



## Research article

# Response surface methodology based optimization for degradation of algin in *Laminaria japonica* feedstuff via fermentation by *Bacillus* in *Apostichopus japonicas* farming

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## ABSTRACT

**Background:** The alga *Laminaria japonica* is the most economically important brown seaweed cultured in China, which is used as food and aquatic animal feedstuff. However, the use of *L. japonica* as a feedstuff in *Apostichopus japonicas* farming is not ideal because *A. japonicas* does not produce enough enzyme activity for degrading the large amount of algin present in *L. japonica*. In this study, semi solid fermentation of the *L. japonica* feedstuff employing a *Bacillus* strain as the microbe was used to as a mean to degrade the algin content in *L. japonica* feedstuff.

**Results:** The *Bacillus* strain, *Bacillus amyloliquefaciens* WB1, was isolated by virtue of its ability to utilize sodium alginate as the sole carbon source. Eight factors affecting growth and algin-degrading capacity of WB1 were investigated. The results of Plackett–Burman design indicated that fermentation time, beef extract, and solvent to solid ratio were the significant parameters. Furthermore, the mutual interaction between the solvent to solid ratio and beef extract concentration was more significant than the other pairs of parameters on algin degradation. Optimal values obtained from Central-Composite Design were 113.94 h for fermentation time, 0.3% (w/v) beef extract and 44.87 (v/w) ratio of solvent to feedstuff. Under optimal conditions, 56.88% of the algin was degraded when a 50-fold scale-up fermentation was carried out, using a 5-L fermenter.

**Conclusions:** This study provides an alternative and economical way to reduce the algin content in *L. japonica* through degradation by WB1, making it a promising potential source of feed for cultured *L. japonica*.

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## 1. Introduction

*Apostichopus japonicas*, which is commonly known as sea cucumber, is a widely cultivated echinoderm species [1] and has long been used as an important fishery resource in China, Russia, Japan, and Korea [2]. In recent years, demands for sea cucumber in the world market have increased rapidly. In a semi-intensive culture, sea cucumbers are fed formulated diets, and powdered brown macroalgae are a necessary component in these diets. *Sargassum thunbergii* and *Sargassum polycystum* are the two traditionally preferred macroalgae. However, rapid growth in the sea cucumber-aquaculture industry has resulted in the overexploitation of these two macroalgal species, which have now become very expensive. Therefore, finding a cheaper and optimal

substitute for *S. thunbergii* and *S. polycystum* is crucial in order to meet the demand of sea cucumber-aquaculture industry in the world [3].

The brown alga *Laminaria japonica*, with its extensive sources and low price in China [4], is widely used in the aquaculture of abalone [5] and sea urchin [6,7]. *L. japonica* contains a high content of algin, and both abalone and urchin have high specific enzyme activities for degrading the algin, which is an important carbon and energy source [8,9]. However, in sea cucumber farming, the use of *L. japonica* as feedstuff presents the problem of high viscosity and stodginess because of sea cucumber has a rather low level of specific enzymes for degrading the large amount of algin with complex structures [10].

Algin is a gelling polysaccharide found in great abundance, because it is part of the cell wall and intracellular material in the *Laminaria* spp. The polysaccharide is composed of (1 → 4)-β-D-mannuronic acid (M) (as well as its C-5 epimer), α-L-guluronic acid (G) units in the form of homopolymeric (MM- or GG-blocks), and heteropolymeric sequences (MG- or GM-blocks) [11,12]. A variety of marine and soil bacteria have been isolated that have the ability to degrade the align of *L. japonica* [8]. Therefore, a feasible and economical strategy to resolve

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the problem associated with the use of *L. japonica* as a feed for sea cucumber is to first convert (via microbial degradation of the feedstuff) the algin into a more usable form for the sea cucumber.

Microbial degradation of algin in *L. japonica* can be carried out via fermentation, but a process optimization is essential to the successful degradation. In the classical “one factor at a time” approach, the fermentation process is optimized by changing one factor at a time while keeping the others at a constant level. This approach is extremely time-consuming and expensive if a large number of factors need to be tested [13]. It also neglects the interaction between factors and does not guarantee the attainment of optimal point, possibly leading to the wrong conclusion [14,15]. Limitation of the single-factor optimization can be eliminated by employing a response surface methodology (RSM), which has the advantage of studying the combined effects of all the factors in a biotechnological process [16], and therefore, allowing the optimal condition of a multivariable system may be determined while saving time and labor by minimizing the number of required experiments [16,17,18,19].

The objective of the present study was to evaluate the feasibility of increasing the digestion and utilization of *L. japonica* feedstuff for sea cucumber through pretreatment by fermentation using a beneficial strain of bacteria. The conditions of the fermentation process were also optimized to allow for maximal degradation of algin.

