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# Optimization of Time and pH Condition for Cell Autoaggregation of *Bifidobacterium pseudocatenulatum* KAKii Using FCCD-Response Surface Methodology

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**Prior to adherence to Caco-2 cell, the strain has to be screened for their hydrophobicity and further autoagregation ability need to be studied. The aim of this study were to screen for hydrophobicity activity of *Bifidobacterium pseudocatenulatum KAKii* and to optimize the best pH and temperature conditions for better autoagregation using FCCD-response surface methodology approached. In this study, cell surface hydrophobicity test of *Bifidobacterium pseudocatenulatum KAKii* was carried out based on microbial adhesion to hydrocarbons (MATH) technique using three hydrocarbon solvents (hexadecane, xylene and toluene). Based on MATH test, the percentage of hydrophobicity (H %) obtained revealed that this local strain was able to manifest a good degree of hydrophobicity however, slightly lower than comparative strain. Further optimization of autoaggregation activity using FCCD-RSM was revealed that the optimum pH and time for strong autoaggregation activity of local probiotic *B. pseudocatenulatum* KAKii was between pH 6.5 and 7.0 at 4h of incubation time, respectively. As a conclusion, this study indicated that cell surface hydrophobicity and autoaggregation ability can be used for preliminary selection and identification of novel probiotic bacteria for further exploitation in food, pharmaceutical and medical industry.**

**Key word:** Bifidobacteria, Hydrophobicity, Autoaggregation, Optimization of time and ph, FCCD-RSM.

The used of food supplements containing probiotic bacteria has been recently considered to protect the human gut from severe microbial shifting that may lead to harmful effect to the host. This living microorganism is able to boost health benefits beyond inherent basic nutrition when administered in appropriate amount[1]. It assists in altering the intestinal bacteria equilibrium, prevent the colonization of pathogens, assist in food digestion, increase the immune response and promote body resistance towards infection[2]. Once these beneficial bacteria adhere on the intestinal surface mucus layer, the immune system will be alarmed causing removal of pathogens and lead to many favorable outcome for human health, especially in cope with disease such as intestinal infection, inflammatory bowel disease and others[3]. The most common strains that been used in probiotic bacteria and can be found in many functional foods and dietary supplements is *Bifidobacterium*[4]. Bifidobacteria are gram positive rods bacteria with characteristic of nonsporulated,

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nonmotile, catalase negative bacteria and required anaerobic or micraerophilic condition to grow[5]. *Bifidobacterium pseudocatenulatum* KAKii, isolated from breast-fed infant feces, has been molecular identified and characterized as a potential probiotic with many beneficial health effects.

In order to achieve effective health benefits of probiotic to the most, adhesion to the intestinal mucosa with extended transit time is important[6]. Hydrophobicity and cell autoaggregation are two parameters that commonly studied for screening potential probiotic strain[19]. The hydrophobic properties of bacterial surfaces assist in the adhesion of bacteria as the bacterial surface properties are commonly associated with attachment to a variety of substrata. This characteristic important in prolong the bacteria survival in the intestines and finally allow it to exerts healthful effect longer[7]. Previous study has reported that the adherence ability of *Lactobacillus* and *Bifidobacteria* strains were strongly associated with cell autoaggregation[8]. This trait is relatively vital in certain niches especially in the human gut ecosystem and it involved cell to cell adherence either cell autoaggregation (between same bacteria strain) or co-aggregation (between genetically different strains)[9]. Certain probiotic bacteria will improve their adherence ability by forming a barrier via autoaggregation or by direct co-aggregate with pathogen in order to obstruct adherence of pathogenic bacteria to intestinal mucosa[10,11,12]. Previous studies have shown the association between the cell autoaggregation and adhesion ability in *B. bifidum*[13] and *B.suis*[14] and the relationship between hydrophobicity and adhesion capability in *B. longum*[8] and some *lactobacilli*[15]. However, the efficient cell autoaggregation can be obtained under good condition such as pH and time of exposure.

