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Original article

## Optimisation of lactic acid fermentation of York cabbage for the development of potential probiotic products

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**Summary** In this study, York cabbage was used as the sole substrate for the lactic acid (LA) fermentation with *Lactobacillus plantarum*. York cabbage was blanched at 95 °C for 12 min to inactivate surface microflora prior to fermentation. To achieve an optimal fermentation condition that would result in higher release of phytochemicals and antioxidant (AO) capacity in the broth, Box-Behnken design integrating a desirability approach was used. A second-order polynomial equation was developed, indicating the effect of solute/liquid ratio, agitation rate and fermentation time on desired parameters. The optimised factors were used for fermenting York cabbage to obtain maximum bacterial growth, LA, polyphenols and AO capacity. There was  $\approx 5 \log \text{ cfu mL}^{-1}$  increment in bacterial growth after fermentation, whereas LA production reached up to  $4.97 \text{ mg mL}^{-1}$ . Results showed that fermentation retains 95–98% and 90–95% of TPC and AOC, respectively. During refrigerated storage (4 °C), the cell numbers, bioactive components and acidity were maintained till 15-day storage.

**Keywords** Antioxidant capacity, HPLC, lactic acid bacteria, polyphenols, response surface methodology, York cabbage.

### Introduction

There are several approaches applied in the food industry or home to render raw vegetable products palatable and extending their shelf life while the original sensory and nutritional properties are maintained as much as possible within the constraints put forward by microbial safety. Among the various processing options, lactic acid (LA) fermentation is considered as a valuable processing method for maintaining and improving the safety, nutritional and sensory properties of vegetables (Buckenhuskes, 1997; Apás *et al.*, 2008; Calo-Mata *et al.*, 2008). In addition, there are reports that the application of lactic acid bacteria (LAB) as probiotics has proven to exert a range of health-promoting activities such as immunomodulation, enhancement of resistance against pathogens and reduction in blood cholesterol levels (Heenan *et al.*, 2002; de Vries *et al.*, 2006).

There is a wide range of vegetable-based fermented products worldwide such as sauerkraut, cucumber pickles, olives; Egyptian pickled vegetables, kim-chi, Thai pak-sian-dong, Chinese hum-choy, Malaysian pickled vegetables and Malaysian tempoyak. A few reports also have emphasised the fermentation of

Brassica vegetables and mainly focused on spontaneous fermentation of white cabbage and their antioxidant (AO) activity (Kusznierewicz *et al.*, 2008; Sun *et al.*, 2009; Martínez-Villaluenga *et al.*, 2012).

Growth and product formation during fermentation by microorganisms can be affected by solid to liquid ratio (S/L ratio), agitation rate and fermentation time. Thus, it is important to optimise the different fermentation parameters to obtain maximum growth of LAB. The 'one-at-a-time-approach' that is frequently used to obtain high yields of the desired metabolic products in a microbial system is extremely time-consuming when a number of factors have to be optimised; and moreover, it disregards the complex interactions between various physiochemical parameters (Abdel-Fattah *et al.*, 2005). Statistical experimental design is an efficient approach to find out the optimal conditions for the targeted response. Response surface methodology (RSM) is a collection of mathematical and statistical techniques for searching optimum conditions of factors for desirable responses, and for evaluating the relative significance of several affecting factors even in the presence of complex interactions (Yin *et al.*, 2010; Zhang & Mu, 2011).

This work is part of an ongoing project to evaluate the potential of Irish Brassica vegetables as a substrate for the development of a probiotic-based product. However, because of the complexity of vegetables, a systematic

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approach is needed to identify the factors, which would allow the growth of LAB initially so that these studies can form a platform for the fermentation of vegetables later with probiotic strains. The use of probiotic bacteria as starter culture is not very common as this might compromise the potential health benefits of the probiotic strain and affect the technological suitability of the strain. York cabbage (Green cabbage) was selected as a model vegetable for this study owing to their abundance in Ireland, high polyphenolic content and AO capacity.

This study was, therefore, aimed at optimisation of LAB fermentation using York cabbage as a substrate. RSM was applied, and a nonlinear response surface model was proposed for the optimisation of fermented York cabbage product with high LAB, LA content, TPC, TFC and AOC. Shelf life of the product was also carried out by evaluating the cell viability, LA content, pH and phytochemical constituents.

