone media. In the previous study, the optimization of the fermentation was done by determining which condition will produce high-performance production of bacteriocin, lactic acid, EPS, and other substances at commercial scale (Rosca *et al.*, 2018). We determined the optimum conditions for lysate production in the two modified MRS media using response surface methodology (RSM), a statistical and mathematical technique used to develop, improve, and optimize processes in which a response of interest is influenced by several variables, in order to maximize that response (Baş and Boyacı, 2007). This method was carried out similarly with the previous study using *Streptococcus macedonicus* MBF10-2 (Andyanti *et al.*, 2019), and we observed the responses of BLIS (Grazia *et al.*, 2017) activity and lysate pH. The pH observation was carried-out as a response because *W. confusa* MBF8-1 previously showed a growth response in the form of changes in sensitivity to antibiotics in various growth conditions, e.g., changes in pH and dextrose carbon source (Malik *et al.*, 2013). Further, we compared the optimization results in MRS Vegitone and soy peptone-modified MRS with those in standard MRS medium.

MATERIALS AND METHODS

Media

We used three types of media in this study, i.e. (i) standard MRS medium: 10 g/L of peptone, 8 g/L of LAB-Lemco (Oxoid, UK), 4 g/L of yeast extract (Difco, USA), 2 g/L of dipotassium hydrogen phosphate, 5 g/L of sodium acetate, 2 g/L of ammonium citrate, 0.2 g/L of magnesium sulfate, 0.05 g/L of manganese sulfate, 0.05% (v/v) Tween 80, and 20 g/L of dextrose; (ii) soy peptone-modified MRS medium: 10 g/L of soy peptone, 8 g/L of LAB-Lemco (Oxoid, UK), 4 g/L of yeast extract (Difco, USA), 2 g/L of dipotassium hydrogen phosphate, 5 g/L of sodium acetate, 2 g/L of ammonium citrate, 0.2 g/L of magnesium sulfate, 0.05 g/L of manganese sulfate, 0.05% (v/v) Tween 80, and 20 g/L of dextrose; and (iii) MRS Vegitone medium: 10 g/L of protease peptone (vegetable peptone), 5 g/L of yeast extract (Difco, USA), 2 g/L of dipotassium hydrogen phosphate, 5 g/L of sodium acetate, 3 g/L of 2-phenylethyl alcohol, 2 g/L of ammonium citrate, 0.1 g/L of magnesium sulfate, 0.05 g/L of manganese sulfate, 0.04 g/L of bromocresol green, 0.004 g/L of captan, 1 mL of polysorbate 80, and 20 g/L of

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dextrose. For agar media, we added Bacto agar (Difco, USA) to a final concentration of 15 g/L for both soy peptone-modified MRS and MRS Vegitone media. Incubation was carried out in anaerobic jars (Oxoid, UK).

Bacterial strains and growth conditions

Bacterial strains used in this study were as follows: *W. confusa* MBF8-1 as lysate-producing bacterium, *Leuconostoc mesenteroides* TISTR 120 as indicator bacterium for BLIS activity assay, and *Bacillus subtilis* ATCC 6633 as negative control bacterium for the lactic acid production determination. All strains were obtained from the culture collection of the Laboratory of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Universitas Indonesia.

Cryo-stocks of all strains were maintained at −80 °C in MRS broth medium–glycerol (1:1). Bacterial strains were routinely confirmed both molecularly using the 16S ribosomal RNA (rRNA) gene and visually by morphology observation and Gram staining. *Weissella confusa* MBF8-1 samples were grown in each media, i.e., standard MRS, MRS Vegitone, and soy peptone-modified MRS, at 30 °C for 24 h in anaerobic jars (Oxoid, UK). *L. mesenteroides* TISTR 120 were grown in standard MRS medium at 30 °C for 24 h in anaerobic jars (Oxoid, UK), and *B. subtilis* ATCC 6633 were grown in nutrient broth (Difco, USA) and nutrient agar (Difco, USA) at 37 °C aerobically. All strains were routinely maintained by growing them in a 7 mL medium at their optimum temperature for 24 h.

Fermentation of *W. confusa* MBF8-1 in standard MRS, MRS Vegitone, and soy peptone-modified MRS media

Liquid cultures for fermentation process were prepared by inoculating colonies of strains to each 10 mL media in reaction tubes at 10% (v/v), followed by incubation at 30 °C for 24 h in anaerobic jars (Oxoid, UK). Next, this seed cultures were inoculated into each 190 mL media in a glass flask, and were incubated at 30 °C for 24 h in micro-aerobic condition without shaking. To monitor bacterial growth, 2 mL of each fermentation culture was collected every hour, and was measured using an ultraviolet–visible (UV–Vis) spectrophotometer (GE Healthcare, Sweden) in triplicate. Finally, cells were harvested by centrifugation at 5000 × *g* at 4 °C for 30 min (Malik *et al.*, 2015) to obtain both cell pellets and supernatant fraction. Cell pellets were stored at −20 °C, while the supernatant were stored at 4 °C. Data of OD_{600} were used to create bacterial growth curves of OD_{600} value versus incubation time.