Commonly, optimum condition of adhesion was carried out using conventional method[16]. Thus study on the optimum condition for cell autoaggregation activity of probiotic using Response Surface Methodology (RSM) is not yet reported elsewhere. RSM is a combination of statistical and mathematical techniques useful for developing, improving and optimising the process. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions[17,18]. The most commonly used response surface design is central composite design. This design is such that it allows the estimation of all the regression parameters required to fit a second order model to a given response.

In this study, we determine the hydrophobicity ability as preliminary step and then optimize the autoaggregation condition (time and pH) of *Bifidobacterium pseudocatenulatum* KAKii using Full Central Composite Design-Response Surface Methodology (FCCD-RSM) approach.

## MATERIALS AND METHODS

### Bacterial strains and growth condition

Four Bifidobacteria strains *Bifidobacterium pseudocatenulatum* KAKii (wild type strain, isolated from infant feces) *B. Pseudocatenulatum* **ATCC 27919**, *B. animalis* 27673, *L. plantarum* L4(commercial strains) were cultivated to early stationary phase in de Man, Rogasa and Sharpe (MRS: Merck, Germany) at 37°C for 24 to 48 hour under anaerobic conditions and maintained in 70% (v/v) glycerol stock until further used. All bacterial cultures used in this study was activated by sub-culturing twice in fresh MRS broth prior to cell surface hydrophobicity and autoaggregation test.

### Cell surface hydrophobicity assay

Cell surface hydrophobicity of isolates was determined according to MATH method describe by[19] with some modification. Three difference hydrocarbon sources were used in

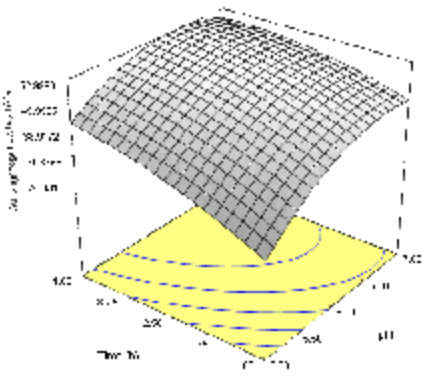

**Fig. 1.** FCCD-RSM for cell autoaggregation ability of *B. pseudocatenulatum* KAKii (A%) from the adjusted quadratic mathematical model.

microbial adhesion to hydrocarbons (MATH) method in order to to identify the consistency of hydrocarbon activity of the strain used. Initially, strains were grown in MRS with L-cyctein broth for 16 to 18 hour at 37°C. Then the cell suspension was centrifuged (2000x *g*, 15 min, 4°C), washed twice in PBS buffer (pH 7.0) and finally suspended in the same buffer. The initial absorbance ($A_0$) at 600 nm of the suspension was adjusted to 0.8 ± 0.01. Hydrophobicity activity was accomplished by the addition of 1.0 mL of solvent used into 5.0 mL of cell suspension in PBS buffer (pH 7.0) with mixing for 2 minutes using vortex mixer. The tubes was incubated for 1 hour at 37°C to allow the phase separation. The lower aqueous phase was carefully removed and absorbance ($A_1$) was recorded at 600 nm. Cell surface hydrophobicity in terms of percent (H%) was calculated using:

$$H\% = (1- A_1/A0) \times 100\% \quad ...(1)$$

**Autoaggregation assay**

Autoaggregation assays were performed according to[8] with some modification. Bacteria were grown in MRS with L-cyctein broth for 16 to 18 hour at 37°C. The cells were harvested by centrifugation (2000x *g*, 15 min, 4°C), washed twice in PBS buffer (pH 7.0) and finally suspended in the same buffer. The effect of pH on autoaggregation was determined at pH 5.0, 6.0 and 7.0 by suspending the bacteria in PBS with the respective pH. The initial absorbance ($A_0$) of the suspension was adjusted to 0.8 ± 0.01 at 600 nm. Cell suspensions (4 mL) were mixed by vortexing for 10 second and autoaggregation was determined during sampling at 1, 2.5 and 4 h of incubation at room temperature. Every sampling hour, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the absorbance ($A_1$) was measured at 600 nm. Auto aggregation percentage will be expressed as A% using the following formula:

$$A\% = ( 1-(A_1/A_0) ) \times 100\% \quad ...(2)$$

**Experimental design and statistical analyses**

Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, version 6.0.6) was used in this study to design the experiment. The optimization of autoaggregation was analysed using Central Composite Design- Response Surface Methodology (CCD-RSM) which is useful in giving rational amount of information for testing the goodness of fit and does not involve large number of design points and thus minimize the overall cost associated with the experiments[20]. In this design, the autoaggeragation (A%) was used as a response in this study and two factors namely pH and time of exposure (hours) with 5 replicates were used at the centre points (Table 1). There were three coded factor levels, -1, 0 and +1 (where -1 corresponded to the low level of each factor, 0 to the middle level, and +1 to the high level).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \varepsilon_i \quad ...(3)$$

The responses (y), was fitted with quadratic polynomial model and subsequently produce the response surface which expressed as Equation 3, where *y* and *x* represent coded independent factors. Meanwhile $\beta_0$ , $\beta_1$ , $\beta_2$ , and $\beta_{22}$ are constant coefficients and is the random error. The center points were repeated five times in order to evaluate the curvature and the experiment replication facilitated the pure error estimation. Followed by that, the significant lack-of-fit of the models was predicted. Twenty six experiments were carried out and in this study (Table 3). Statistical analyses for the data obtained were performed using MINITAB statistical software (MINITAB 2006). In order to assess the adequacy of the model (Equation 3), verification was performed by using an optimized level of pH condition and time of exposure will be tested under the same experiments condition to verify the predicted value of the statistical model.

## RESULTS AND DISCUSSION

**Cell surface hydrophobicity assay**

Based on the result obtained, the highest hydrophobicity percentage recorded among the tested probiotic bacteria was by *B. pseudocatenulatum* **ATCC 27919** (33.62% ±0.29) when toluene was used as hydrocarbon source (Table 2). *B. pseudocatenulatum* **ATCC 27919** was

**Table 1.** The factors and response used in the optimization of cell autoaggregation usinf FCCD-RSM

| Factor | Coded factor | | | Response |
|---|---|---|---|---|
| | -1 | 0 | +1 | |
| pH | 5 | 6 | 7 | Cell auto |
| Time (h) | 1 | 2.5 | 4 | aggregation (A%) |

maintained the highest percentage of hydrophobicity activity among all the strains in all hydrocarbon sources. Meanwhile *B. pseudocatenulatum* KAKii showed a promising hydrophobicity percentage (27.88±0.63) compare to *B. animalis* 27673 and *L. plantarum* L4 when tested with xylene. It can be suggested that *B. pseudocatenulatum* KAK**ii** strain was able to manifest a good degree of hydrophobicity, although slightly lower than comparative strain *B. pseudocatenulatum* 27616. This indicated that *B. pseudocatenulatum* KAK**ii** can be a potential probiotic strain as this trait have been previously describe by[8] to have relationship with adhesion ability of probiotic strain. Hydrophobicity play a significant role in bacterial adhesion as microbial behaviour is controlled by physiochemical properties of the cell wall[21]. The adhesion ability to host intestinal epithelial and mucus was very crucial for the transient probiotic to colonize[22] and this characteristic important in prolong the bacteria survival in the intestines and finally allow it to exerts healthful effect longer[7].

