Optimization of medium composition by response surface methodology for improving biomass yield of *Tetragenococcus halophilus* CH6-2

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Abstract
Tetragenococcus halophilus CH6-2 was proven to positively affect the sensory characteristics of fish sauce. This study aims to optimize the medium components for enhancing the biomass of T. halophilus CH6-2 for starter culture production using a statistical method. A minimum-run resolution IV factorial design was used to screen the medium component that significantly affected biomass yield. Three components, $MgSO_4$, K_2HPO_4 and glucose, were selected for further optimization study using Box-Behnken design. The optimal concentration after optimization was 2.58 g/L $MgSO_4.7H_2O$, 6.09 g/L K_2HPO_4 and 12.4 g/L glucose.

The obtained OD600 and cell dry weight on the optimized medium were 1.87- and 2.51-fold increased, reaching 3.993 ± 0.034 and 1.450 ± 0.018 mg/mL respectively. The viable cell counts in the optimized medium reached 11.56 log CFU/mL in 24 hours of cultivation which was a two-log increase compared with 9.55 log CFU/mL after 48 hours in the MRS medium. In conclusion, higher biomass could be obtained in a shorter time in the optimized medium than in the MRS medium.

Keywords: Cell density, medium component, optimization, *Tetragenococcus halophilus*, response surface methodology.

Introduction

Vietnamese traditional fish sauce is produced by fermenting previously mixed fish and salt at a ratio of 3 to 1 for a very long period, from 10 to 18 months. Besides the long fermentation time, using indigenous enzymes and microorganisms leads to an unstable product of inadequate quality. Attempting to shorten production time by using commercial protease alone results in fish sauce with low sensory quality. Therefore, using a starter culture to stabilize the quality and improving the organoleptic properties of commercial enzymatic hydrolyzed fish sauce are desirable.

Microorganisms such as *Virgibacillus* sp., *Staphylococcus* sp. and *T. halophilus* were used as a starter culture. They were reported to produce an active volatile compounds profile, which could improve the sensory quality of the fish sauce[5,31,34]. *T. halophilus* was isolated from salt-fermented products such as fermented Thai fish sauce (nam pla)[29], Indonesian Soy Mash (Kecap) fermentation[27], Indonesian shrimp paste "terasi"[12] and Korean traditional salted-fermented food Myeolchi (anchovy)-jeotgal[10]. *T. halophilus* creates a profile of flavoring components in fermented salted food[15,30,32,36].

In addition, *T. halophilus* might possess some beneficial health-related properties such as immunoregulatory effect[13], probiotic potential[11] and Ameliorating Development of Atopic Dermatitis[24]. *T. halophilus* CH6-2 isolated from Cat Hai fish mash in Vietnam was reported previously [22]. According to our previous study, *T. halophilus* CH6-2 tolerated 25% NaCl concentration, produced aroma-active compounds and exhibited intracellular aminopeptidase activities[14], suppressing histamine accumulation (personal data), thus it seems to be a potential starter culture for fish sauce fermentation. For that reason, the enhanced cell density of *T. halophilus* CH6-2 is required to satisfy the needs of an industrial scale. The *T. halophilus* CH6-2 was reported to grow best at pH 7, temperature of 35°C, NaCl concentration from 5% to 7% and reached maximal OD600 after 72 hours of cultivation in Man de Rogosa Sharp (MRS) medium[14].

However, the study on the effect of the growth medium component on the growth of *T. halophilus* is still modest. Like other lactic acid bacteria (LAB), *T. halophilus* requires complex media supplementing nucleotides, amino acids, salts and many other nutrients for their growth. The general growth media for *T. halophilus* in reported studies were M17[3,7,28], MRS and tryptic soy broth (TSB) with the common components such as tryptone, peptone, yeast extract, $MgSO_4$, K_2HPO_4 and glucose[10,12,33]. The OD600 values of two *T. halophilus* strains were 0.61 ± 0.01 and 0.58 ± 0.01 after 5 days of cultivation on MRS medium [12] or of four others were 1.1-1.2 after 10 days on TBS media, corresponding to maximal $8.4x10^7$ CFU/mL[10].

Despite being used as the standard growth media for lactic acid bacteria, MRS is not always in optimal composition or concentration for specific LAB strains. The reason could be due to the lack or excess of nutrients and essential minerals, as well as preferences of different carbon sources of different LAB. The optimized culture media should satisfy several criteria such as cost-effectiveness, high product yield and short fermentation time[3].

Various strategies exist to optimize the medium composition to increase biomass yield. The classical statistical method is monothetic analysis (one factor at a time OFAT), in which

all variables are kept constant except one. OFAT is an easy and simple approach. However, it requires more runs at a high number of factors and the interaction effect could not be investigated[16]. Meanwhile, other statistical methods including Minimum-Run Resolution IV and Box-Behnken Design, can overcome those limitations and are therefore preferred. This study aims to find the optimal medium by statistical method for improving the biomass of *T. halophilus* CH6-2.

Material and Methods

Bacterial strain and chemicals: The *T. halophilus* CH6-2 (GenBank assession number MW139248.1) strain was isolated from the fish mash[22]. Stock cultures were preserved at -80 °C in MRS broth containing 25% (v/v) glycerol. Before use, the strain from stock cultures was streaked onto an MRS agar plate and a single colony was subcultured in MRS medium at 35ºC for 48 hours. Chemicals for MRS and our developed optimized media of bacteriological grade were purchased from LAB (UK), Merck (Germany) and Samchun (Korea).

Biomass determination: Biomass was assumed to be proportional to the optical density at 600 nm (OD600). The viable cell was counted on Petri dishes containing MRS medium supplementing 50 g/L NaCl. One unit of OD600 of *T. halophilus* CH6-2 corresponded to 0.357 ± 0.022 mg/mL cell dry weight. Each experiment was conducted in triplicate and repeated twice.

Inoculum preparation and cultivation: Fresh inoculum of *T. halophilus* CH6-2 strains was added to an Erlenmeyer flask containing MRS medium at a ratio of 1% (v/v) (Table 1) and was cultivated at 35 °C for 48 h under static conditions. Biomass was collected by centrifugation at 6000 rpm for 15 min at 4 °C and was washed twice with sterilized distilled NaCl 0.9% and subsequently transferred into a flask containing cultivation media with composition as indicated in the text to get initial OD600 of 0.1 (corresponds to an initial cell density of $1.1x10^7$ CFU/mL). The inoculated culture was incubated in a static condition at 35ºC. The OD600 was determined after 72 hours of cultivation.

Effect of medium composition on the growth of *T. halophilus* CH6-2 using OFAT: Two media MRS, modified K1 according to Zacharof and Lovitt[35] (Table 1), were first used for the cultivation of *T. halophilus* CH6-2. The inoculum and cultivation were prepared as described above. The OD600 was determined after 72 hours of cultivation at static conditions. Components are removed from the K1 medium one by one (CH_3COONa, K_2HPO_4, $Na_3C_6H_5O_7$, Tween 80, $MgSO_4$, $MnSO_4$) (Table 1) and the effect of their omission on OD600 was evaluated. The inoculum and cultivation were conducted as described above. The OD600 was determined after 72 hours of cultivation at static conditions.