Indicator of *W. confusa* MBF8-1 lactic acid production

To measure the production of lactic acid, we determined the pH value of the supernatants during fermentation. Ten mL of liquid cultures was inoculated into 190 mL of each medium and incubated at 30 °C for 24 h in an anaerobic jar, and 5 mL of fermentation medium was collected every 6 h. Supernatants were collected by centrifugation at 5000 × *g* at 4 °C for 30 min, and pH were measured using pH meter (Hanna, Japan). The procedure was repeated with *B. subtilis* ATCC 6633 as negative comparison bacteria for lactic acid production. The pH measurement was done in triplicate.

Bacteria cell lysis optimization

Optimization of cell lysis was performed using two methods; mechanical by ultra-sonication technique and enzymatic using lysozyme. For the mechanical method, cell pellets were suspended in phosphate buffer (pH 7.4) containing 50 mM phenylmethyl sulfonyl fluoride (PMSF), and were homogenized by using a vortex. Ultrasonication conditions were set as follows: duty cycle of 0.5 sec, with variations of amplitude (20%, 30%, and 40%), cycles (10, 15, and 20 times), and duration (60, 75 and 90 sec), with 15-sec intervals, and cell amount (OD_{600} = 28.8, 15, and 7.8). For the enzymatic method, cell pellets were suspended in lysozyme solution (2 mg/mL in phosphate buffer pH 7.4 contained 50 mM PMSF) (Cappannella *et al.*, 2016) and phosphate buffer (pH 7.4), and were incubated at 37 °C for optimum lysozyme activity (Garrett and Grisham, 2016). The number of lysed cells produced by both methods was observed using a microscope (Primo Star Zeiss ZPS-0058, Germany).

BLIS activity assay and pH measurement of *W. confuse* MBF8-1 lysate produced in standard MRS medium

The lysate pH was measured using a pH meter (Hanna, Japan). BLIS activity assay of *W. confuse* MBF8-1 lysate was conducted performing disk diffusion method (Balouiri *et al*., 2016). An amount of 500 µg/mL ampicillin solution as positive control and phosphate buffer (pH 7.4) as a negative control were used. *L. mesenteroides* TISTR 120 was chosen as a test microorganism. BLIS activity was determined by measuring the zone of inhibition diameter using calipers.

Optimization of *W. confuse* MBF8-1 lysate production in MRS Vegitone and soy peptone modified-MRS media using RSM

Optimization of lysate production in MRS Vegitone and soy peptone-modified MRS media was done using RSM with a central composite design (CCD) in Design Expert 7.0 Software. The optimized factors measured were nitrogen source (soy peptone and proteose peptone), dextrose concentrations, and fermentation time. Observation of the responses (BLIS activity and lysate pH) were carried-out by using CCD to find the optimum conditions of *W. confusa* MBF8-1 lysate production in three media used. The optimum conditions when using standard MRS medium were taken as the center point: 2% dextrose, 1% protease peptone, and 10 h fermentation for MRS Vegitone medium and 8 h fermentation for soy peptone-modified MRS medium. Each factor was set to contain five levels (Tables 1 and 2), and replication was performed at the center point.

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Table 1: Levels of optimized factors used for the optimization of *W. confusa* MBF8-1 lysate production in MRS Vegitone medium using RSM.

Optimized factors	Coded level				
	–α	–1	0	+1	+α
Dextrose (%)	1	1.5	2	2.5	3
Soy peptone (%)	0.5	0.75	1	1.25	1.5
Fermentation time (h)	4	6	8	10	12

Table 2: Levels of optimized factors used for the optimization of *W. confusa* MBF8-1 lysate production in soy peptone modified-MRS medium using RSM.

Optimized factors	Coded level				
	–α	–1	0	+1	+α
Dextrose (%)	1	1.5	2	2.5	3
Proteose peptone (vegetable) (%)	0.5	0.75	1	1.25	1.5
Fermentation time (h)	6	8	10	12	14

The experimental design applied comprised 20 trials, and the value of responses was determined in triplicate. Second-order polynomial coefficients were calculated using Design Expert software ver. 7.0.0 (Stat-Ease Inc., USA). Statistical analysis of the model selected was performed using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Bacterial growth curves

Weissella confusa MBF8-1 culture grown in standard MRS medium (Figure 1a) were monitored by OD_{600} value of cell culture. Lag phase was observed for 1 h at OD_{600} =0.186, while log phase was subsequently started an hour after at OD_{600} = 0.289. The growth reached the stationary phase after 10 h of incubation at OD_{600}= 3.270. When *W. confusa* MBF8-1 culture was grown in MRS Vegitone medium (Figure 1b), the lag phase was maintained for 3 h, while the log phase was completed in an hour shortened, 8 h, the stationary phase was reached a bit later (at 12 h of fermentation) than those in standard MRS medium.

The culture of *W. confusa* MBF8-1 grown in soy peptone modified-MRS medium (Figure 1c) passed the lag phase after 1 h of fermentation, followed by the log phase began at 2 h to 8 h of fermentation, followed by the bacterial stationary phase at 9 h of fermentation until the end of the log phase.