**Optimization of cell autoaggregation test**

The experimental design and result for the optimization of pH and time for cell autoaggregation test of *B. pseudocatenulatum* KAKii using Full Central Composite Design-RSM

**Table 2.** Cell surface hydrophocity (H%) of *Bifidobacterium pseudocatenulatum* KAKI and comparative strain to xylene, hexadecane and toluene

| Bacteria | Hydrophobicity (H%) | | |
|---|---|---|---|
| | Xylene | Hexadecane | Toluene |
| *B. pseudocatenulatum* KAKii | 27.88±0.48[a] | 22.80±0.25[a] | 25.40±0.58[a] |
| *B. pseudocatenulatum* ATCC 27919 | 31.90±0.54[b] | 29.75±0.15[b] | 33.62±0.76[b] |
| *B. animalis* 27673 | 25.47±0.47[c] | 22.84±0.41[a] | 23.71±0.64[a] |
| *L. plantarum* L4 | 24.31±0.40[d] | 21.57±0.76[a] | 23.57±0.34[a] |

Mean (± standard deviation) of result from three separate experiments
* Value in the same column with different letters were significantly different (P<0.01)

**Table 3.** Experimental design and results using Central Composite Design-RSM for autoaggregation test of *Bifidobacterium pseudocatenulatum* KAKii

| Run | Factors | | | | Response |
|---|---|---|---|---|---|
| | pH (A) | | Time (B) | | Auto |
| | Coded value | Actual value | Coded value | Actual value | aggregation % (A%) |
| 1 | 0 | 6.00 | 0 | 2.50 | 50.05 |
| 2 | +1 | 7.00 | 0 | 2.50 | 46.99 |
| 3 | -1 | 5.00 | +1 | 4.00 | 42.20 |
| 4 | -1 | 5.00 | 0 | 2.50 | 35.99 |
| 5 | +1 | 7.00 | +1 | 4.00 | 52.32 |
| 6 | 0 | 6.00 | 0 | 2.50 | 45.79 |
| 7 | 0 | 6.00 | +1 | 4.00 | 49.42 |
| 8 | 0 | 6.00 | -1 | 1.00 | 41.47 |
| 9 | -1 | 5.00 | -1 | 1.00 | 23.55 |
| 10 | +1 | 7.00 | -1 | 1.00 | 42.44 |
| 11 | 0 | 6.00 | 0 | 2.50 | 49.69 |
| 12 | 0 | 6.00 | 0 | 2.50 | 51.26 |
| 13 | 0 | 6.00 | 0 | 2.50 | 47.46 |

Values represent average of twice replication

(FCCF-RSM) are shown in Table 3. Based on the result, it shows that the range of cell autoaggregation obtained was from 23.55% to 52.32%. Result has revealed that the highest autoaggregation (52.32%) obtained when the cell autoaggregation condition were set at pH 7 with 4 hour of incubation time. Meanwhile the lowest value (23.55%) was recorded at pH 5 with l hour of incubation time.

Further regression analysis on the experiment data was carried out and cell autoaggregation activity of *Bifidobacterium pseudocatenulatum* KAKii can be described. From the equation (Eq. 4), it can be suggested that both factors (pH and time of incubation) were presented significance to the response ($P < 0.01$). The increment of 6.67and 6.08 for pH and time of incubation unit respectively, would gave a significance impact to the cell autoaggregation activity.

$$y = 48.57 + 6.67A + 6.08B - 6.37A^2 - 2.42B^2 - 2.19AB \quad ...(4)$$

The quadratic model ANOVA analysis as shown in Table 4 has revealed that the model used was significance ($P < 0.01$) and can be acceptable. The $R^2$ suggests the sample variation of 94.63% for cell autoaggregation (y) and this shows that only about 5.37% of total variation is not explained by the model. Thus it indicates good agreement between the experimental and predicted values for the response. The lack of fit indicates the inability of the model to represent data in the experimental domain at points, which are not found in the regression[23]. The lack of fit value of 1.40 denotes the model was insignificant ($P > 0.05$), suggesting that the model fitted well to the data in the experimental region.