Screening medium component by factorial design: Minimum runs resolution IV (Min Run Res IV) factorial design was used for screening the variables that significantly affect the cell density of *T. halophilus* CH6-2. From preliminary screening results, seven medium components including (A) glucose, (B) yeast extract (YE), (C) K_2HPO_4, (D) $MgSO_4.7H_2O$, (E) casamino acid, (F) tryptone and (G) peptone corresponding to seven variables were selected (Table 2) to investigate their effect on response value OD600 (Y). These seven variables are represented at three coded levels (-1, 0, +1) (Table 2).

Table 1
Compositions of the studied media

	Concentration (g/L)	
Component	MRS	K1
Pepton	10	-
Meat Extract	10	-
Yeast Extract	5	20
CH_3COONa	5	10
K_2HPO_4	2	5
$Na_3C_6H_5O_7$	10	10
Tween 80	1	1
$MgSO_4.7H_2O$	0.1	0.2
$MnSO_4.4H_2O$	0.05	0.02
Glucose	20	20
NaCl	50	50
pH	7	7

The experimental design with 18 runs including two replicates at the center point, the real values of variables and corresponding response values (OD600) are listed (Table 4). NaCl was fixed at 50 g/L in all runs. The cultivation was conducted in a static condition at 35ºC and the OD600 was determined after 72 hours of cultivation. The factors significantly affecting the cell density were selected for subsequent optimization experiments.

Response surface methodology (RMS): The most significant variables (i.e. medium component) from the screening study by Min Run Res IV factorial design were further evaluated by RSM using Box-Behnken Design BBD to find their optimal concentration as well as to estimate their effect and their interaction effect on cell density. The design matrix of BBD with three variables, which were A ($MgSO_4.7H_2O$), B (K_2HPO_4) and C (glucose) respectively, led to a set of 15 experiments, including three repeated at central points. Each independent variable was set at three coded levels (-1, 0, +1). The actual and response values of BBD are shown in table 3. The response value (Y) represented OD600.

Statistical analysis: The design matrix for minimum run and BBD experiments was set up and analyzed using Design Expert Software Version 13.0. A One-way Analysis of Variance was performed using SPSS 26 (IBM USA) to determine the differences in the means in OFAT experiments. Means were considered statistically significant when $p < 0.05$.

Table 2
Real and coded values of variables used in Minimum runs resolution-IV

Code	Parameter (g/L)	Low (-1)	Central (0)	High (+1)
A	Glucose	5.00	15.00	25.00
B	YE	5.00	15.00	25.00
C	K_2HPO_4	2.00	5.00	8.00
D	$MgSO_4.7H_2O$	0.20	2.60	5.00
E	Casaminoacid	5.00	15.00	25.00
F	Tryptone	5.00	15.00	25.00
G	Peptone	5.00	15.00	25.00

Table 3
Coded and real values of independent variables used in BBD

Code	Independent Factors (g/L)	Levels		
		-1	0	+1
A	$MgSO_4.7H_2O$	0.00	5.00	10.00
B	K_2HPO_4	3.00	5.00	7.00
C	Glucose	5.00	10.00	15.00

Table 4
Experimental design and response values of Minimum runs resolution-IV design.

Run	A	B	C	D	E	F	G	OD_{600}
1	25	5	2	5	25	5	5	2.270 ± 0.220
2	5	25	8	0.2	5	25	25	2.976 ± 0.124
3	25	25	8	5	25	25	25	3.271 ± 0.246
4	5	5	2	5	25	25	25	2.862 ± 0.101
5	25	25	2	5	5	25	5	3.178 ± 0.054
6	5	25	8	5	25	5	5	3.920 ± 0.182
7	15	15	5	2.6	15	15	15	3.437 ± 0.025
8	5	5	8	0.2	25	5	25	2.195 ± 0.029
9	15	15	5	2.6	15	15	15	3.556 ± 0.111
10	5	25	2	0.2	25	25	5	2.968 ± 0.075
11	5	5	8	5	5	25	5	2.950 ± 0.096
12	25	25	8	0.2	5	5	5	3.907 ± 0.003
13	25	25	2	0.2	25	5	25	2.744 ± 0.142
14	25	5	2	0.2	5	25	25	2.766 ± 0.038
15	5	5	2	0.2	5	5	5	1.387 ± 0.030
16	25	5	8	5	5	5	25	2.933 ± 0.231
17	25	5	8	0.2	25	25	5	2.845 ± 0.024
18	5	25	2	5	5	5	25	2.788 ± 0.274

A: glucose, B: yeast extract, C: K_2HPO_4, D: $MgSO_4.7H_2O$, E: casamino acid, F: tryptone, G: peptone

Results and Discussion

Effect of medium composition on cell density of *T. halophilus* CH6-2: MRS is considered the common medium for culturing LAB. However, MRS is unsuitable for industrial use because of the cost, complexity and potential health risks of beef extract, poultry peptone and low cell density. Therefore, attempts are continually made to find a new suitable media for each lactic acid bacteria group. Figure 1 revealed that the K1 medium promoted better cell growth among the two-culture media evaluated and the OD600 is almost 1.27-fold higher than that on the MRS medium. Despite complex organic nitrogen components including meat extract, yeast extract, peptone and plenty of other components, MRS gave the lower OD600 value, suggesting the excess of some nutrients but also the lack of some others.

The K1 medium with a two-fold increase of $MgSO_4$, K_2HPO_4 and CH_3COONa concentrations (Table 1) but of lower concentration and less type of organic nitrogen (only 20 g/L yeast extract compared with 25 g/L combined yeast and meat extract, peptone) gave the higher OD600 value revealing the role of these components. The developed medium K1 was reported to assist well in the growth of all three investigated *Lactobacilli*[35]. Both manganese ion and magnesium ion were shown to contribute to the growth of *Lactobacillus rhamnosus* FTDC 8313[18]. To understand the effect of each component of the K1 medium on the growth

of *T. halophilus* CH6-2, each component except for the main carbon source glucose and nitrogen source (yeast extract) was omitted from the K1 medium (Figure 2).

The results of figure 2 revealed that only the omission of K_2HPO_4 and $MgSO_4$ had a strong negative effect on the growth; the OD600 value of *T. halophilus* CH6-2 decreased by two- and 1.67-fold respectively, compared with that of the K1 medium. The omission of $MnSO_4$ and Tween 80 had almost no effect on the growth. The omission of $Na_3C_6H_5O_7$ resulted in a reduction of less than 1.15 times in OD600.