Bacterial growth curves show bacterial growth phases, so that cell harvest times can be known when the time when BLIS activity and pH value of *W. confusa* MBF8-1 lysate is assumed at their optimum state. The OD_{600} of the growth medium is assumed to be proportional to the cell amount in the culture media (Stevenson *et al*., 2016).

It presumed that the differences in the growth phases of *W. confusa* MBF8-1 in standard MRS and MRS Vegitone media are due to the differences in composition between the two media. Bacto peptone and meat extract are the nitrogen sources in standard MRS medium; while MRS Vegitone medium contain only proteose peptone

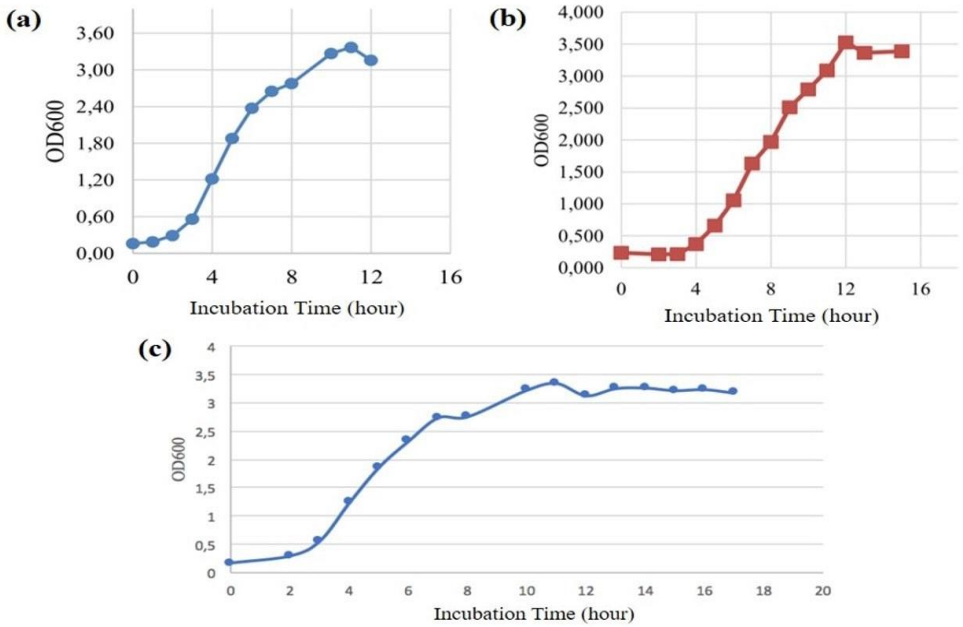

Figure 1: *W. confusa* MBF8-1 growth curves in (a) standard MRS, (b) MRS Vegitone and (c) soy peptone modified-MRS media. The OD of the medium were measured on three sample replicates.

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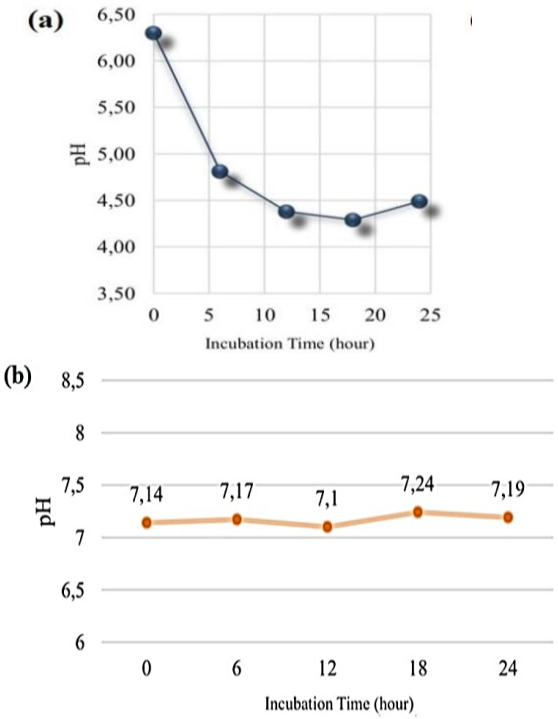

Figure 2: pH of the supernatant pH versus incubation time of (a) *W. confusa* MBF8-1 in standard MRS medium and (b) *B. subtilis* ATCC 6633 in standard MRS medium. The pH value of supernatant was measured on three sample replicates.

as the nitrogen source. Nitrogen is a constituent of amino acids, nucleic acid nucleosides, and coenzymes that affect the growth and metabolism of microorganisms. In addition, the amount of $MgSO_4$ as a mineral source in MRS Vegitone medium is less than that in standard MRS medium. It assumed that because of this lower nutrition supply in MRS Vegitone medium compared to that in standard MRS medium, the lag time was longer to reach stationary phase (Maier *et al*., 2009).