**Table 4.** ANOVA and regression analysis for the response of autoaggregation test (y): optimization of pH (A) and time (B).

| Source of variation | Sum of squares or coefficient estimate | DF[a] | Mean square or standard error | F value | *P* value |
|---|---|---|---|---|---|
| Model[b] | 695.94 | 5 | 139.19 | 24.66 | 0.0003 |
| Residual | 39.51 | 7 | 5.64 | | |
| Lack of fit | 20.27 | 3 | 6.76 | 1.40 | 0.3644 |
| Pure error | 19.25 | 4 | 4.81 | | |
| Total | 735.46 | 12 | | | |
| Factor | | | | | |
| Intercept | 48.57 | 1 | 0.99 | | |
| A | 6.67 | 1 | 0.97 | 47.26 | 0.0002[d] |
| B | 6.08 | 1 | 0.97 | 39.29 | 0.0004[d] |
| $A^2$ | -6.37 | 1 | 1.43 | 19.88 | 0.0029[d] |
| $B^2$ | -2.42 | 1 | 1.43 | 2.86 | 0.1345 |
| AB | -2.19 | 1 | 1.19 | 3.41 | 0.1075 |

[a]DF : degree of freedom, [b] $R^2$= 94.63%, [c]A,pH ,B, time(h), [d]Significant at á=0.01.

**Table 5.** The validation of the optimized condition *for cell autoaggregation of B. pseudocatenulatum* KAKii

| Bacterial strain | *B. pseudocatenulatum* KAKii |
|---|---|
| Predicted value (A%) | 52.90[a] |
| Experimental value (A%) | 51.22[a] |
| Error (%) | 3.18 |

* Value in the same column with different letters were significantly different (P<0.01)

Figure 1 shows the three-dimensional response surface model reveal that pH and time (h) have major effect toward the cell autoaggregation ability of *B. pseudocatenulatum* KAKii. The response surface produced was in convex form with a peak maximum for the autoaggregation test which can be used to find an optimal value for both factors. The corresponding value of pH and time were fixed as "in range" form whereas the autoaggregation percent (A %) was fixed at its maximum. It has been observed that as

the time increased from pH 6.5 to 7, the autoaggregation ability of the bacteria reached to its maximum value. Previous study shows that the autoaggregation of *Lactobacillus* strains exhibited high autoaggregation ability after 5 hour of incubation at pH 7 showing that increased in incubation time have improved autoaggregation ability (Tuo *et al*., 2013)[24].

Cell adhesion is a multistep process involving contact of the bacteria cell membrane and interacting surface. Previous study has reported that the adherence ability of *Lactobacillus* and *Bifidobacteria* strains were strongly associated with cell autoaggregation[8]. Probiotic strain with the ability to autoaggregate and hydrophobic cell surface could be adhering better to the intestinal cells[12].

**Validation of the optimized condition for cell autoaggregation of *Bifidobacterium pseudocatenulatum* KAKii**

The combination of pH 6.3 (A), and time at 4h (B), based on the optimum point obtained from response surface methodology was predicted to yield 52.97% of cell autoaggregation (y). At this point, the autoaggregation percent (A %) was at its maximum value (Table 5). This prediction was confirmed using validation experiment in which maximum of 51.22% was successfully acquired from replication experiments. Although the experimental value was lower compare to predicted value, no statistical difference (P> 0.01) was obtained showing that experimental value fitted well to the predicted results.

## CONCLUSION

Results from this study have revealed that strain *B. pseudocatenulatum* KAKii strain can be explored as potential probiotic strain as it able to manifest a good degree of hydrophobicity. In addition, optimization of pH and time condition for cell autoaggregation using response surface methodology (RSM) greatly improved the autoaggregative percentage of this strain. The quadratic model was found sufficient for the optimization of both conditions for cell autoaggregation. The optimal condition with pH 6.3, and incubation time at 4h gave an optimum *B. pseudocatenulatum* KAKii strain autoaggregation ability. Our findings also indicate that the cell surface hydrophobicity along with the capability to autoaggregate, can be applied for preliminary screening to categorize potentially adherent bacteria appropriate for commercial proposes.

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