## Materials and methods

### Plant materials and their preparation

Fresh Irish York cabbage was purchased from a local supermarket in Dublin in January 2011. Fifteen to 18 York cabbage heads (20–22 kg) were randomly selected and trimmed off their outer leaves and stem. The heads were then divided into four segments, and the central core was removed. The segments were chopped into  $0.5 \times 5\text{--}6$  cm pieces, using a vegetable cutting machine. A pooled batch of about 15 kg cabbage was stored under dark refrigerated conditions ( $4^\circ\text{C}$ ) as the raw material.

### Culture and Inoculum preparation

*Lactobacillus plantarum* ATCC 8014 was purchased from Medical Supply Company, Dublin, Ireland. The culture was maintained at  $-70^\circ\text{C}$  in 20% glycerol stocks and grown in Man Rossa de Sharpe [MRS (Scharlau Chemie, Barcelona, Spain)] broth at  $37^\circ\text{C}$ . For the preparation of the inoculum, 25 mL of sterile MRS broth was inoculated with 1 mL of thawed stock culture and incubated at  $37^\circ\text{C}$  for 12–14 h. This was then serially diluted 100 times to obtain working culture containing 5–6 log CFU mL cells as determined by plate counts.

### Fermentation based on Box-Behnken design (BBD)

To statistically optimise the York cabbage fermentation and to evaluate main effects, interaction effects and quadratic effects of the three factors (S/L ratio, agitation rate and fermentation time) on the growth of *L. plantarum*, a design with three factors and three levels including five replicates at the centre point was used

(Table 1). To optimise the fermentation, appropriate amount of cabbage that was prepared according to section (Plant materials and their preparation) was mixed with water as per the nutrient illustration (Table 1) in order to achieve the required S/L ratio. Before fermentation, York cabbage was blanched in a 250 mL conical flask at  $95^\circ\text{C}$  for 12 min (Jaiswal *et al.*, 2012a). The flasks containing York cabbage and water [hereafter called York cabbage broth (YCB)] were blanched and inoculated with 5% inoculum (5 mL) upon cooling. Uninoculated flask was kept as control for the respective batch of experiments. The flasks were incubated at  $37^\circ\text{C}$  at their respective agitation rate (0, 100 or 200 rpm). Three flasks were harvested at the times specified by the software, and the supernatant was analysed for pH, viable cell count, LA, TPC, TFC, FRAP and DPPH.

### Viable cell counts

Viable cell counts in the YCB (log CFU mL<sup>-1</sup>) were determined by the standard plate method with MRS medium. Dilution of 1 mL broth was carried out in 9 mL MRD to plate the suitable dilution. The plates were incubated at  $37^\circ\text{C}$  for 36–48 h for cell enumeration.

### Analytical procedure

Each sample of the fermented broth was centrifuged at 10 000 rpm for 15 min at  $4^\circ\text{C}$ . The supernatant was used for all the analysis.

### TPC, TFC and HPLC-DAD analysis of polyphenols

TPC, TFC and HPLC-DAD analysis was carried out according to the existing method in our laboratory (Jaiswal *et al.*, 2012b). For TPC, results were expressed as micro gram gallic acid (Sigma-Aldrich, Steinheim, Germany) equivalents per ml ( $\mu\text{g GAE mL}^{-1}$ ), while for TFC, results were expressed as  $\mu\text{g quercetin (Sigma-Aldrich) equivalents per ml (}\mu\text{g QE mL}^{-1}\text{)}$  of sample. For the HPLC-DAD, the chromatograms were monitored at 280 nm hydroxybenzoic acid (HBA), 320 nm hydroxycinnamic acids (HCA), 360 nm (flavones and

Table 1 Process variables and level in BBD

Independent variables	Coded symbols	Coded variable level		
		-1	0	+1
Solid to liquid ratio (w/v)	$X_1$	0.05	0.15	0.25
Fermentation time (hours)	$X_2$	8	22	36
Agitation rate (rpm)	$X_3$	0	100	200

Level and code of independent variables, agitation rate, fermentation time and solid to liquid ratio used for BB experimental design.

flavonols) and 520 nm (anthocyanins); and complete spectral data were recorded in the range of 220–600 nm.