On the other hand, the growth phase of *W. confusa* MBF8-1 grown in soy peptone modified-MRS medium showed a similar condition as that in standard MRS medium. This is due to the fact that peptone derived from animal or meat contains >10% nitrogen, while soy peptone (vegetable peptone) contains 8.7% nitrogen (Atlas, 2010). The difference lies in the percentage of nitrogen, which is only ≈1.3% difference between soy peptone modified-MRS medium and standard MRS medium.

Indicator of lactic acid production increase in supernatants

Weissella confusa MBF8-1 cultures were grown in standard MRS medium (Figure 2a), the pH decreased from 6.30 to 4.81 from 0 to 6 h and then continued until 18 h of fermentation, indicating that *W. confusa* MBF8-1 produces more lactic acid and confirmed it belongs to the LAB group. In the case of *B. subtilis* ATCC 6633 (Figure 2b), the pH was stable, with a narrow range between 7.14 and 7.19, from 0 to 24 h as this bacterium served as negative control in this study.

Bacterial cell lysis optimization

Bacterial cell lysis using the mechanical method produced fewer lysed cells as observed using a microscope (Figure 3b). The optimum conditions for this method were as follows: duty cycle 0.5 sec, 15 cycles, 40% amplitude, 75 sec duration with 15 sec rest in every cycle, and OD_{600} ≈8. The lysis solution composition was 1 mL of phosphate buffer (pH 7.4) and 5 µL of PMSF. By contrast, the number of lysed cells produced by the enzymatic method was higher as also observed using a microscope (Figure 3c).

The mechanical (ultrasonication) method is commonly used for Gram-positive bacterial cell lysis. However, this method is not considered effective for bacterial cell lysis because it is time-consuming and the number of lysed

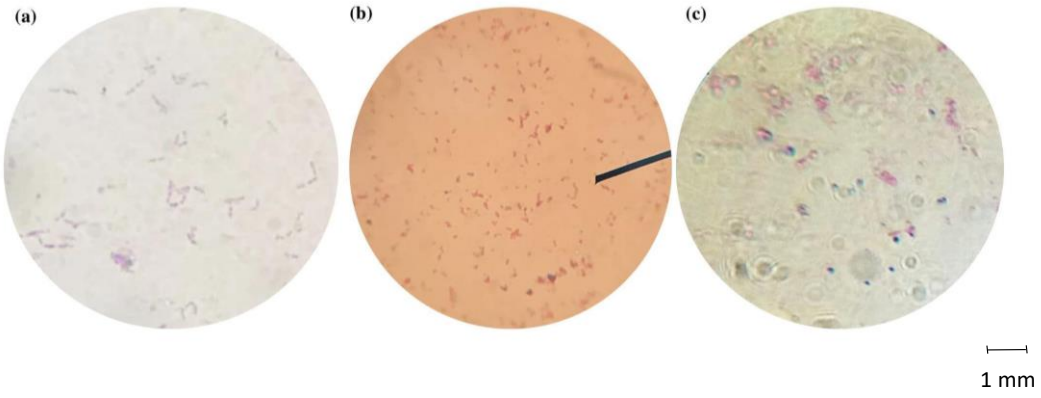

Figure 3: Microscopic observation of *W. confusa* MBF8-1 cell lysis. (a) Before lysis, (b) After lysis using ultrasonication (mechanical method) and (c) After lysis using lysozyme (enzymatic method; 1000× magnification). Red colored-cells represent lysed cells.

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cells is low, both visually and microscopically. Lysozyme (used in the enzymatic method) is an enzyme that can hydrolyze polysaccharides (Garrett and Grisham, 2016). The number of lysed cells is higher, and it is less time-consuming than the mechanical method. Therefore, the enzymatic method using lysozyme is the preferred method for bacterial cell lysis.

BLIS activity assay and pH value measurement of *W. confusa* MBF8-1 lysate produced in standard MRS medium

From the triplicate measurement of *W. confusa* MBF8-1 in standard MRS medium, the lowest lysate pH (7.26) was obtained at 8 h of fermentation as the most optimum condition. In addition, BLIS activity assay showed that the inhibition zone diameter increased until 8 h of fermentation, where the largest inhibition zone diameter (7.85 mm) was observed, and the bacteria were in the late-log phase (data not shown). The purpose of pH measurement of lysate is to confirm and ensure that cells have been appropriately lysed. Bacterial cell lysis occurs when the pH is lower than that of the solution for lysis (phosphate buffer pH 7.4).

Previous studies have shown that bacteriocin produced by *W. confusa* MBF8-1 inhibits the growth of *L. mesenteroides* TISTR 120 and *L. mesenteroides* MBFWR3, as well as *M. luteus*, *L. lactis*, *W. cibaria* and *W. confusa* (Sari *et al.*, 2011). Therefore, *L. mesenteroides* TISTR 120 was chosen as the test microorganism for BLIS activity assay. It was also reported that bacteriocin production increases when bacteria are in the log phase and reaches its maximum in the late-log phase (Goh and Philip, 2015).