## 2. Materials and methods

### 2.1. Microorganisms

The algin-degrading bacterial strain was isolated from the sea mud of Xiaoyaowan Bay (Dalian, China) using medium containing sodium alginate as the sole carbon source [20]. The risk of disease associated with the isolated algin-degrading bacterium was tested by injecting the sea cucumber with 100 µL of a high dose of the bacterial suspension (10<sup>9</sup> CFU mL<sup>-1</sup>) at the site of the coelom. Morphological, physiological, and biochemical characterizations of the strain was performed according to Bergy's Manual of Systematic Bacteriology. Genetic characterization was achieved by 16S rDNA sequence analysis as described by Merrifield et al. [20,21]. Analysis of 16S rDNA was performed through NCBI BLAST.

### 2.2. Preparation of seed culture

Bacterial cells from a -80°C frozen stock were first cultured in marine agar (2216E) containing alginate, and the culture was then used as the inoculum for the degradation of *L. japonica* feedstuff under semi-sold fermentation. For preparation of the seed culture, bacterial cells from a fresh 2216E plate were inoculated into a 250 mL flask containing 50 mL liquid 2216E medium. The flask was incubated at 28°C for 24 h with agitation at 150 rpm. The final concentration of the seed culture was adjusted to 1 × 10<sup>9</sup> CFU mL<sup>-1</sup>. The viability of the cells was evaluated by the plate counting method. The experiment was performed in triplicate.

**Table 1**  
Experimental levels of screened variables in Plackett–Burman design solvent.

Symbol code	Variables	Low level (-)	High level (+)
X <sub>1</sub>	Fermentation period (h)	72	96
X <sub>2</sub>	NaCl (% w/v)	2.5	3.0
X <sub>3</sub>	Maltose (% w/v)	0.15	0.25
X <sub>4</sub>	Beef extract (% w/v)	0.2	0.3
X <sub>5</sub>	Initial pH	6.5	8.0
X <sub>6</sub>	Ratio of solvent to material (v/w)	30	40
X <sub>7</sub>	Inoculum size (% v/v)	5	10
X <sub>8</sub>	Temperature (°C)	25	30
X <sub>9</sub> , X <sub>10</sub> , X <sub>11</sub>	virtual factors	-	+

### 2.3. Semi solid substrate fermentation for algin degradation

Solid powdered *L. japonica* feedstuff was filtered through a 200-mesh sieve. The sieved powder was added to a 250 mL flask containing water and an additional carbon source as well as a nitrogen source and other nutrients and minerals (composition in g L<sup>-1</sup>: KH<sub>2</sub>PO<sub>4</sub>, 1.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; KCl, 0.5; L-phenylalanine, 2 × 10<sup>-3</sup>; MnSO<sub>4</sub>, 5 × 10<sup>-3</sup>; CuSO<sub>4</sub>, 0.16 × 10<sup>-3</sup>; FeSO<sub>4</sub>, 0.15 × 10<sup>-3</sup>). The initial pH of the mixture was adjusted to 7.0, and the mixture was then autoclaved at 120°C for 20 min. The cooled semi-solid medium was inoculated with the seed culture. This semi-solid substrate fermentation was then carried out under the conditions specified by the experimental design described below.

### 2.4. Experimental design for algin degradation

#### 2.4.1. Screening of significant parameters affecting algin degradation

Additional carbon source (glucose, sucrose, starch, maltose or glycerol) and nitrogen source (tryptone, beef extract, NH<sub>4</sub>NO<sub>3</sub>, carbamide or (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) were added one to the *L. japonica* medium at a concentration of 0.2% (w/v) each. Various parameters of the medium (initial pH value, NaCl content and ratio of solvent to material) and fermentation conditions (fermentation period, inoculum size and temperature) were tested, and the significant parameters were identified by Plackett–Burman design. Each parameter was examined at a high level (coded +1) and a low level (coded -1) as described in Table 1, which were obtained from the pre-experiment, and the screening was carried out in 12 experimental runs of batch fermentation process without feeding strategy.

#### 2.4.2. Optimization of significant parameters for algin degradation

The three significant parameters (X<sub>1</sub>: fermentation period; X<sub>4</sub>: beef extract; X<sub>6</sub>: ratio of solvent to material) obtained from the screening process achieved with Plackett–Burman design were optimized using a Central Each significant parameter was assessed at five levels (-2, -1, 0, +1, +2), with five replicates at the center points, as shown in Table 2. A total of 20 experiments were conducted, and the results were used to fit the polynomial model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_4 X_4 + \beta_6 X_6 + \beta_{11} X_1^2 + \beta_{44} X_4^2 + \beta_{66} X_6^2 + \beta_{14} X_1 X_4 + \beta_{16} X_1 X_6 + \beta_{46} X_4 X_6 \quad [\text{Equation 1}]$$