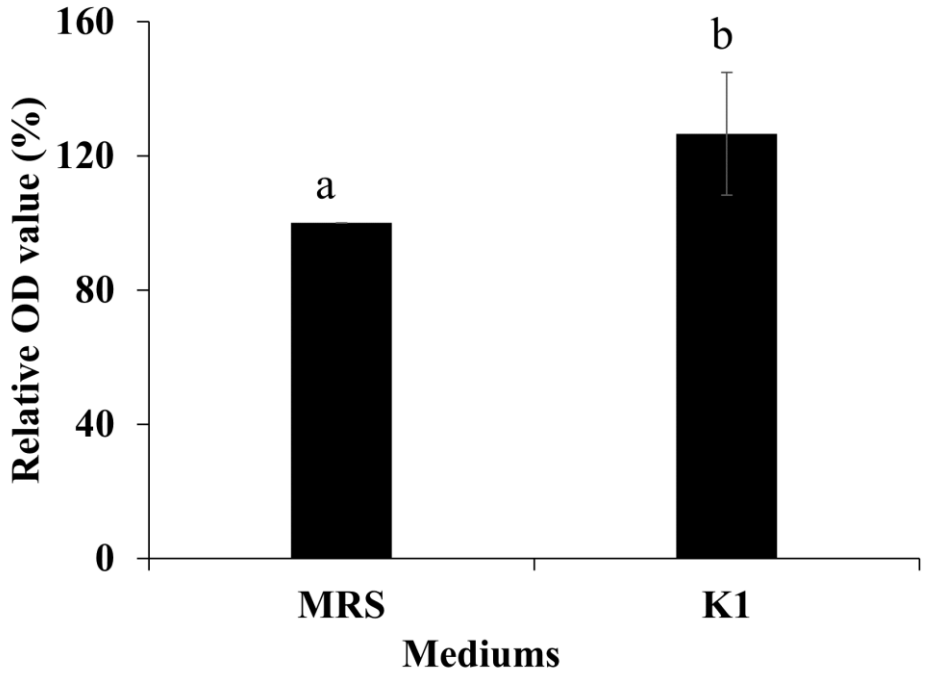

Figure 1: The OD600 of *T. halophilus* CH6-2 on MRS and K1 media after 72 hours of cultivation. The OD600 value obtained at MRS medium corresponding to 2.02 was considered 100%. Different letters indicate a significant difference between the experimental values ($p < 0.05$).

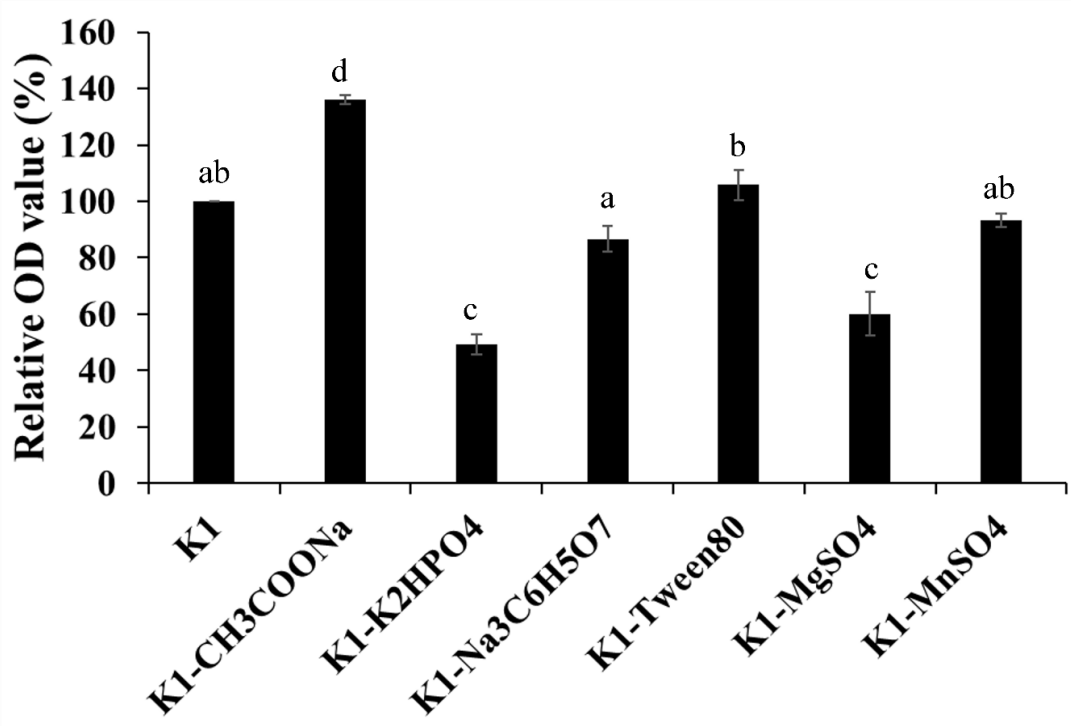

Figure 2: Effect of each omitted K1 medium component on the growth of *T. halophilus* CH6-2. The OD600 value obtained at the K1 medium 2.56 was considered 100%. Different letters indicate a significant difference between the experimental values ($p < 0.05$)

Meanwhile, the omission of CH_3COONa even resulted in a 1.36 times increase in OD600 value, showing its negative role in the growth of *T. halophilus* CH6-2. $Na_3C_6H_5O_7$ and CH_3COONa are commonly used buffers in LAB media. The omission of sodium acetate caused the growth reduction of *Lb. plantarum* because of the rapid decrease in pH[6].

The *T. halophilus* grows more slowly than *Lactobacillus* and does not cause pH to decline rapidly; thus, $Na_3C_6H_5O_7$ alone might be enough for buffering. Manganese ion was crucial to the growth and development of LAB[26]. Moreover, Mg^{2+} could assist the development of bacterial cell walls as it is required for *de novo* synthesis and assembly of peptidoglycan[4]. Besides, ions Mn^{2+} and Mg^{2+} are exchangeable in the metal binding sites of many proteins[9].

Ion Mg^{2+} appears to be the only essential metal ion for growing *Lb. delbrueckii* ssp. *lactic*[8] or *Streptococcus thermophilus*[17]. K_2HPO_4 acts as a buffer salt in microbial growth media and is the sole source of phosphorus. ATP energy generation, DNA and RNA synthesis and many other biochemical processes require phosphorus. K_2HPO_4 was reported to provide K^+ and phosphate for microbial growth[21]. Tween 80 was shown to stimulate the growth of LAB[19] and other microorganisms while insignificantly affecting *Lactobacillus casei* YIT 9018[23] and negatively affecting the growth of *P. acidilactici* TP-6[20]. Therefore, it can be assumed that not all LABs grow well in the medium, supplementing Tween 80.

From the above results, the two medium components $MgSO_4$, K_2HPO_4 that showed a strong effect on the cell density of *T. halophilus* CH6-2, the carbon source glucose, the nitrogen source yeast extract and three other organic nitrogen sources (reported to support the growth of LAB namely casamino acid, peptone, tryptone) were chosen for factorial design to screen the medium component that significantly improves the cell density of *T. halophilus* CH6-2.