#### Determination of individual sugars, total sugar content and organic acids

Total sugars in the centrifuged test samples were estimated by the phenol-sulphuric acid method (duBois *et al.*, 1956). Analysis of organic acid and individual sugars was done on an Alliance HPLC (Waters, e2695 Separation module) equipped with an auto sampler and controller with dual pump. The detection system consisted of a Waters-486 UV detector (210 nm) and Waters-410 differential refractometer connected in a series. A 20  $\mu\text{L}$  of sample was injected into a compartment set at 65  $^{\circ}\text{C}$  containing Rezex ROA-Organic acid H+ (8%) (350  $\times$  7.8 mm; Phenomenex, UK) column fitted with a guard column (50  $\times$  7.8 mm; Phenomenex) at a flow rate of 0.6 mL min using 0.005 M  $\text{H}_2\text{SO}_4$  as the mobile phase. Respective standards were used to identify and quantify sugars and organic acids contents in the samples.

#### Antioxidant capacity analysis

In this study, two different methods [2,2-Diphenyl-1-picrylhydrazyl free radical scavenging capacity (DPPH RSC) and Ferric reducing AO potential (FRAP) assay were used for the estimation of total AO capacity of the fermented YCB. The analysis was carried out as reported elsewhere (Rajauria *et al.*, 2010). For the DPPH RSC, ascorbic acid was used as a reference compound, and the results were expressed as micro gram ascorbic acid equivalents per ml ( $\mu\text{g AscE mL}^{-1}$ ) YCB. Trolox (Sigma-Aldrich) was used as a standard for FRAP assay, and the results were expressed as micro gram Trolox equivalents per mL ( $\mu\text{g TE mL}^{-1}$ ) of YCB.

#### Statistical analysis

All the experiments were carried out in triplicate and replicated twice unless stated. Results are expressed as mean values  $\pm$  standard deviation (SD). Data from the BBD were subjected to a second-order multiple regression analysis using least-squares regression to obtain the parameter estimated for the mathematical model. The regression analysis and analysis of variance (ANOVA) were carried out using the STATGRAPHICS Centurion XV. Values of  $P < 0.05$  were considered as statistically significant.

#### Results and Discussion

Before the statistical optimisation of different parameters, preliminary analysis was carried out with various S/L ratios and fermentation time, and on the basis of these results, 0.05–0.25% (w/v) for S/L ratio and (8–36 h) fermentation time was chosen to perform the

present factorial analysis. Results showed that both S/L ratio and fermentation time had significant effects on bacterial growth and LA production. It was also evident that some other organic acids were also produced during the fermentation such as propionic acid and citric acid. It is worth remarking that the total sugar content reduced continuously as the bacterial population increased with fermentation time, which ranging from 25.00 to 56.45% after 8–36 h of fermentation. Among the individual sugars, glucose and fructose were traced in the YCB. A concentration of 0.44  $\text{mg mL}^{-1}$  glucose was estimated at S/L ratio of 0.05%, which increased to 1.90  $\text{mg mL}^{-1}$  as the S/L ratio increased to 0.25%. A similar trend was observed for fructose, which increased from 0.72 to 2.90  $\text{mg mL}^{-1}$  as the S/L ratio increased from 0.05 to 0.25%. It was observed that fermentation led to a sharp depletion in the glucose level in YCB as there was no glucose traced after 36 h of fermentation, whereas at 22 h fermentation, only S/L ratio of 0.25 showed traces of glucose (0.07  $\text{mg mL}^{-1}$ ). A similar trend was also shown by fructose as after 22 h of fermentation as S/L ratio of 0.25% showed 0.43  $\text{mg mL}^{-1}$ .

Fermentation resulted in a slight reduction in the TPC, TFC and AO capacity, but the change was not significant. Earlier studies have reported that strictly controlled fermentation by some isolated strains of lactic bacteria resulted in no change in the AO potency of final sauerkraut compared to fresh vegetable (Tolonen *et al.*, 2004). Nonetheless, the results obtained in this study were encouraging as LAB grew well in the YCB and the phytochemical content, and AO capacity was almost retained after fermentation.