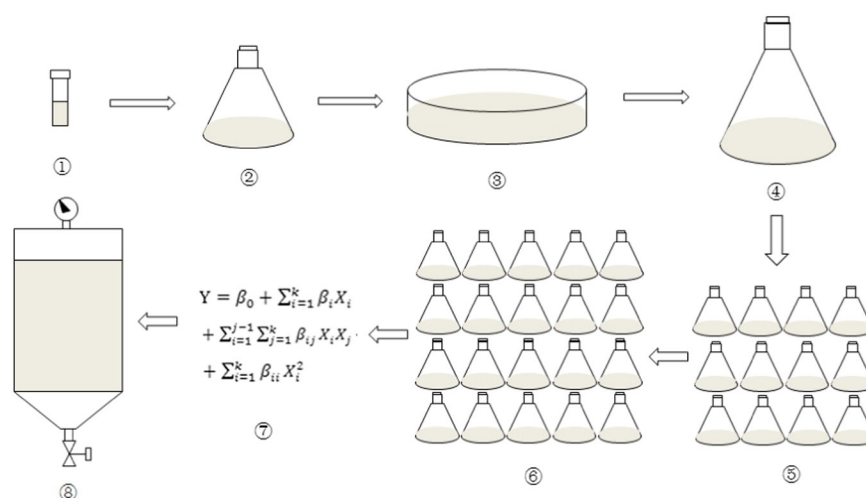
where Y is the predicted response, X<sub>1</sub>, X<sub>4</sub> and X<sub>6</sub> are the independent variables, β<sub>0</sub> is the intercept term, β<sub>1</sub>, β<sub>4</sub> and β<sub>6</sub> are the linear coefficients, β<sub>11</sub>, β<sub>44</sub> and β<sub>66</sub> are the quadratic coefficients, and β<sub>14</sub>, β<sub>16</sub> and β<sub>46</sub> are the interactive coefficient estimates. The optimal levels of each variable for maximal algin degradation were obtained by solving the regression equation and analyzing the response surface and contour plots.

### 2.5. Experimental verification

In order to verify the accuracy of the model, scale-up experiments were carried out using the optimized medium composition and culture conditions in a 5-L fermenter (Biotech-3BG, Shanghai Baoxin Bio-Engineering Equipment CO., LTD., China). The batch fermentation was conducted at a stirring speed of 150 rpm, an aeration rate of

**Table 2**  
Experimental range of three variables at five levels in Central-Composite design.

Variables	Coded levels				
	-2	-1	0	1	2
X <sub>1</sub> : Fermentation period (h)	48	72	96	120	144
X <sub>4</sub> : Beef extract (% w/v)	20	30	40	50	60
X <sub>6</sub> : Ratio of solvent to material (v/w)	0.1	0.2	0.3	0.4	0.5



**Fig. 1.** The schematic of the experimental. (1) WB1 strain stored at  $-80^{\circ}\text{C}$ ; (2) revive WB1 in marine medium (2216E) with alginate at  $28^{\circ}\text{C}$  for 24 h 150 rpm; (3) spread on marine agar (2216E) with alginate at  $28^{\circ}\text{C}$  for 24 h; (4) incubate in 2216E medium with agitation as the same condition described in (2), then adjust the concentration to  $1 \times 10^9$  CFU  $\text{mL}^{-1}$ ; (5) Plackett–Burman design (based on the parameters in Table 2 and Table 4); (6) Central-Composite design (based on Table 3 and Table 6); (7) regression equation; (8) scale-up experiments verification.

0.4 vvm and a tank pressure of 0.03 Mpa without feeding strategy. Changes in algin content during the progress of fermentation were determined by the calcium acetate method [22]. The progress of the optimizing model was carried out as described in Fig. 1.

## 2.6. Scanning electron microscopy

To observe the morphology of the algin-degrading bacterium in the presence of *L. japonica* feedstuff, bacterial cells harvested at the logarithmic phase of the growth curve of the fermentation were washed twice in physiological saline and then subjected to scanning electron microscopy analysis. The cell sample was first fixed by immersion in 2.5% (v/v) glutaraldehyde for 12 h at  $4^{\circ}\text{C}$ , and then washed with phosphate buffer (0.1 M, pH 7.0) for 1 h at room

temperature. It was further dehydrated in a graded 20–100% (v/v) ethanol series and was dried under vacuum at room temperature. The dried sample was coated on a double-sided conducting adhesive tape pasted onto a metallic stub and subjected to gold covering ( $\sim 100^{\circ}\text{A}$ ) [23]. Finally, it was examined under a scanning electron microscope (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) at 20 kV.