Optimization of *W. confusa* MBF8-1 lysate production in MRS Vegitone and soy peptone modified-MRS media using RSM

Optimization of *W. confusa* MBF8-1 lysate production using RSM facilitated the analysis of the interaction of three optimized factors (dextrose concentration, proteose peptone concentration, and fermentation time) with the responses observed, and in determining the optimum conditions for each factor. The experimental design of the three optimized factors and the CCD results in MRS Vegitone medium and soy peptone modified-MRS are presented in Tables 3 and 4, respectively.

For both responses (BLIS activity and lysate pH), the quadratic model was selected as the best model, based on model fit analysis, which could describe the interaction between the three optimized factors and the responses. Tables 5 and 6 show the ANOVA results of the response surface model fitting for BLIS activity and lysate pH, respectively, in MRS Vegitone medium.

From all data obtained, a solution condition was resulted by Design Expert 7.0 software, and the optimum conditions for *W. confusa* MBF8-1 lysate production in MRS Vegitone medium were as follows: 1.50% dextrose, 0.75% proteose peptone, and 11.75 h of fermentation, with an inhibition zone diameter of 7.41 mm and lysate pH 7.36. The desirability of this condition was 0.765. However, the result of BLIS activity was lower, and lysate pH was higher in MRS Vegitone medium compared to the standard MRS medium.

Table 3: Experimental design of three optimized factors and CCD result in MRS Vegitone medium.

Std.	Dextrose concentration (A) (%)	Proteose peptone concentration (B) (%)	Fermentation time (C) (h)	BLIS activity (mm)	Lysate pH
1	1.50	0.75	8	7.10	7.38
2	2.50	0.75	8	7.18	7.55
3	1.50	1.25	8	7.45	7.45
4	2.50	1.25	8	7.35	7.50
5	1.50	0.75	12	7.50	7.33
6	2.50	0.75	12	7.35	7.35
7	1.50	1.25	12	7.53	7.37
8	2.50	1.25	12	7.27	7.36
9	1.00	1.00	10	7.27	7.35
10	3.00	1.00	10	7.33	7.37
11	2.00	0.50	10	7.40	7.38
12	2.00	1.50	10	7.37	7.35
13	2.00	1.00	6	6.60	7.41
14	2.00	1.00	14	6.67	7.39
15	2.00	1.00	10	7.28	7.53
16	2.00	1.00	10	7.42	7.48
17	2.00	1.00	10	7.47	7.44
18	2.00	1.00	10	7.53	7.46
19	2.00	1.00	10	7.48	7.46
20	2.00	1.00	10	7.77	7.49

For BLIS activity (by inhibition zone, data not shown), ANOVA results (Table 5) showed that the model component was significant because of a very low probability value ($p > F = 0.0101$), indicating a small chance (1.01%) that a "model *F*-value" of this size could occur because of noise. For this model, p was <0.0500, indicating that the model terms were highly significant. The component that had a significant result was C^2 ($p \leq 0.0001$). Therefore, ANOVA results showed that the three optimized factors had no significant effect on BLIS activity.

For lysate pH (Table 6), ANOVA results showed that the model component was significant because of a low probability value ($p > F = 0.0406$), indicating a small chance (4.06%) that a "model *F*-value" of this size could occur because of noise. For this model also, p was <0.0500, indicating that the model terms were highly significant. The components that had a significant result were C, A^2, and B^2. Therefore, ANOVA results showed that fermentation time (factor C, $p = 0.0216$) significantly affected lysate pH.

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Table 4: Experimental design of three optimized factors and CCD results in soy peptone modified-MRS medium.

Std.	Dextrose concentration (A) (%)	Soy peptone concentration (B) (%)	Fermentation time (C) (h)	BLIS activity (mm)	Lysate pH
1	1.50	0.75	6	6.00	6.00
2	2.50	0.75	6	7.28	7.28
3	1.50	1.25	6	7.23	7.23
4	2.50	1.25	6	7.56	7.56
5	1.50	0.75	10	6.28	6.28
6	2.50	0.75	10	6.00	6.00
7	1.50	1.25	10	6.00	6.00
8	2.50	1.25	10	6.16	6.16
9	1.00	1.00	8	6.00	6.00
10	3.00	1.00	8	6.00	6.00
11	2.00	0.50	8	6.00	6.00
12	2.00	1.50	8	6.80	6.80
13	2.00	1.00	4	7.10	7.10
14	2.00	1.00	12	7.60	7.60
15	2.00	1.00	8	7.17	7.17
16	2.00	1.00	8	7.93	7.93
17	2.00	1.00	8	8.10	8.10
18	2.00	1.00	8	7.60	7.60
19	2.00	1.00	8	7.80	7.80
20	2.00	1.00	8	8.10	8.10

The mathematic equation describing the relationship between optimized factors and BLIS activity in MRS Vegitone medium is:

$$y = 7.52 - 0.019(A) + 0.025(B) + 0.044(C) - 0.037(AB) - 0.050(AC) - 0.071(BC) - 0.035(A^2) - 0.014(B^2) - 0.20(C^2)$$

where y is BLIS activity and A, B, and C are coded values of dextrose, proteose peptone, and fermentation time, respectively.