Screening of significant medium components affecting the cell density of *T. halophilus* CH6-2 strain by Minimum-Run Resolution IV factorial design: Min Run Res IV is usually employed for screening the variables for further optimization by RSM. Seven variables (A) glucose, (B) yeast extract (YE), (C) K_2HPO_4, (D) $MgSO_4.7H_2O$, (E) casamino acid, (F) tryptone and (G) peptone were screened. The coded and real values of variables are presented in table 2. The results showed that the response values OD600 of experiments varied over a relatively wide range from 1.39 to 3.92 (Table 4). ANOVA of the factorial design showed that the model was statistically significant (p <0.0001) (Table 5). The variables B-YE, C-K_2HPO_4, D- $MgSO_4.7H_2O$, A-Glucose and F-Tryptone were statistically significant (p <0.0001), suggesting their prominent effect on the growth.

The Pareto chart showed the influential level of each factor on OD600 through the t-value of effect (Figure 3). All highly significant variables had a positive effect, which means that the increase in their concentration will lead to an increase in cell density. YE had the most prominent effect, followed by K_2HPO_4, $MgSO_4$, glucose and tryptone (Figure 3). Despite the positive effect, tryptone showed a lower effect than YE and thus was excluded from further optimization.

Among four nitrogen sources, YE, casamino acid, tryptone and peptone, YE possessed the highest t-value. Yeast extract is known to be rich in vitamins, minerals and amino acids and is an important nitrogen source for LAB[1,2]. Despite being ranked in the third position, the effect of $MgSO_4$ was much lower than that of K_2HPO_4 and YE. Thus, from the screening study, four medium components, K_2HPO_4, YE, $MgSO_4.7H_2O$ and glucose, were selected for further optimization study by BBD.

Table 5
ANOVA in Minimum runs resolution-IV design

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Model	10.24	14.00	0.7314	91.59	< 0.0001	Significant
A-Glucose	0.44	1.00	0.44	55.00	< 0.0001	
B-Yeast Extract	3.71	1.00	3.71	464.62	< 0.0001	
C-K_2HPO_4	1.49	1.00	1.49	186.91	< 0.0001	
D-$MgSO_4.7H_2O$	0.57	1.00	0.57	71.80	< 0.0001	
E-Casaminoacid	0.0009	1.00	0.0009	0.12	0.74	
F-Tryptone	0.38	1.00	0.38	46.97	< 0.0001	
G-Peptone	0.12	1.00	0.12	14.96	0.001	
AD	1.80	1.00	1.80	225.44	< 0.0001	
Curvature	1.16	1.00	1.16	144.99	< 0.0001	
Residual	0.16	20.00	0.008			
Lack of Fit	0.0004	1.00	0.0004	0.04	0.84	Not significant
Pure Error	0.16	19.00	0.0084			
Cor Total	11.56	35.00				

R^2 = 0.9646; adjusted R^2 = 0.9739; predicted R^2 = 0.9515

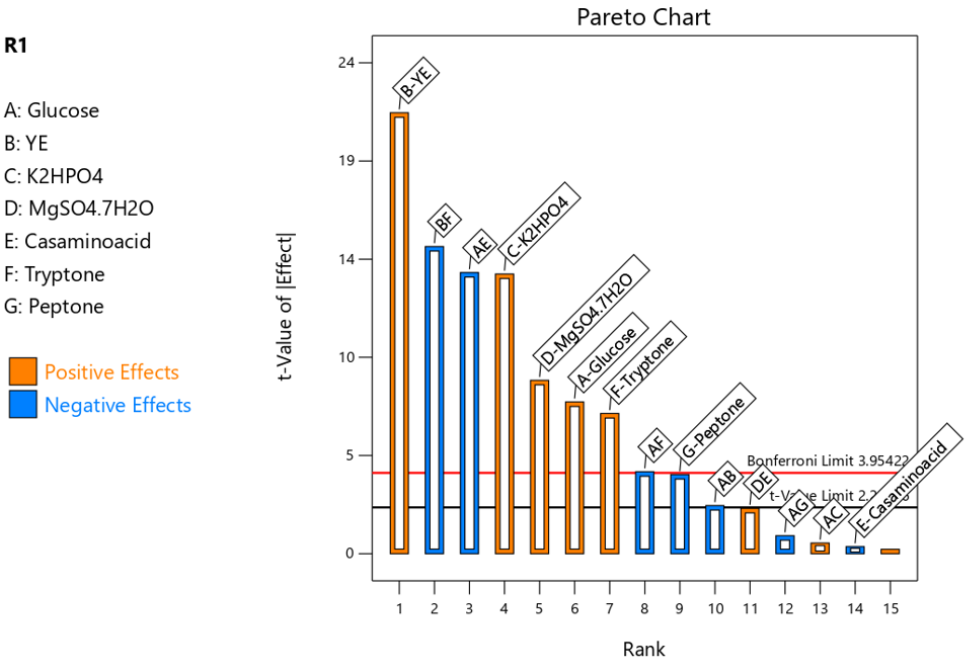

Figure 3: Pareto chart of the standardized effects. The blue bar indicates the negative effect. A-glucose; B- yeast extract; C- K_2HPO_4; D- $MgSO_4.7H_2O$; E - Casaminoacid; F- Tryptone; G- Peptone.

Optimization of medium component concentration by BBD: Before optimization of the medium component by BBD, the concentration range of the medium component was investigated. *T. halophilus* CH6-2 growth behavior was evaluated at various YE, K_2HPO_4, glucose and $MgSO_4.7H_2O$ concentrations. It could be seen that YE had a prominent effect on OD600, as was observed previously (Figure 3). The OD600 value increased more than two-fold when YE concentration increased from 5 g/L to 55 g/L. Further increase of YE concentration resulted in a steady increase of OD600 but at a lower rate (Figure 4A). Higher YE concentration is undesired due to economic reasons. Therefore, a YE concentration of 55 g/L was fixed for the BBD study.

The OD600 value increased two-fold at 3 g/L K_2HPO_4 compared to the medium without K_2HPO_4 (Figure 4B). At the K_2HPO_4 concentration range from 3 g/L to 7 g/L, the OD600 value did not appear to change much. Higher K_2HPO_4 of 10 g/L even reduced the OD600 value. Thus, the K_2HPO_4 range from 3 g/L to 7 g/L was chosen for the BBD study.