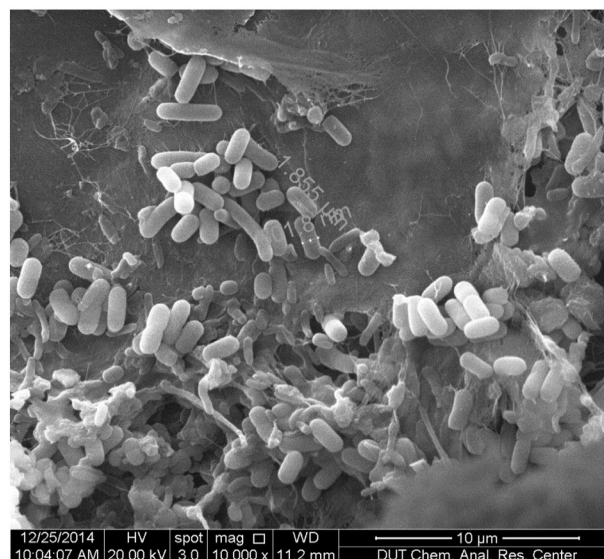
## 2.7. Statistical analysis

All experiments were carried out in triplicate. The experimental design, statistical analysis of the data and building of the quadratic model were performed with the statistical software package “Design Expert 8.0.5b”. Isoresponse contour and three-dimensional surface plots were generated to describe the interaction between parameters and to find optimal values for each parameter. The results were expressed as means  $\pm$  SEs.

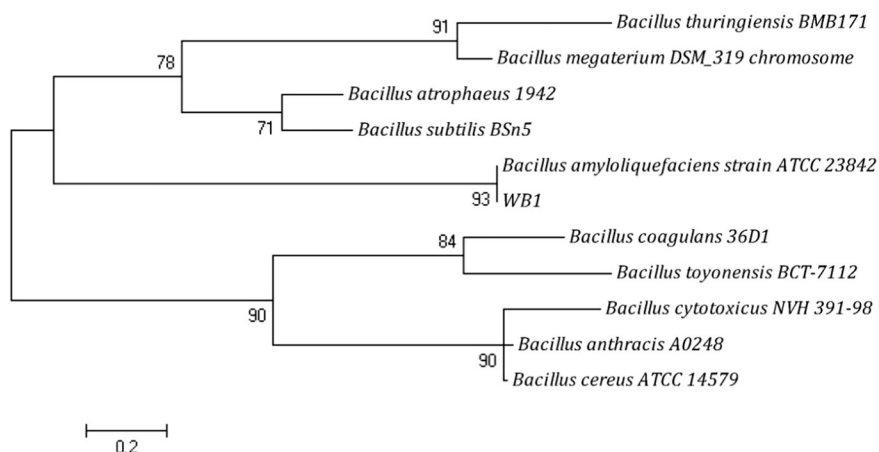
**Table 3**

Morphological, physiological, and biochemical characteristics of the algin-degrading bacterial strain isolated from sea mud.

Parameters	Result
Reactions	
Gram staining	Gram positive
Cell morphology	Rod
Motility	+
Catalase	+
Methyl red	-
Voges–Proskauer	+
Citrate	+
Nitrate	+
Urease	-
Gelatin	-
Starch	+
Casein	+
Glucose	-
H <sub>2</sub> S	+
Lactose	+
Sucrose	+
Cellobiose	-
Raffinose	-
Trehalose	+
Mannose	+
Inulin	+
Adonitol	+
Mannitol	+
Sorbitol	+
Inositol	+
Salicin	-
Esculin	+



**Fig. 2.** Scanning electron micrograph of *B. amyloliquefaciens* WB1 in the presence of *L. japonica* feedstuff.



**Fig. 3.** Phylogenetic analysis of the algin-degrading bacteria isolated from sea mud. The phylogenetic tree was obtained by neighbor-joining analysis of 16S rDNA sequences. It shows the position of the algin-degrading strain in relation to the other *Bacillus* sp. Numbers on the branch indicate the bootstrap confidence value (100 replicates). The scale bar represents 0.2 substitutions per nucleotide position.

### 3. Results

#### 3.1. Characterization of algin-degrading strain WB1

The morphological, physiological, and biochemical characteristics of the algin-degrading bacterial strain are presented in Table 3. Scanning electron microscopy (SEM) analysis revealed rod shaped cells in the logarithmic phase of the growth curve (Fig. 2). 16S rDNA homology analysis and phylogenetic tree revealed a close relationship between the bacterial strain and other *Bacillus* sp. with a sequence similarity of 93% with *Bacillus amyloliquefaciens* ATCC 23842 (Fig. 3). Based on the results of 16S rDNA homology analysis, and together with the morphological, physiological, and biochemical characteristics, the algin-degrading strain was identified as a *Bacillus* sp. and designated as *B. amyloliquefaciens* WB1. It was deposited in China General Microbiological Culture Collection Center (Beijing, China) with a strain number CGMCC No. 8873.