The mathematic equation describing the relationship between optimized factors and lysate pH in MRS Vegitone medium is:

$$y = 7.48 + 0.017(A) - 0.031(C) - 0.017(AB) - 0.026\ (AC) + (3.750E-003)BC - 0.028(A^2) - 0.027(B^2) - 0.019(C^2)$$

Table 5: ANOVA results of the response surface model fitting for BLIS activity in MRS Vegitone medium.

Source	Sum of squares	Df[a]	Mean square	*F*-value	*p*-value (Prob>*F*)
Model	1.16	9	0.13	4.93	0.0101*
A	5.625E-003	1	5.625E-003	0.21	0.6532
B	1.000E-002	1	1.000E-002	0.38	0.5507
C	0.031	1	0.031	1.17	0.3052
AB	0.011	1	0.011	0.43	0.5273
AC	0.020	1	0.020	0.76	0.4030
BC	0.040	1	0.040	1.53	0.2443
A^2	0.031	1	0.031	1.19	0.3010
B^2	5.209E-003	1	5.209E-003	0.20	0.6653
C^2	1.02	1	1.02	39.08	<0.0001**
Residual	0.26	10	0.026		
Lack of fit	0.14	5	0.027	1.06	0.4739
Pure error	0.13	5	0.025		
Cor total	1.43	19			
R^2	0.8162		Adj R^2		0.6508

A: Dextrose; B: Proteose Peptone (Vege); C: Fermentation time
[a]Degrees of freedom
*Significant at $p = 0.05$; **Significant at $p = 0.01$

where y is the lysate pH.

A good quadratic model gives a significant value to the response ($p<0.1$), insignificant lack of fit ($p>0.05$), and mutually supportive predicted R^2 and adjusted R^2 (i.e., the difference does not exceed 0.2), and the value of adequate precision is >4.

Meanwhile, the equation describing the relationship between optimized factors and BLIS activity in soy peptone modified-MRS medium becomes:

$$y = 7.73 + 0.093A + 0.19B - 0.16C - 0.064(AB) - 0.22(AC) - 0.20(BC) - 0.47A^2 - 0.37B^2 - 0.14C^2$$

where y is BLIS activity, and A, B, and C are coded values of dextrose, soy peptone, fermentation time, respectively.

This quadratic model is also good because it gives a significant value to the response ($p<0.1$) and insignificant lack of fit ($p>0.05$), and the value of adequate precision is >4.

In addition, the equation that describes the relationship between optimized factors and lysate pH in soy peptone modified-MRS medium becomes:

$$y = 7.30 - 0.010A - 2.500E - 003B + 3.750E - 003C + 0.028(AB) - 7.500E - 003(AC) - 1.000E - 002(BC) + 0.053A^2 + 0.027B^2 + 0.068C^2$$

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Table 6: ANOVA results of response surface model fitting for lysate pH in MRS Vegitone medium.

Source	Sum of squares	Df[a]	Mean square	*F*-value	*p*-value (Prob>*F*)
Model	0.062	9	6.844E-003	3.24	0.0406*
A	4.556E-002	1	4.556E-003	2.16	0.1727
B	0.000	1	0.000	0.000	1.0000
C	0.016	1	0.016	7.40	0.0216*
AB	2.450E-003	1	2.450E-003	1.16	0.3068
AC	5.512E-003	1	5.512E-003	2.61	0.1373
BC	1.125E-004	1	1.125E-004	0.053	0.8222
A^2	0.019	1	0.019	9.11	0.0129*
B^2	0.018	1	0.019	8.70	0.0145*
C^2	9.001E-003	1	9.001E-003	4.26	0.0659
Residual	0.021	10	2.113E-003		
Lack of fit	0.016	5	3.23E-003	3.25	0.1108
Pure error	4.971E-003	5	9.942E-004		
Cor total	1.43	19			
R^2	0.8162		Adj R^2		0.6508

A: Dextrose; B: Proteose Peptone (Vege); C: Fermentation time
[a]Degrees of freedom
*Significant at $p = 0.05$

where *y* is lysate pH.

The ANOVA results of the response surface model fitting for BLIS activity in soy peptone modified-MRS modified-MRS medium are shown in Table 7. On the basis of the numerical optimization results, the desirability of optimum conditions for *W. confusa* MBF8-1 lysate production in soy peptone modified-MRS medium was 0.853. However, the result of BLIS activity was lower and lysate pH was slightly higher in soy peptone modified-MRS medium compared to standard MRS medium. The inhibition zones of *W. confusa* MBF8-1 lysate in soy peptone modified-MRS medium are shown in Figure 4.

The ANOVA results of response surface model fitting for lysate pH in soy peptone modified-MRS medium are shown in Table 8. The equation of the mathematical model for lysate pH response was a quadratic equation with R^2= 0.7504. On the basis of the variance test, we obtained a significant model, which means that the response of lysate pH with a value of 0.0370 was given a significantly different treatment. The value of lack of fit obtained was insignificant, with p= 0.3237 (p>0.05). Adjusted R^2 and predicted R^2 were 0.5257 and −0.3781, respectively, and the value of precision was 5.333. The graphic result from RSM are shown in Figures 5 and 6.