The OD600 value was almost 1.71-fold increased at the glucose concentration of 10 g/L compared with without glucose, reaching 2.340 ± 0.150 (Figure 4C). The pH values, which reflect the metabolism of lactic acid bacteria, were lower at higher OD600 values (Data were not shown). The results suggested that a suitable glucose concentration could promote the growth of *T. halophilus* CH6-2. The growth inhibition of *T. halophilus* CH6-2 at higher glucose concentration could be explained by the excessive lactic acid production[25]. Therefore, a glucose range from 5 g/L to 15 g/L was chosen for the BBD study. An increase of $MgSO_4.7H_2O$ concentration to 5 g/L resulted only in a slight increase in the OD600 value (Figure 4B). Further increase led to a decrease of OD600, especially at 20 g/L (about a 20% decrease in OD600 value). Interestingly, the OD600 value reached the highest value of 2.757 ± 0.183 in the experiment screening for the $MgSO_4$ range due to the synergic effect of glucose and $MgSO_4$, suggesting that further optimization by response surface methodology is needed whereas the interaction between variables could be seen. Thus, the $MgSO_4.7H_2O$ range from 0 g/L to 10 g/L was chosen for the BBD study.

After choosing the concentration range of $MgSO_4.7H_2O$ from 0 to 10 g/L, K_2HPO_4 from 3 to 7 g/L, glucose from 5 to 15 g/L and fixing YE at 55 g/L, a Box-Behnken design was applied for optimization (Table 3). Table 6 presents the experimental design consisting of 15 runs with the real values of variables and the obtained response OD600 values. The OD600 values appeared to change less than in factorial design, from 3.183 to 3.986, suggesting that the concentration of medium components was already close to optimal due to the previous optimization experiment. The highest OD600 was observed in the experiment with a concentration of 10 g/l glucose, 5 g/l $MgSO_4.7H_2O$ and 5 g/l K_2HPO_4.

Regression analysis of the data presented in table 7 resulted in the quadratic polynomial equation describing the cell density OD600 (Y) as a function of the actual levels of $MgSO_4.7H_2O$ (A), K_2HPO_4 (B) and glucose concentration (C):

$Y = 3.9500 + 0.0946A + 0.0637B + 0.1794C – 0.0923AB – 0.0952AC + 0.0325BC – 0.1505A^2 – 0.0606B^2 – 0.2472C^2$.

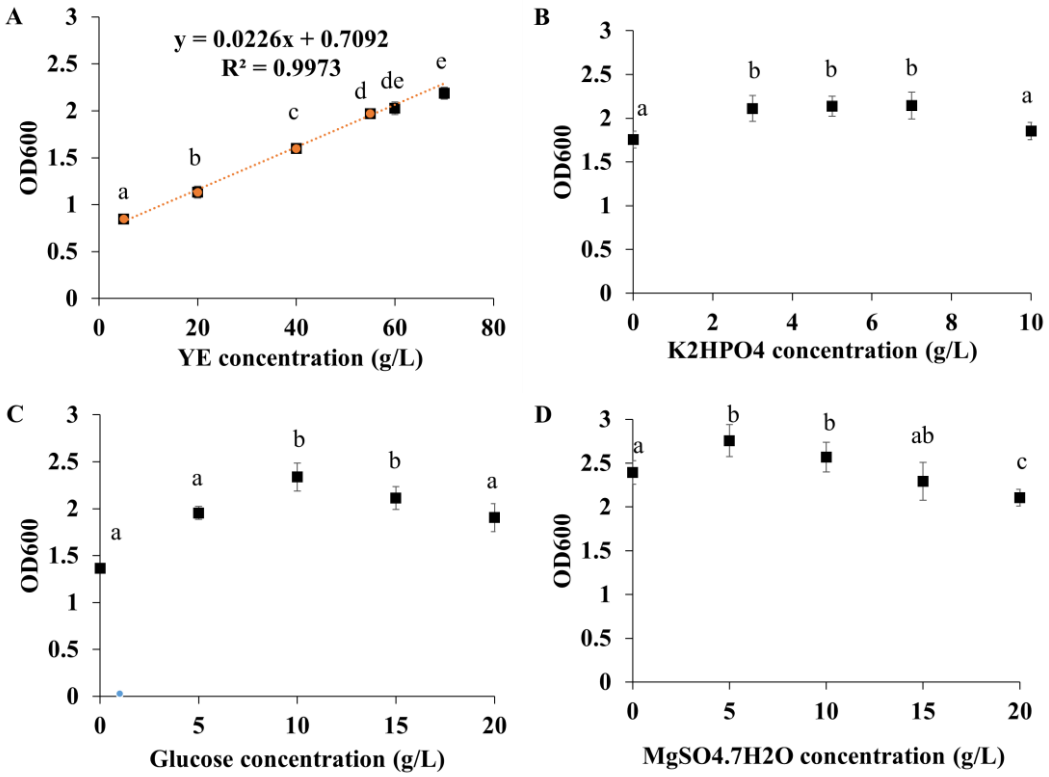

Figure 4: Effect of (A) YE, (B) K_2HPO_4, (C) glucose and (D) $MgSO_4.7H_2O$ concentrations on OD600 of *T. halophilus* CH6-2, different letters indicate a significant difference between the experimental values ($p < 0.05$).
(A): YE concentrations of 5, 20, 40, 55, 60 and 70 g/L were added to the medium containing 20 g/L $MgSO_4.7H_2O$;
(B): K_2HPO_4 concentrations of 0, 3, 5, 7 and 10 g/L were added to the medium containing 20 g/L $MgSO_4.7H_2O$, 50 g/L YE;
(C): Glucose concentrations of 0, 5, 10, 15 and 20 g/L were added to the medium containing 20 g/L $MgSO_4.7H_2O$, 50 g/L YE;
(D): $MgSO_4.7H_2O$ concentrations of 0, 5, 10, 15 and 20 g/L were added to the medium containing 20 g/L $MgSO_4.7H_2O$, 50 g/L YE; 10 g/L glucose. All media contain 50 g/L NaCl

Table 6
BBD matrix with real values of variables and corresponding response values

Run	$MgSO_4.7H_2O$	K_2HPO_4	glucose	OD600
1	0	5	5	3.183 ± 0.030
2	5	3	5	3.468 ± 0.088
3	0	3	10	3.460 ± 0.132
4	5	7	5	3.516 ± 0.087
5	0	7	10	3.787 ± 0.042
6	10	5	15	3.737 ± 0.069
7	10	5	5	3.515 ± 0.040
8	10	3	10	3.881 ± 0.074
9	5	5	10	3.955 ± 0.066
10	5	7	15	3.886 ± 0.037
11	5	3	15	3.709 ± 0.026
12	5	5	10	3.934 ± 0.070
13	10	7	10	3.839 ± 0.088
14	5	5	10	3.968 ± 0.062
15	0	5	15	3.822 ± 0.105

ANOVA (Table 7) shows that the model is significant (p = 0.0003). The determination coefficient (R^2) is high (0.9763) indicating that the model could fully elucidate data variability. The adjusted determination coefficient (adjusted R^2) is also high (0.9407), suggesting a high agreement between the experimental and predicted values. The "lack of

fit" was insignificant, indicating the present equation's sufficiency.