#### 3.2. Effects of different supplementary nutrients on algin degradation

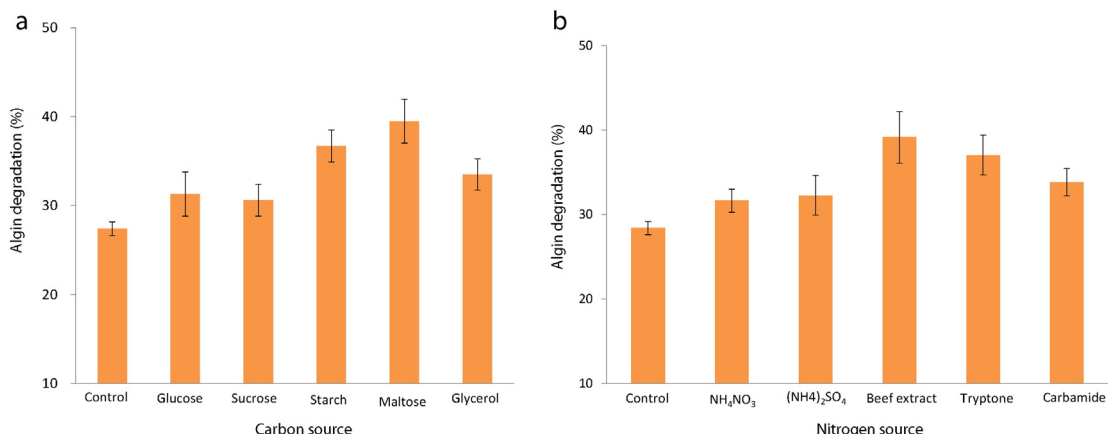
The effects of additional carbon and nitrogen sources at concentrations of 0.2% (w/v) on the degradation of algin are shown in Fig. 4. Maltose and beef extract served as the best supplemental carbon and nitrogen sources, respectively. Therefore, maltose and beef extract were used as additional nutrients for the degradation of algin in subsequent experiments aiming to optimize the degradation process.

#### 3.3. Screening of the significant parameters using Plackett–Burman design

A total of eight parameters were screened for their effects on algin degradation in semi-solid fermentation using Plackett–Burman design (Table 4). The results showed that the degradation process was rather inefficient, with the amount of algin degraded varying from 41.72% to 59.46%. This highlighted the importance of further optimization to attain higher efficiency for the degradation process. Table 5 shows the resulting effects of these parameters on algin degradation and the analyses of their significance, where it is clear that positive coefficients of  $X_1$ ,  $X_4$ ,  $X_5$ ,  $X_6$ , and  $X_8$  resulted in direct effect on the response Y, while the negative coefficients produced an inverse effect on algin degradation. Factors with  $P$ -values of less than 0.05 were considered to have a significant effect on the response. Fermentation period ( $X_1$ ), beef extract ( $X_4$ ) and ratio of solvent to solid material ( $X_6$ ) were consequently determined to be the significant parameters for the degradation of algin via semi-solid fermentation.

#### 3.4. Further optimization of the screened variables using Central-Composite design

Following the initial Plackett–Burman screen, Central-Composite design, one of the commonly used analytical methods in RSM, was used to determine the optimal levels of the three significant parameters. The design matrix and corresponding results are given in



**Fig. 4.** Effects of different supplementary carbon sources (a) and nitrogen sources (b) on algin degradation by *B. amyloliquefaciens* WB1.

**Table 4**  
Results of the experimental design for screening by using Plackett–Burman design in twelve trials.

Runs	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	X <sub>4</sub>	X <sub>5</sub>	X <sub>6</sub>	X <sub>7</sub>	X <sub>8</sub>	X <sub>9</sub>	X <sub>10</sub>	X <sub>11</sub>	Algin degradation (%)
1	+	+	-	+	+	+	-	-	-	+	-	59.46
2	-	+	+	-	+	+	-	-	-	-	+	41.72
3	+	-	+	+	-	+	+	+	-	-	-	58.60
4	-	+	-	+	+	-	+	+	+	-	-	47.64
5	-	-	+	-	+	+	-	+	+	+	-	43.90
6	-	-	-	+	-	+	+	-	+	+	+	53.32
7	+	+	-	-	+	-	+	+	+	+	+	52.28
8	+	+	-	-	-	+	-	+	+	+	+	56.34
9	+	+	+	-	-	-	+	-	+	+	-	42.50
10	-	+	+	+	-	-	-	+	-	+	+	47.62
11	+	-	+	+	+	-	-	-	+	-	+	55.56
12	-	-	-	-	-	-	-	-	-	-	-	42.13

**Table 6.** Regression analysis was performed to fit the response function with the experimental data and to investigate the simultaneous influence of the three selected variables. As shown in **Table 7**, the model *F*-value is 10.14 with a low probability value [ $(P_{\text{model}} > F) < 0.001$ ], indicating a 0.1% chance that a “Model *F*-value” could occur due to noise. This demonstrated that the model was highly significant. The fitness of the model was verified by the determination coefficient ( $R^2$ ), which was calculated to be 0.9013, indicating that 90.13% of the sample variation in the algin degradation was attributed to independent variables. The lack of fit was non-significant ( $P > 0.05$ ), which indicated the high reliability of the quadratic. Furthermore, the lower value of the coefficient of variation ( $CV = 5.60\%$ ) implied better precision and reliability for the experiments. According to the coefficients of the regression equation listed in **Table 8**, the empirical relationship between algin degradation and independent variables could be described by [Equation 2]:

$$Y = 57.82 + 3.31X_1 + 1.50X_4 + 2.09X_6 - 0.36X_1X_6 + 1.32X_1X_4 - 2.93X_4X_6 - 2.65X_1^2 - 1.90X_6^2 - 3.31X_4^2 \text{ [Equation 2]}$$