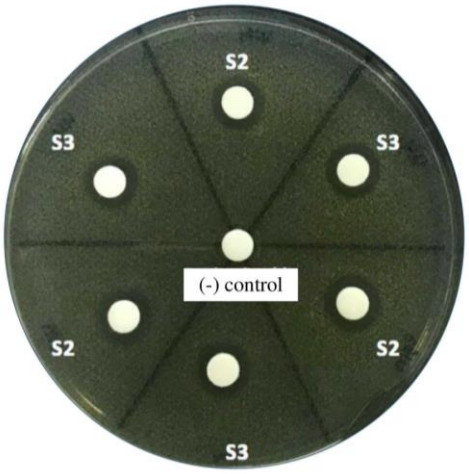

Figure 4: Inhibition zones of *W. confusa* MBF8-1 lysate in soy peptone modified-MRS medium. S2: Sample replicate 2; S3: Sample replicate 3. (-) control: Phosphate buffer pH 7.4

Table 7: ANOVA results of the second-order response surface model fitting for BLIS activity in soy peptone modified-MRS medium.

Source	Sum of squares	Df[a]	Mean square	*F*-value	*p*-value (Prob>*F*)
Model	9.51	9	1.06	3.77	0.0251
A	0.14	1	0.14	0.50	0.4976
B	0.56	1	0.56	2.00	0.1882
C	0.43	1	0.43	1.54	0.2424
AB	0.033	1	0.033	0.12	0.7404
AC	0.37	1	0.37	1.34	0.2746
BC	0.33	1	0.33	1.19	0.3017
A^2	5.67	1	5.67	20.25	0.0011
B^2	3.53	1	3.53	12.62	0.0053
C^2	0.47	1	0.47	1.69	0.2222
Residual	2.80	10	0.28		
Lack of fit	2.17	5	0.43	3.43	0.1011
Pure error	0.63	5	0.13		
Cor total	12.31	19			
R^2	0.7725		Adj R^2		0.5677

A: Dextrose; B: Soy peptone (Vege); C: Fermentation time
[a]Degrees of freedom

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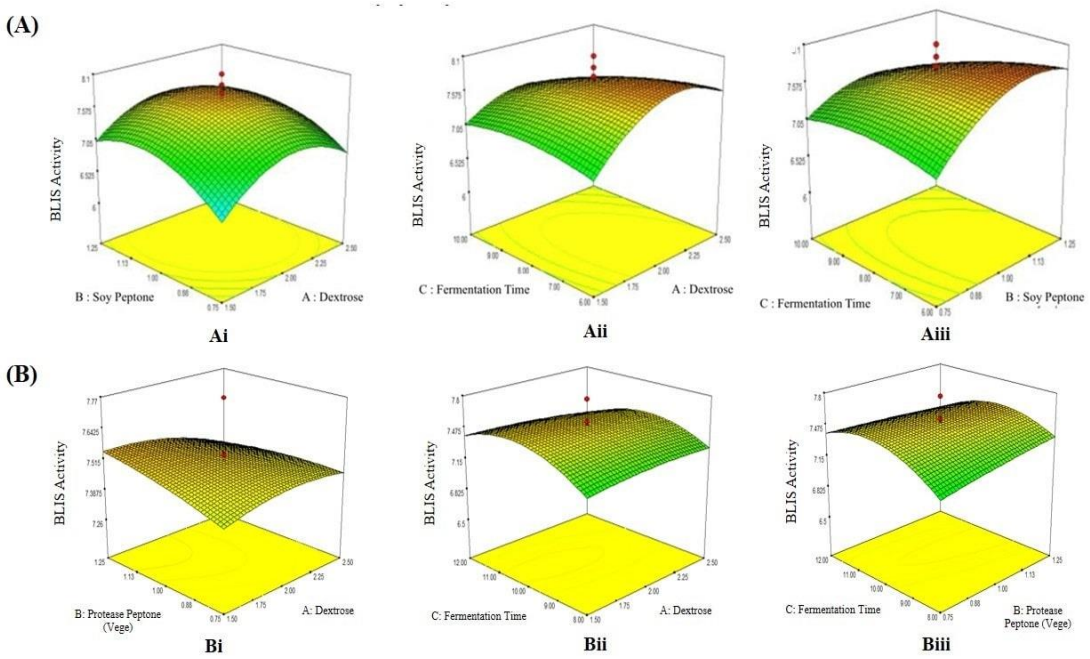

Figure 5: Interaction between independent factors for BLIS activity presented as 3-dimensional graphs. (A) Dextrose, soy peptone, and fermentation duration; (B) Dextrose, proteose peptone vegetable (Vegitone), and fermentation duration. (Ai) Effects of dextrose and soy peptone; (Aii) Effects of dextrose and duration of fermentation; (Aiii) Effects of soy peptone and duration of fermentation; (Bi) Effects of dextrose and proteose peptone vegetable (Vegitone); (Bii) Effects of dextrose and duration of fermentation; (Biii) Effects of proteose peptone vegetable (Vegitone) and duration of fermentation.