The low probability value of linear and quadratic terms of K_2HPO_4 ($p < 0.05$) showed its strong effect on the cell density. On the other hand, the low probability value of interaction terms AB, BC and AC ($p < 0.05$) indicates that an interaction between $MgSO_4$ and K_2HPO_4, K_2HPO_4 and glucose as well as $MgSO_4$ and glucose strongly affects the growth of *T. halophilus* CH6-2. The interaction between K_2HPO_4 and glucose showed the most prominent effect (p =0.0042), suggesting only a minor change in their concentration bringing significant alteration of cell density. The result confirmed the efficacy of RSM over OFAT approaches in investigating the interaction between variables.

Figure 5 represented 2D contour plots of interactions between variables of the model. Figure 5A shows the interaction effect between $MgSO_4$ and K_2HPO_4 concentration on the cell density. $MgSO_4.7H_2O$ concentration from 2.0 g/L to 10 g/L and K_2HPO_4 in the range from 3.58 g/L to 7.58 g/L resulted in an OD600 value above 3.9. On the other hand, glucose concentration from 9 g/L to 15 g/L and $MgSO_4.7H_2O$ from 1.36 g/L to 9.36 g/L also resulted in an OD600 value above 3.9 (Figure 5B).

Furthermore, glucose concentration from 9 g/L to 15 g/L and the K_2HPO_4 concentration ranging from 4.07 g/L to 8.07 g/L resulted in OD600 above 3.9 (Figure 5C). Thus, maximum OD600 was reached at a combination concentration of $MgSO_4.7H_2O$, K_2HPO_4 and glucose of 2.58 g/L, 6.09 g/L and 12.44 g/L respectively.

Experiments performed under optimized conditions proposed by the model resulted in OD600 of 3.996 ± 0.042, which was correlated with the predicted value of 3.999, justifying the validity of the response model. Figure 6 presents the time course of the OD600 value, cell dry weight and log CFU/mL of *T. halophilus* CH6-2 in optimized, K1 and MRS medium under the same cultivation conditions.

Results showed that the OD600 value and cell dry weight reached the maximum at 72 hours of cultivation on all three media. The maximal OD600 on the optimized medium was 3.993, corresponding to the dry biomass weight of 1.450 mg/mL, whereas they were 2.815 and 0.84 mg/mL on the K1 medium, 2.139 and 0.578 mg/mL on the MRS medium respectively (Figure 6A and 6B).

Thus, after optimization, the OD600 and cell dry weight were 1.87 and 2.51 times increased compared with the MRS medium. Similarly to the OD600 and cell dry weight, the viable cell density determined by the plate count method after 72 hours of cultivation which was shown to be a 2.25-fold increase from $2.65x10^9$ CFU/mL to $5.95x10^9$ CFU/mL (Figure 7) which was much higher than in the reported study of $8.4x10^7$ CFU/mL[10].

It should be noted that the viable cell count reached the highest at 24 hours of cultivation time on the optimized medium whereas it was 48 hours of cultivation for the K1 and MRS media. The highest viable cell numbers were 11.56, 10.74 and 9.55 log CFU/mL on the optimized, K1 and MRS media respectively. Thus, the optimized media gave more than two log increase in viable cells compared with the MRS medium; meanwhile, the K1 gave only one log increase. Furthermore, the optimized medium comprises of only four components instead of ten in the MRS medium or eight in the K1 medium, thus simplifying the medium preparation.

Table 7
ANOVA of the quadratic model in BBD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.7305	9	0.0812	27.45	0.0003	Significant
A-$MgSO_4$	0.0771	1	0.0771	26.09	0.0022	
B-K_2HPO_4	0.0325	1	0.0325	10.99	0.0161	
C-glucose	0.2773	1	0.2773	93.79	< 0.0001	
AB	0.0341	1	0.0341	11.52	0.0146	
AC	0.0423	1	0.0423	14.31	0.0092	
BC	0.0042	1	0.0042	1.43	0.2773	
A^2	0.0865	1	0.0865	29.27	0.0016	
B^2	0.0140	1	0.0140	4.75	0.0721	
C^2	0.2332	1	0.2332	78.89	0.0001	
Residual	0.0177	6	0.0030			
Lack of Fit	0.0094	3	0.0031	1.12	0.4636	Not significant
Pure Error	0.0084	3	0.0028			
Cor Total	0.7482	15				

R^2 = 0.9676; adjusted R^2 = 0.9385; predicted R^2 = 0.9837

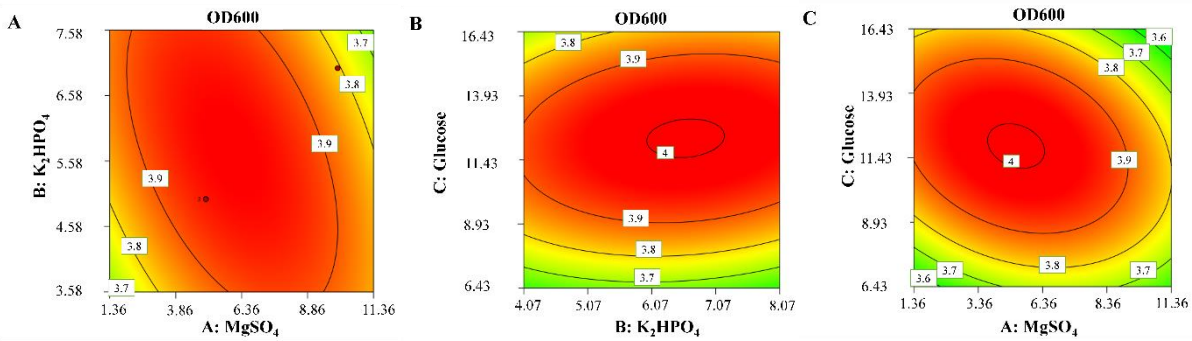

Figure 5: Response surface curve showing the interaction effect between (A) K_2HPO_4 and $MgSO_4$, (B) glucose and K_2HPO_4, (C) $MgSO_4$ and glucose on OD600 of *T. halophilus* CH6-2

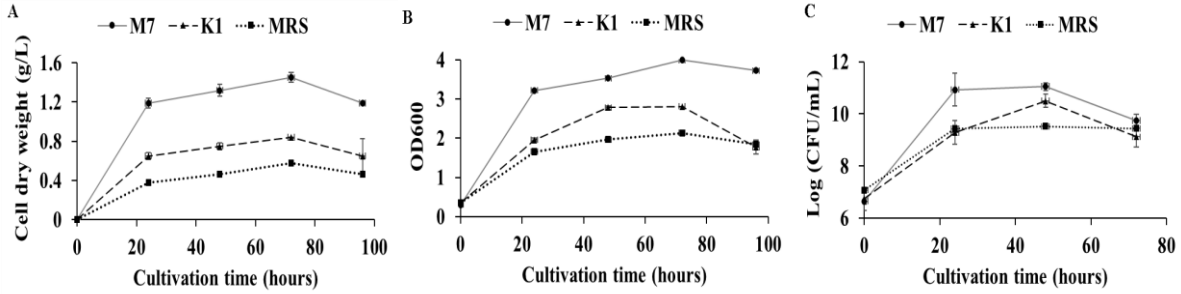

Figure 6: The time course of (A) cell dry weight, (B) the OD600 value and (C) log CFU/mL of *T. halophilus* CH6-2 in the optimized, K1 and MRS medium.