Where *Y* is the algin degradation (predicted response), and  $X_1$ ,  $X_4$  and  $X_6$  are the coded values of the fermentation period, beef extract concentration, and ratio of solvent to solid, respectively. Among the model terms,  $X_1$ ,  $X_6$ ,  $X_1^2$ ,  $X_4^2$ ,  $X_6^2$  and  $X_4X_6$  were found to be significant ( $P < 0.05$ ).

In order to show the interaction of the significant variables and provide a visual interpretation of the locations of the optimal conditions, three-dimensional response surface graphs and contour plots (**Fig. 5a-f**) were generated for two factors at a time, with the third factor held at its intermediate levels (zero). The shape of the corresponding contour plot indicates whether or not the mutual interactions between the independent parameters are significant. Through this analysis, the degradation of algin in *L. japonica* was found to be significantly affected by the fermentation time and solvent to solid ratio (**Fig. 5a**). The degradation of algin increased with increases in fermentation time and ratio of solvent to solid until a maximum

**Table 5**  
Estimated effect, coefficients, and significance analyses for the screened variables in Plackett–Burman design.

Code	Coefficient	Effect	<i>P</i> -value	significance
X <sub>1</sub>	4.03	40.19	0.0079	Yes
X <sub>2</sub>	-0.88	1.89	0.2614	No
X <sub>3</sub>	-1.77	7.76	0.0682	No
X <sub>4</sub>	2.13	11.25	0.0436	Yes
X <sub>5</sub>	4.167E-003	4.288E-005	0.9952	No
X <sub>6</sub>	3.61	32.20	0.0108	Yes
X <sub>7</sub>	-0.75	1.37	0.3247	No
X <sub>8</sub>	0.97	2.34	0.2223	No

**Table 6**  
Results of experimental design matrix for further optimization by using Central-Composite design in twenty trials.

Runs	X <sub>1</sub>	X <sub>4</sub>	X <sub>6</sub>	Predicted algin degradation (%)	Actual algin degradation (%)
1	-1	-1	-1	41.07	39.87
2	1	-1	-1	45.78	44.26
3	-1	-1	1	48.47	48.06
4	1	-1	1	58.45	54.84
5	-1	1	-1	50.66	50.62
6	1	1	-1	53.92	50.68
7	-1	1	1	46.34	44.21
8	1	1	1	54.89	52.44
9	-2	0	0	40.57	40.64
10	2	0	0	53.82	57.41
11	0	0	-2	40.39	41.57
12	0	0	2	48.75	51.23
13	0	-2	0	47.19	48.74
14	0	2	0	53.21	55.32
15	0	0	0	57.82	58.85
16	0	0	0	57.82	61.33
17	0	0	0	57.82	59.25
18	0	0	0	57.82	57.34
19	0	0	0	57.82	57.36
20	0	0	0	57.82	57.42

level was attained. Further increase in fermentation time beyond 108 h only had a subtle effect on the degradation. Similar to the effects of fermentation time and solvent to solid ratio, increases in beef extract concentration also increased the degradation of algin, but the rate of increase was rather subtle around the longer periods of fermentation time. Increases in algin degradation were also obtained with increases in solvent to solid ratio over the lower range of beef extract concentrations (**Fig. 5c**). However, at the high range of beef extract concentrations, increases in solid to solvent ratio had a negative effect on the degradation of algin. This indicated that significant interaction occurred between solvent to solid ratio and beef extract concentration. Furthermore, the elliptical nature of the contour plots of **Fig. 5f** also indicates that the mutual interaction between the solvent to solid ratio and beef extract concentration was more significant than the mutual interaction between solvent to solid ratio and fermentation time or fermentation time and beef extract, with respect to algin degradation.

Maximum algin degradation and optimal values of the test parameters were obtained by solving [Equation 2]. The analysis yielded 0.3% (w/v) beef extract, a solvent to solid ratio of 44.87 (v/w) and 113.94 h of fermentation time as the optimal values for these variables. The predicted amount of algin degraded calculated using these optimal values was 59.53% as opposed to 41.72–59.46% before optimization.