#### Statistical optimisation

##### Effect of process variables on bacterial growth

The effect of all the studied factors on the growth of *L. plantarum* is shown in Table 2. Experimental results for *L. plantarum* growth were fitted to a full quadratic second-order polynomial equation by applying multiple regression analysis (Eqn 1).

$$\begin{aligned}
 &+ 4.357 + 24.975 \times X_1 + 0.193 \times X_2 + 0.0047 \\
 &\times X_3 - 55.425 \times X_1^2 - 0.1607 \times X_1 \times X_2 - 0.0027 \\
 &\times X_1 \times X_3 - 0.00215 \times X_2^2 - 0.000042 \times X_2 \\
 &\times X_3 - 0.000014 \times X_3^2
 \end{aligned} \tag{1}$$

when the values of  $X_1$ – $X_3$  were substituted in the aforementioned equation, the predicted log CFU  $\text{mL}^{-1}$  for *L. plantarum* was obtained. To determine the significance of different variables and their interactions, Pareto charts and  $P$ -values were used. The length of the bars is proportional to the absolute magnitude of the

**Table 2** BB experimental designs for three independent variables, experimental and predicted values for total bacterial count, lactic acid production, TPC, TFC, AOC as DPPH scavenging capacity and FRAP

$X_1$ (w/v)	$X_2$ (hours)	$X_3$ (rpm)	Total bacterial counts (log CFU mL <sup>-1</sup> )		Lactic acid production (mg/ml)		Total phenolic content (mg GAE mL <sup>-1</sup> )		Total flavonoid content (mg QE mL <sup>-1</sup> )		DPPH scavenging capacity (mg AscE mL <sup>-1</sup> )		FRAP (TE mL <sup>-1</sup> )	
			Exp	Predicted	Exp	Predicted	Exp	Predicted	Exp	Predicted	Exp	Predicted	Exp	Predicted
0.25	36	100	10.26	9.98	4.72	4.96	202.25	200.51	174.38	180.55	268.59	256.08	8.97	9.08
0.25	22	200	9.40	9.50	3.60	3.49	182.95	183.40	178.13	169.77	228.59	239.92	8.60	8.59
0.15	8	200	8.42	8.27	1.14	1.16	132.50	132.71	125.00	121.72	190.50	167.00	6.37	6.47
0.15	22	100	9.69	8.74	2.97	2.88	134.83	132.78	11.25	124.50	184.49	197.37	6.47	6.54
0.15	8	0	8.26	8.08	1.24	1.36	140.58	139.29	128.13	125.94	180.59	179.40	6.38	6.48
0.05	22	200	8.78	8.65	1.32	1.54	67.20	65.25	61.88	71.33	96.39	107.37	3.20	3.21
0.25	22	0	9.34	9.48	4.72	4.50	185.43	187.38	178.13	168.68	258.02	247.04	8.77	8.76
0.15	22	100	9.72	9.74	2.81	2.88	144.71	132.78	130.00	124.50	218.59	197.37	6.47	6.54
0.15	22	100	9.67	9.74	2.94	2.88	122.22	132.78	130.63	124.50	193.06	197.37	6.48	6.54
0.05	8	100	6.81	7.09	0.27	0.03	70.80	72.54	75.00	68.83	106.87	119.38	3.44	3.33
0.15	22	100	10.00	9.74	3.01	2.88	127.17	132.78	120.00	124.50	189.73	197.37	6.52	6.54
0.15	36	0	10.03	10.18	4.05	4.03	142.25	140.04	133.13	136.41	146.58	170.08	6.86	6.76
0.25	8	100	8.40	8.45	1.03	1.13	176.96	176.30	133.13	144.77	254.54	257.71	8.70	8.61
0.05	36	100	9.57	9.52	1.15	1.05	62.85	63.50	73.75	62.11	133.30	121.13	3.26	3.35
0.15	36	200	9.95	10.13	3.46	3.34	143.84	145.13	138.13	140.32	175.25	176.44	6.79	6.69
0.05	22	0	8.61	8.51	1.32	1.43	65.22	64.77	64.38	72.74	117.63	106.30	3.12	3.13
0.15	22	100	8.60	9.74	2.67	2.88	134.98	132.78	130.63	124.50	201.00	197.37	6.74	6.54