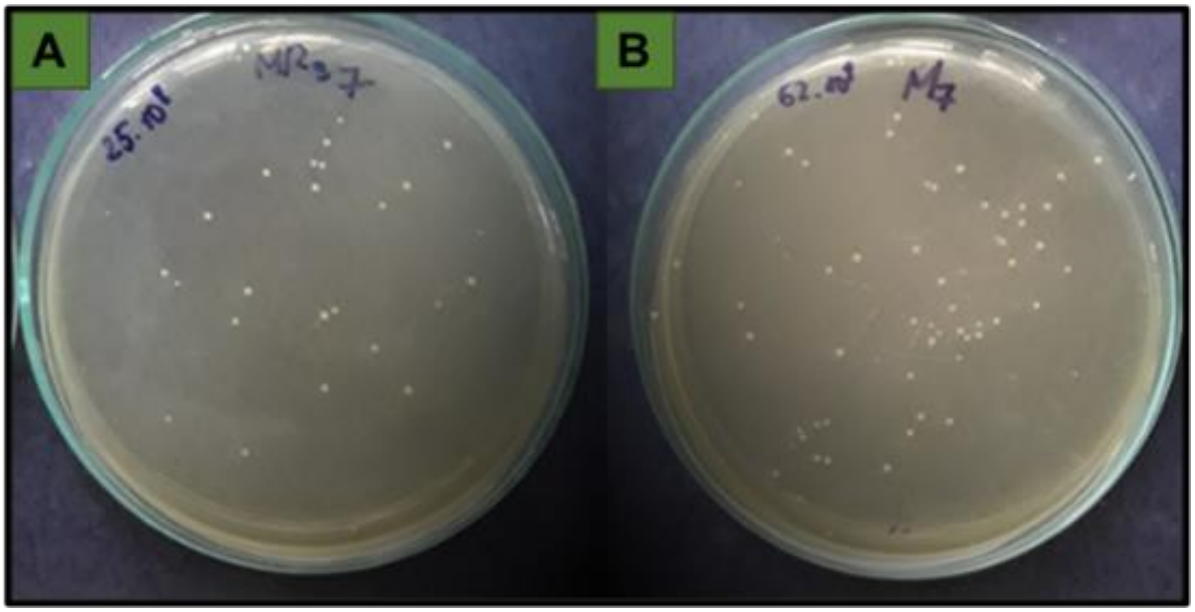

Figure 7: The cell density determination of culture broth by plate count method after 72 hours of cultivation on the (A) MRS and (B) optimized medium M7.

Despite a significantly higher viable cell number, the amount of YE used for the optimized medium was more than two-fold increase compared with the K1 medium, thus limiting its use for industrial scale and requiring further study.

Conclusion

This study is on optimizing medium components for improving the cell density of *T. halophilus*. The medium components consisting of K_2HPO_4, yeast extract, glucose and $MgSO_4$ were shown to have a prominent effect on the cell density of *T. halophilus* CH6-2. Yeast extract showed the most influential effect on *T. halophilus* CH6-2 cell density from four nitrogen sources including yeast extract, tryptone, casamino acid and peptone. The obtained optimized medium in this study comprised of 12.4 g/L glucose, 55 g/L yeast extract, 6.09 g/L K_2HPO_4 and 2.58 g/L $MgSO_4.7H_2O$. The OD600 and cell dry weight of *T. halophilus* CH6-2 in the optimized medium were 1.87 - and 2.51-fold increase, reaching 3.993 and 1.45 mg/mL after 72 hours of cultivation.

In addition, the viable cell counts on the optimized medium reached the highest in 24 hours instead of 48 hours of cultivation. Moreover, a two-log increase of viable cell counts was obtained (11.56 log CFU/mL compared with 9.55 log CFU/mL on MRS). Compared to MRS, the optimized medium comprises of only four components instead of ten, simplifying the medium preparation.

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References

1. Aasen I.M., Møretrø T., Katla T., Axelsson L. and Storrø I., Influence of complex nutrients, temperature and pH on bacteriocin production by Lactobacillus sakei CCUG 42687, *Appl Microbiol Biotechnol*, **53(2)**, 159-166 **(2000)**

2. Calderon M., Loiseau G. and Guyot J.P., Nutritional requirements and simplified cultivation medium to study growth and energetics of a sourdough lactic acid bacterium Lactobacillus fermentum Ogi E1 during heterolactic fermentation of starch, *J Appl Microbiol*, **90(4)**, 508-516 **(2001)**

3. Chun B.H., Han D.M., Kim K.H., Jeong S.E., Park D. and Jeon C.O., Genomic and metabolic features of *Tetragenococcus halophilus* as revealed by pan-genome and transcriptome analyses, *Food Microbiol*, **83**, 36-47 **(2019)**

4. El Zoeiby A., Sanschagrin F. and Levesque R.C., Structure and function of the Mur enzymes: development of novel inhibitors, *Mol Microbiol*, **47(1)**, 1-12 **(2003)**

5. Fukami K., Funatsu Y., Kawasaki K. and Watabe S., Improvement of fish-sauce odor by treatment with bacteria isolated from the fish-sauce mush (moromi) made from frigate mackerel, *J Food Sci*, **69(2)**, 45-49 **(2004)**

6. Hayek S.A., Gyawali R., Aljaloud S.O., Krastanov A. and Ibrahim S.A., Cultivation media for lactic acid bacteria used in dairy products, *J Dairy Res*, **86(4)**, 490-502 **(2019)**

7. He G., Wu C., Huang J. and Zhou R., Acid tolerance response of *Tetragenococcus halophilus*: A combined physiological and proteomic analysis, *Process Biochem*, **51(2)**, 213-219 **(2016)**

8. Hébert E.M., Raya R.R. and de Giori G.S., Nutritional requirements of *Lactobacillus delbrueckii* subsp. lactis in a chemically defined medium, *Current Microbiology*, **49(5)**, 341-345 **(2004)**

9. Jakubovics N.S. and Jenkinson H.F., Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria, *Microbiol*, **147(7)**, 1709-1718 **(2001)**

10. Jeong A.K., Zhuang Y., Venkatesh P., Hyun-Jin K. and Jeong Hwan K., Properties of *Tetragenococcus halophilus* strains isolated from Myeolchi (anchovy)-jeotgal, *Microbiol Biotechnol Lett*, **46(4)**, 313-319 **(2018)**