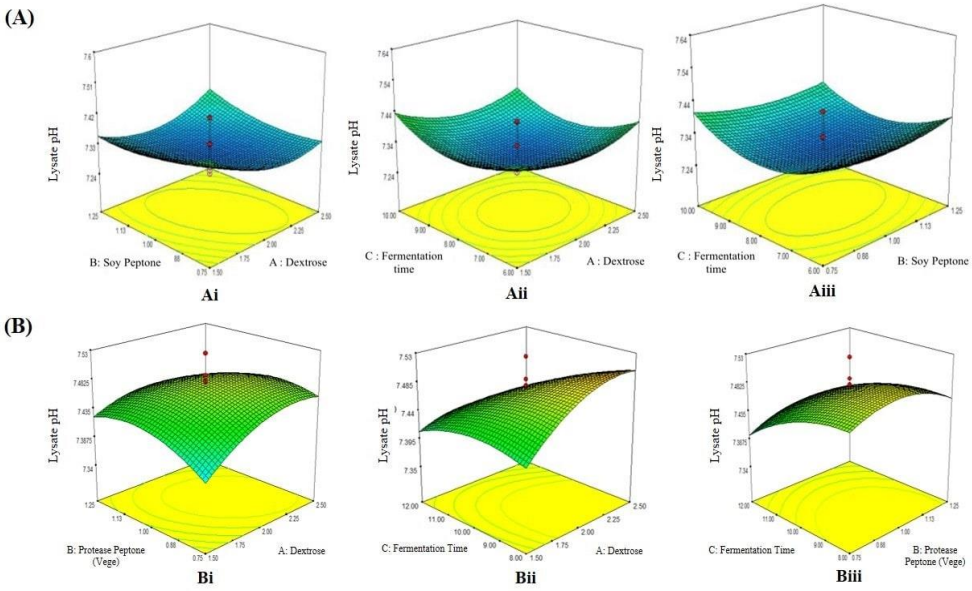

Figure 6: Interaction between independent factors for lysate pH presented as 3-dimensional graphs. (A) dextrose, soy peptone, and fermentation duration; (B) dextrose, proteose peptone vegetable (Vegitone), and fermentation duration. (Ai) Effects of dextrose and soy peptone; (Aii) Effects of dextrose and duration of fermentation; (Aiii) Effects of soy peptone and duration of fermentation; (Bi) Effects of dextrose and proteose peptone vegetable (Vegitone); (Bii) Effects of dextrose and duration of fermentation; (Biii) Effects of proteose peptone vegetable (Vegitone) and duration of fermentation.

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Table 8: ANOVA results of response surface model fitting for lysate pH in soy peptone modified-MRS medium.

Source	Sum of squares	Df[a]	Mean square	*F*-value	*p*-value (Prob> *F*)
Model	0.16	9	0.018	3.34	0.0370
A	1.600E-003	1	1.600E-003	0.29	0.6004
B	1.000E-004	1	1.000E-004	0.018	0.8951
C	2.250E-004	1	2.250E-004	0.041	0.8433
AB	6.050E-003	1	6.050E-003	1.11	0.3176
AC	4.500E-004	1	4.500E-004	0.082	0.7801
BC	8.000E-004	1	8.000E-004	0.15	0.7101
A^2	0.070	1	0.070	12.84	0.0050
B^2	0.018	1	0.018	3.25	0.1016
C^2	0.120	1	0.120	21.16	0.0010
Residual	0.055	10	5.469E-003		
Lack of fit	0.033	5	6.631E-003	1.54	0.3237
Pure error	0.022	5	4.307E-003		
Cor total	0.220	19			
R^2	0.7504		Adj R^2	0.5257	
			Predicted R^2	-0.3781	
			Value of precision	5.333	

A: Dextrose; B: Soy peptone; C: Fermentation time
[a]Degrees of freedom

The optimum fermentation conditions for *W. confusa* MBF8-1 lysate production in two modified MRS media are as follows: MRS Vegitone (1.50% dextrose, 0.75% proteose peptone, and 11.75 h fermentation) and soy peptone modified-MRS (2.05% dextrose, 1.05% soy peptone, and 7.53 h fermentation). The potential inhibition zone diameter and pH of the lysate produced are as follows: 7.41 mm and 7.36, respectively (MRS Vegitone) and 7.80 mm and 7.30, respectively (soy peptone modified-MRS). The pH of *W. confusa* MBF8-1 lysate is slightly lower (7.26), and BLIS activity is higher (7.85 mm) using standard MRS medium (2% dextrose, 1% peptone, and 8 h fermentation) when compared to the two modified MRS media.

CONCLUSION

The pH and BLIS activity of *W. confusa* MBF8-1 lysate produced using soy peptone modified-MRS as fermentation medium showed about the same result as the standard MRS. Thus, soy peptone modified-MRS can be used as an alternative plant-based growth medium for *W. confusa* MBF8-1 lysate production.

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