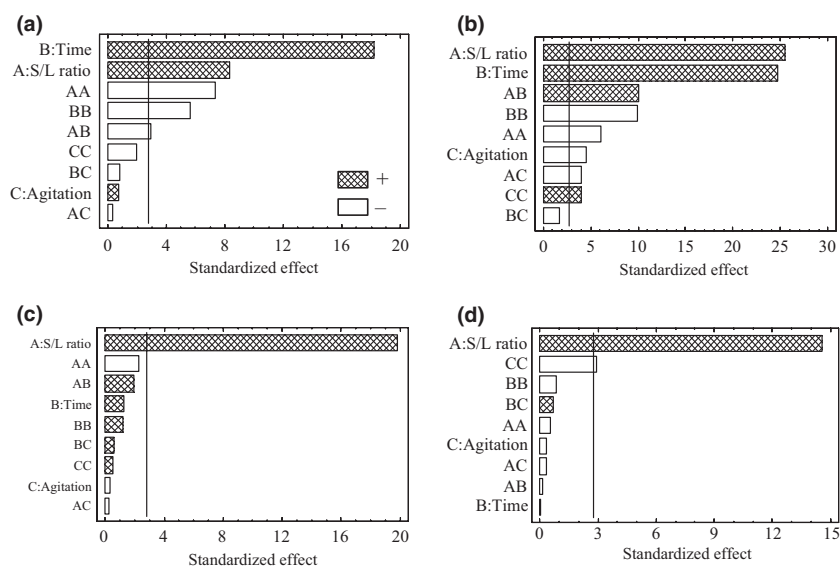
$X_1$ : solid to liquid ratio;  $X_2$ : fermentation time (hours);  $X_3$ : agitation rate; GAE, gallic acid equivalent; QE, quercetin equivalent; AscE, ascorbic acid equivalent; TE, Trolox equivalent.

estimated effects coefficients, while the dashed line represents the minimal magnitude of statistically significant effects (95% of the confidence interval) with respect to response. Pareto chart (Fig. 1a) shows that among the variables used in this study,  $X_1$  (S/L ratio) and  $X_2$  (fermentation time) had significant effect on bacterial growth. The  $P$ -values also indicated the interaction strength of each parameter. The smaller the  $P$ -values are, the bigger the significance of the corresponding coefficient. Corresponding  $P$ -values also suggest that these two parameters are significantly different from zero at the 95.0% confidence level. Therefore, they can act as limiting factors and a small variation will alter the growth of *L. plantarum* to a considerable extent.

The lack-of-fit test is designed to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The value of 0.0868 for lack of fit implies that it was not significant comparing to the pure error and that the model equation was adequate for predicting the *L. plantarum* growth. The fitness of the model was further confirmed by a satisfactory value of  $R^2$ , which was calculated to be 0.966, indicating that 96.60% of the variability in the response could be predicted by the model. The adjusted  $R^2$  statistic was 92.23%. The standard error of the estimate is a measure of the accuracy of predictions. The standard error of the estimate shows the SD of the residuals to be 0.154. Mean

absolute error (MAE) measures how close the predictions are to the eventual outcomes. The MAE of 0.136 was the average value of the residuals.

Furthermore, the predicted log CFU mL<sup>-1</sup> by the final quadratic model, along with the corresponding values observed, is given in Table 2. The agreement between the log CFU mL<sup>-1</sup> predicted by the model and the experimental data is very strong as shown by a high value of  $R^2$  (0.966). The three-dimensional response surfaces were generated to study the interaction between the three factors tested and to visualise the combined effects of factors on the growth of LAB (not shown). The effect of interaction of the three components on growth of *L. plantarum* was tested by contour plots for all possible combinations of factors, keeping one factor constant at a time. The interactions between the variables can be inferred from the shapes of the contour plots (Yu *et al.*, 2008). Circular contour plots indicate that the interactions between the variables are negligible. In contrast, elliptical ones indicate the evidence of the interactions. An increase in log cfu mL<sup>-1</sup> was seen with an increase in the fermentation time and S/L ratio up to 36 h and 0.17, respectively. The response value becomes constant with a further increase in the S/L ratio. Similarly, contour plots between time and agitation showed a positive effect on fermentation time until 36 h, whereas there was no effect was seen as the rate of agitation increased.



**Figure 1** Pareto chart showing the effect of solid to liquid ratio (w/v), agitation rate (rpm) and fermentation time (h) on (a) bacterial counts, (b) lactic acid production, (c) TPC, (d) DPPH scavenging capacity. Line crossing the bar represents significant effect ( $P < 0.05$ ) of process parameters.