### 3.5. Experimental validation of the optimized conditions

*L. japonica* powder (50 g) supplemented with 0.2% (w/v) maltose and 0.3% (w/v) beef extract was mixed with 2.24 L water containing minerals as stated above. The mixture was adjusted to pH 7.5 and fermentation was carried out with 8% inoculum at 30°C for 113.94 h in a 5-L fermenter. The average value of algin degradation obtained

**Table 7**  
Analysis of variance (ANOVA) of the quadratic model for the Central-Composite design.

Source	DF	SS	MS	<i>F</i> -value	<i>P</i> -value > <i>F</i>
Model	9	760.15	84.46	10.14	0.0006
Residual	10	83.26	8.33		
Lack of fit	5	67.47	13.49	4.27	0.0685
Total	19	843.41			

CV = 5.60%;  $R^2 = 0.9013$ ; Adeq precisor = 8.851; SS, sum of squares; DF, Degrees of freedom; MS, Mean square.

**Table 8**  
Coefficients and test of significance for the quadratic model for the Central-Composite design.

Term	Coefficient	Standard error	t-value	P-value
Constant	57.82	1.15	10.14	0.0006
X <sub>1</sub>	3.31	0.72	21.09	0.0010
X <sub>4</sub>	1.50	0.72	4.35	0.0635
X <sub>6</sub>	2.09	0.72	8.39	0.0159
X <sub>1</sub> X <sub>4</sub>	-0.36	1.02	0.12	0.7315
X <sub>1</sub> X <sub>6</sub>	1.32	1.02	1.67	0.2248
X <sub>4</sub> X <sub>6</sub>	-2.93	1.02	8.23	0.0167
X <sub>1</sub> <sup>2</sup>	-2.65	0.58	21.28	0.0010
X <sub>4</sub> <sup>2</sup>	-1.90	0.58	10.94	0.0079
X <sub>6</sub> <sup>2</sup>	-3.31	0.58	31.10	0.0002

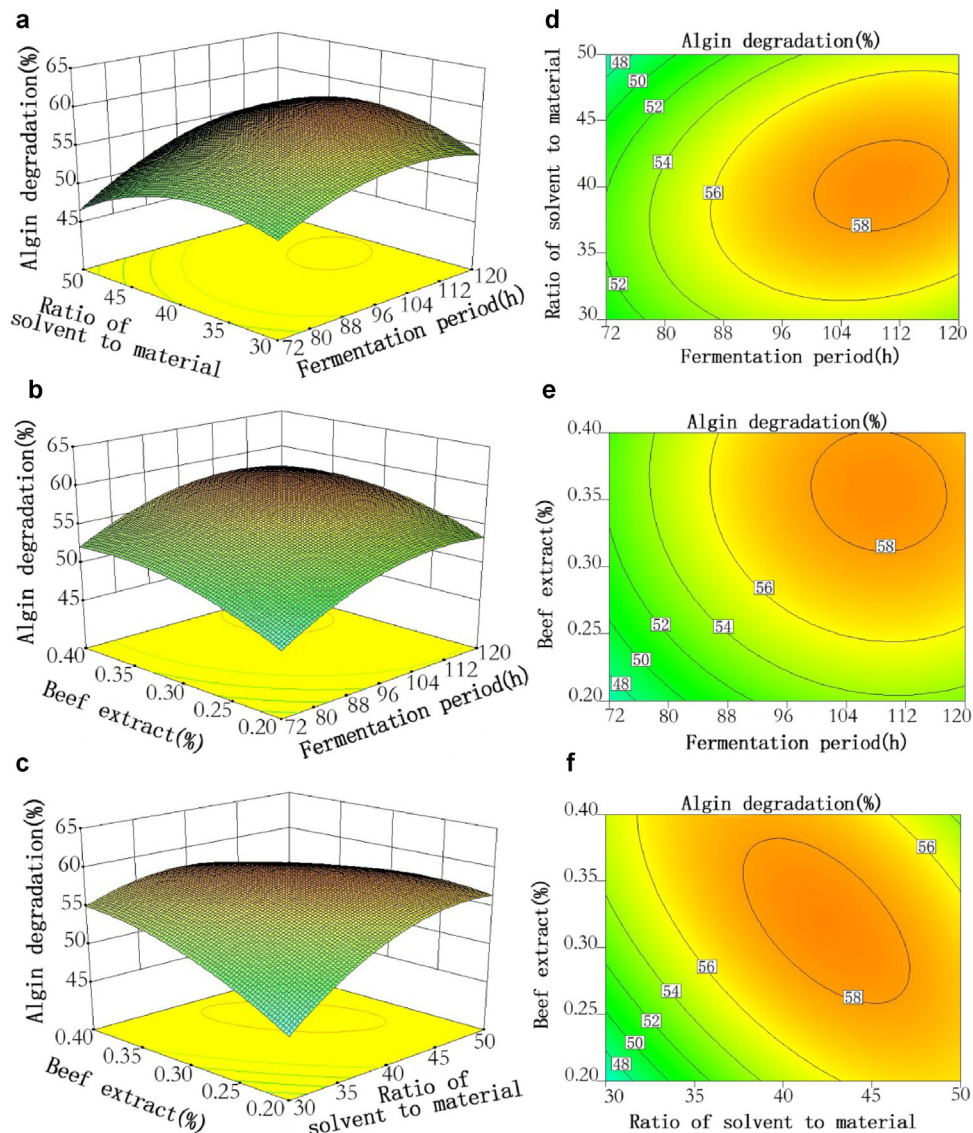
experimentally was 56.88%, which was very similar to the predicted yield (59.53%), thus confirming the validity of [Equation 2] and an optimal point for the degradation of algin in semi-solid fermentation.