11. Kim D.H., Kim S.A., Jo Y.M., Seo H., Kim G.Y., Cheon S.W., Yang S.H., Jeon C.O. and Han N.S., Probiotic potential of *Tetragenococcus halophilus* EFEL7002 isolated from Korean soy Meju, *BMC Microbiol*, **22(1)**, 149 **(2022)**

12. Kobayashi T., Kajiwara M., Wahyuni M., Kitakado T., Hamada-Sato N., Imada C. and Watanabe E., Isolation and characterization of halophilic lactic acid bacteria isolated from "terasi" shrimp paste: a traditional fermented seafood product in Indonesia, *The Journal of General and Applied Microbiology*, **49(5)**, 279-286 **(2003)**

13. Kumazawa T., Nishimura A., Asai N. and Adachi T., Isolation of immune-regulatory *Tetragenococcus halophilus* from miso, *PloS One*, **13(12)**, e0208821 **(2018)**

14. Le T.H., Nguyen N.T., Le T.M.C. and Nguyen T.M.T., Characterization of *Tetragenococcus halophilus* from Vietnamese fish sauce, *Vietnam J Sci Technol*, **57(5)**, 544-550 **(2019)**

15. Lee J.H., Heo S., Jeong K., Lee B. and Jeong D.W., Genomic insights into the non-histamine production and proteolytic and lipolytic activities of *Tetragenococcus halophilus* KUD23, *FEMS Microbiol Lett*, **365(1)**, 1-4 **(2017)**

16. Lee Y.M., Kim J.S. and Kim W.J., Optimization for the maximum bacteriocin production of *Lactobacillus brevis* DF01 using response surface methodology, *Food Sci Biotechnol*, **21(3)**, 653-659 **(2012)**

17. Letort C. and Juillard V., Development of a minimal chemically-defined medium for the exponential growth of *Streptococcus thermophilus*, *J Appl Microbiol*, **91(6)**, 1023-1029 **(2001)**

18. Lew L.C., Liong M.T. and Gan C.Y., Growth optimization of *Lactobacillus rhamnosus* FTDC 8313 and the production of putative dermal bioactives in the presence of manganese and magnesium ions, *J Appl Microbiol*, **114(2)**, 526-535 **(2013)**

19. Li J.Y., Zhang L.W., Du M., Han X., Yi H.X., Guo C.F., Zhang Y.C., Luo X., Zhang Y.H., Shan Y.J. and Hou A.J., Effect of tween series on growth and Cis-9, trans-11 conjugated linoleic acid production of *Lactobacillus acidophilus* F0221 in the presence of bile salts, *International Journal of Molecular Sciences*, **12(12)**, 9138-9154 **(2011)**

20. Lim Y.H., Foo H.L., Loh T.C., Mohamad R. and Abdul Rahim R., Rapid evaluation and optimization of medium components governing tryptophan production by pediococcus acidilactici tp-6 isolated from malaysian food via statistical approaches, *Molecules (Basel, Switzerland)*, **25(4)**, 779 **(2020)**

21. MacLeod R.A. and Snell E.E., Some mineral requirements of the lactic acid bacteria, *J Biol Chem*, **170(1)**, 351-365 **(1947)**

22. Nga T.T., Tra D.T. and Ha L.T., Isolation of halophilic lactic bacteria *Tetragenococcus halophilus* from Vietnamese fish sauce, *Vietnam J Sci Technol*, **55(5A)**, 186-194 **(2017)**

23. Oh S., Rheem S., Sim J., Kim S. and Baek Y., Optimizing conditions for the growth of *Lactobacillus casei* YIT 9018 in tryptone-yeast extract-glucose medium by using response surface methodology, *Applied and Environmental Microbiology*, **61(11)**, 3809-3814 **(1995)**

24. Ohata E., Yoshida S., Masuda T., Kitagawa M., Nakazawa T., Yamazaki K. and Yasui H., *Tetragenococcus halophilus* MN45 ameliorates development of atopic dermatitis in atopic dermatitis modelNC/Nga mice, *Food Sci Technol Res*, **17(6)**, 537-544 **(2011)**

25. Papagianni M., Metabolic engineering of lactic acid bacteria for the production of industrially important compounds, *Comput Struct Biotechnol J*, **3(4)**, e201210003 **(2012)**

26. Raccach M., Manganese and Lactic Acid Bacteria, *Journal of Food Protection*, **48(10)**, 895-898 **(1985)**

27. Roling W. and van Verseveld H.W., Characterization of *Tetragenococcus halophila* populations in Indonesian soy mash (Kecap) fermentation, *Applied and Environmental Microbiology*, **62(4)**, 1203-1207 **(1996)**

28. Saum S.H. and Müller V., Salinity-dependent switching of osmolyte strategies in a moderately halophilic bacterium: glutamate induces proline biosynthesis in *Halobacillus halophilus*, *J Bacteriol*, **189(19)**, 6968-6975 **(2007)**

29. Thongthai C., McGenity T.J., Suntinanalert P. and Grant W.D., Isolation and characterization of an extremely halophilic archaeobacterium from traditionally fermented Thai fish sauce (nam pla), *Lett Appl Microbiol*, **14(3)**, 111-114 **(1992)**

30. Udomsil N., Chen S., Rodtong S. and Yongsawatdigul J., Improvement of fish sauce quality by combined inoculation of *Tetragenococcus halophilus* MS33 and *Virgibacillus* sp. SK37, *Food Control*, **73**, 930-938 **(2017)**

31. Udomsil N., Rodtong S., Choi Y.J., Hua Y. and Yongsawatdigul J., Use of *Tetragenococcus halophilus* as a starter culture for flavor improvement in fish sauce fermentation, *J Agric Food Chem*, **59(15)**, 8401-8408 **(2011)**

32. Udomsil N., Rodtong S., Tanasupawat S. and Yongsawatdigul J., Proteinase-producing halophilic lactic acid bacteria isolated from fish sauce fermentation and their ability to produce volatile compounds, *Int J Food Microbiol*, **141(3)**, 186-194 **(2010)**

33. Yao S., Zhou R., Jin Y., Huang J. and Wu C., Effect of co-culture with *Tetragenococcus halophilus* on the physiological characterization and transcription profiling of *Zygosaccharomyces rouxii*, *Food Res Int*, **121**, 348-358 **(2019)**

34. Yongsawatdigul J., Rodtong S. and Raksakulthai N., Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures, *J Food Sci*, **72(9)**, M382-M390 **(2007)**

35. Zacharof M.P. and Lovitt R.W., Partially chemically defined liquid medium development for intensive propagation of industrial fermentation *Lactobacilli* strains, *Ann Microbiol*, **63(4)**, 1235-1245 **(2013)**

36. Zhang L., Zhang L. and Xu Y., Effects of *Tetragenococcus halophilus* and *Candida versatilis* on the production of aroma-active and umami-taste compounds during soy sauce fermentation, *J Sci Food Agric*, **100(6)**, 2782-2790 **(2020)**.

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