#### Effect of process variables on lactic acid production

The second-order polynomial equation (Eqn 2) obtained to predict the LA production is:

$$\begin{aligned}
 & -1.958 + 16.573 \times X_1 + 0.172 \times X_2 - 0.0015 \\
 & \times X_3 - 41.0 \times X_1^2 + 0.501 \times X_1 \times X_1 - 0.028 \\
 & \times X_1 \times X_1 - 0.0035 \times X_1^2 - 0.001 \times X_1 \times X_1 \\
 & + 0.001 \times X_1^2 \quad (2)
 \end{aligned}$$

A comparison of predicted and experimentally obtained values can be seen in Table 2. As can be visualised from the Pareto chart, eight factors have  $P$ -values  $< 0.05$ , indicating that they are significantly different from zero at the 95.0% confidence level. Thus, all the factors are important for the LA production and a small variation can alter the content. The value of 0.0774 for lack of fit implies that it is not significant comparing to the pure error and that the model equation was adequate for predicting the LA. The  $R^2$  statistic indicates that the model as fitted explains 98.78% of the variability in LA production. The adjusted  $R^2$  statistic is 97.23%. The standard error of the estimate shows the SD of the residuals was 0.139. The MAE of 0.129 was the average value of the residuals. The three-dimensional response surface also confirms the interaction (elliptical-shaped contours) between the factors studied. An increase in LA production was seen with an increase in the fermentation time up to 36 h and S/L ratio up to 0.25 (not shown). Similarly, contour plots between time and agitation showed a positive effect of both these factors on  $\log \text{cfu mL}^{-1}$  up to 36 h and 90 rpm, respectively.

#### Effect of process variables on phytochemical content

The second-order polynomial equations for TPC (Eqn 3) and TFC (Eqn 4) are the following:

$$\begin{aligned}
 & + 51.945 + 769.857 \times X_1 - 1.917 \times X_2 - 0.06995 \\
 & \times X_3 - 957.975 \times X_1^2 + 5.936 \times X_1 \times X_2 - 0.112 \\
 & \times X_1 \times X_3 + 0.0256 \times X_2^2 + 0.0017 \times X_2 \times X_3 \\
 & + 0.0002 \times X_3^2 \quad (3)
 \end{aligned}$$

$$\begin{aligned}
 & + 52.55 + 626.3 \times X_1 - 0.768 \times X_2 - 0.174 \times X_3 \\
 & - 1045.0 \times X_1^2 + 7.589 \times X_1 \times X_2 + 0.063 \times X_1 \\
 & \times X_3 + 0.001 \times X_2^2 + 0.001 \times X_2 \times X_3 + 0.001 \times X_3^2 \quad (4)
 \end{aligned}$$

Values of  $X_1$ - $X_3$  were substituted in the aforementioned equation, and comparison of predicted and experimentally obtained values is presented in Table 2. Corresponding Pareto chart for TPC (Fig. 1c) and TFC (not shown) showed that in both cases, only S/L ratio has significant ( $P > 0.05$ ) influence on phytochemicals.  $P$ -value for lack of fit in the ANOVA table was greater or equal to 0.05 (0.9657 for TPC and 0.1526 TFC), and the model appeared adequate for the observed data at the 95.0% confidence level. In the case of TPC, the  $R^2$  indicates that the model as fitted explains 98.96% of the variability. The adjusted  $R^2$  was 97.63%. The standard error of the estimate shows the SD of the residuals to be 8.58, and the MAE of 2.64 is the average value of the

residuals. Results showed that increase in S/L ratio increased the TPC; whereas there was no effect of the other studied factors. While in the case of TFC,  $R^2$  and adjusted  $R^2$  were 95.34% and 89.34%, respectively. The standard error of the estimate shows the SD of the residuals to be 8.68. The MAE of 6.92 was the average value of the residuals. Three-dimensional contour plots also confirmed that there was no interaction between variables and increase in S/L ratio clearly had a significant effect on the TPC and similar trends observed for TFC.