#### 4. Discussion

Similar to *S. thunbergii*, which is the optimal traditional feedstuff for sea cucumber, *L. japonica* also belongs to the brown algae, and widely

used as a feedstuff in aquaculture. Compared with *S. thunbergii*, *L. japonica* has a huge economic advantage as feedstuff for *A. japonica* because of its abundant source and lower cost. The high level of algin in *L. japonica* cell walls, which is a framework of cellulose microfibrils embedded in a continuous three-dimensional alginate network, consisting of calcium-bridged poly(G) blocks and entangled poly(M) chains [24]. It enables the algae to withstand the great force of sea current. Unfortunately, this also limits the practical use of *L. japonica* as high quality feedstuff in the sea cucumber farming.

In response to different nutrient conditions and the impact of the environments, different genes may be expressed that function to harness energy available under adverse conditions [25]. Many of the algin-degrading bacteria associated with brown algal fronds, soil, and sea sand normally produce a mixture of carbohydrate-degrading enzymes that includes agarose, laminarase, and cellulase, as well as alginate lyase [8]. This mixture of carbohydrate-degrading enzymes allows these organisms to efficiently harness all of the energy available, either as the sole or as a secondary carbon source. This implies that under stress, limited nutrition or starvation, or simply in an alginate-rich environment, algin can serve as a primary source of food for these bacteria.



**Fig. 5.** Response surfaces (a–c) and contour plots (d–f) of interaction between fermentation period, beef extract and ratio of solvent to material on algin degradation *B. amyloliquefaciens* WB1.

Faced with the need to maintain a sustainable high quality feedstuff in a resource-challenged environment, an alternative cost-effective feeding strategy would be to improve the added value of *L. japonica* feedstuff. Enhancement of the nutritive value of *L. japonica* feedstuff via fermentation could increase the bio-availability of nutrients, reduce or remove the component that is difficult to utilize or to convert it into a more readily utilized form. Most of the previous studies on algin degradation have focused on the isolation and purification of alginate lyase for commercial purposes [26,27,28,29,30]. Alginate lyase is induced when algin-degrading bacteria are grown in the presence of algin [8]. The present work paid close attention to the effect of viable beneficial algin-degrading bacteria on the degradation of algin in *L. japonica* feedstuff. The goal of this work was to explore the possibility of converting *L. japonica* feedstuff into high quality feed for possible use in sea cucumber farming. The bacterial strain, *B. amyloliquefaciens* WB1, isolated from sea mud could be considered as a food-grade microorganism [31], since reports from various studies have demonstrated the use of *Bacillus* sp. as probiotics for improving the survival and growth of marine animals, as well as for enhancing their resistance to disease under aquaculture conditions [32,33,34,35].

The use of statistical models to optimize the fermentation process has become increasingly popular in present-day biotechnology, due to the applicability and suitability of this approach for a wide range of studies [36]. RSM is a valuable method that has been successfully applied to improve the production of primary and secondary metabolites in different fermentation processes [37,38]. In this study, after screening the medium composition for algin degradation by single factor experiments, a RSM model was employed to optimize the fermentation parameters for maximum algin degradation in semi-solid fermentation. Among the eight parameters tested, only three (fermentation time, beef extract concentration, and solvent to solid ratio) were identified as significant parameters for algin degradation. Fermentation time was an important parameter in terms of the accumulation of fermentative metabolites for the whole semi-solid fermentation process to attain maximum algin degradation. It has been reported that the biosynthesis of alginate lyase requires a number of extracellular signaling peptides [8], and beef extract provided as a supplementary nitrogen source, could easily be utilized by the microorganism to synthesize those peptides that could stimulate algin degradation. The initial ratio of solvent to solid-material is also a major critical factor in the semi-solid fermentation system because it determines the growth of the microbe and the yield of the product [39]. Low solvent content can retard cell growth and enzyme production [40]. Proper ratio of solvent to solid-material would provide an ideal microenvironment for supporting growth and enhancing metabolite production. The validity of [Equation 2] obtained through this systematic analysis was confirmed by the use of a 50-fold scale-up fermentation process, which gave maximal algin degradation rate as high as 56.88%, very similar to the predicted value of 59.53%.

Based on the present findings, semi-solid fermentation carried out using *L. japonica* feedstuff as a substrate and the algin-degrading beneficial bacteria *B. amyloliquefaciens* WB1 as the microbial strain could offer an alternative cost-effective and process for sea cucumber farming in China because of the abundant source and low cost of *L. japonica* in China.

#### Conflict of interest

The authors declare no conflict of interest.

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