#### Effect of process variables on AO capacity

A quadratic second-order polynomial equation was obtained for DPPH by applying multiple regression analysis (Eqn 5) and predicted polynomial model for DPPH:

$$\begin{aligned} &+ 58.9048 + 820.072 \times X_1 + 0.962 \times X_2 + 0.315 \\ &\times X_3 - 343.679 \times X_1^2 - 0.6038 \times X_1 \times X_2 \\ &- 0.205 \times X_1 \times X_3 - 0.027 \times X_2^2 + 0.0034 \\ &\times X_2 \times X_3 - 0.0019 \times X_3^2 \end{aligned} \quad (5)$$

A comparison of predicted and experimentally obtained values can be seen in Table 2. Corresponding  $P$ -values showed ( $X_1$ ) (S/L ratio) and ( $X_3 \times X_3$ ) (agitation  $\times$  agitation) to be significant model terms with  $P$ -values  $< 0.0001$  and  $0.0441$ , respectively, demonstrating that the models were highly significant. The  $P$ -value of  $0.1003$  implied that the lack of fit was insignificant relative to the pure error owing to noise. The  $R^2$  which was 93.06% represented satisfactory correlation between actual values and predicted ones. The adjusted  $R^2$  value was 84.15%. The standard error of the estimate shows the SD of the residuals to be 13.28. The MAE of 11.36 was the average value of the residuals.

The software generated the following regression equation, which demonstrates the empirical relationship between S/L ratio, fermentation time and agitation for maximum FRAP value in terms of coded units:

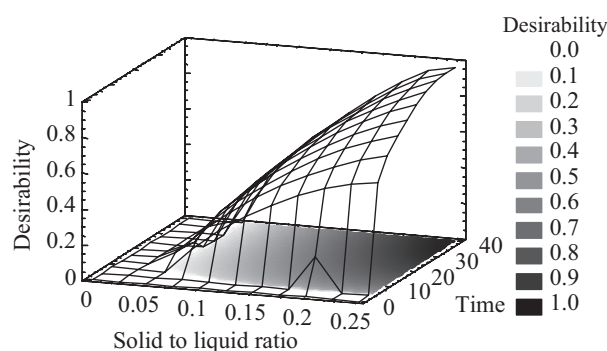
$$\begin{aligned} &+ 1.35669 + 43.1971 \times X_1 - 0.0284082 \times X_2 \\ &+ 0.00202071 \times X_3 - 56.05 \times X_1^2 + 0.0803571 \\ &\times X_1 \times X_2 - 0.006 \times X_1 \times X_3 + 0.0059693 \\ &\times X_2^2 - 0.00001 \times X_2 \times X_3 - 0.0001 \times X_3^2 \end{aligned} \quad (6)$$

The ANOVA of the quadratic regression models for all the parameters showed that the three effects have  $P$ -values  $< 0.05$ , indicating that they were significantly different from zero at the 95.0% confidence level. The  $R^2$

and adjusted  $R^2$  values of the model were 99.78% and 99.50%, respectively, and there was no significance in the lack of fit ( $P = 0.2488$ ) in the model. The standard error of the estimate shows the SD of the residuals to be 0.115. The MAE of 0.074 was the average value of the residuals. Because the  $P$ -value was  $> 5.0\%$ , there was no indication of serial autocorrelation in the residuals at the 5.0% significance level. This indicated that the models could be used to predict responses. The quadratic models generated for the system found the S/L ratio to be significant ( $P < 0.05$ ) for maximum FRAP. Pareto chart for DPPH scavenging capacity (Fig. 1e) and FRAP (not shown) confirmed that S/L ratio has a linear effect on DPPH scavenging capacity and FRAP value. Response surface contour plots of DPPH scavenging capacity and FRAP indicate that the significant interactions between S/L ratio and AOC, fermentation time and agitation were not significant factors.

#### Optimisation of the fermentation conditions

As the aim was to achieve higher bacterial growth, LA production, phytochemical content and AO capacity, the goal was set to 'maximise' with importance '5'. The values of responses were converted to a desirability function. The desirability values of the minimum and maximum yields were configured as 0 and 1, respectively. Applying the desirability function with all pre-selected goals for each factor gave the specific value for all responses, which are presented in Fig. 2. The optimised factors were as follows: fermentation time 36 h, S/L ratio  $0.25 \text{ g mL}^{-1}$  and agitation rate 100 rpm. The software optimised bacterial growth was  $9.98 \text{ cfu mL}^{-1}$ , LA production  $4.97 \text{ mg mL}^{-1}$ , TPC  $200.50 \text{ } \mu\text{g GAE mL}^{-1}$ , TFC  $180.51 \text{ } \mu\text{g QE mL}^{-1}$  and AOC  $256.05 \text{ } \mu\text{g AscE mL}^{-1}$  (DPPH AO system) and  $9.08 \text{ } \mu\text{g TE mL}^{-1}$  (FRAP AO system).



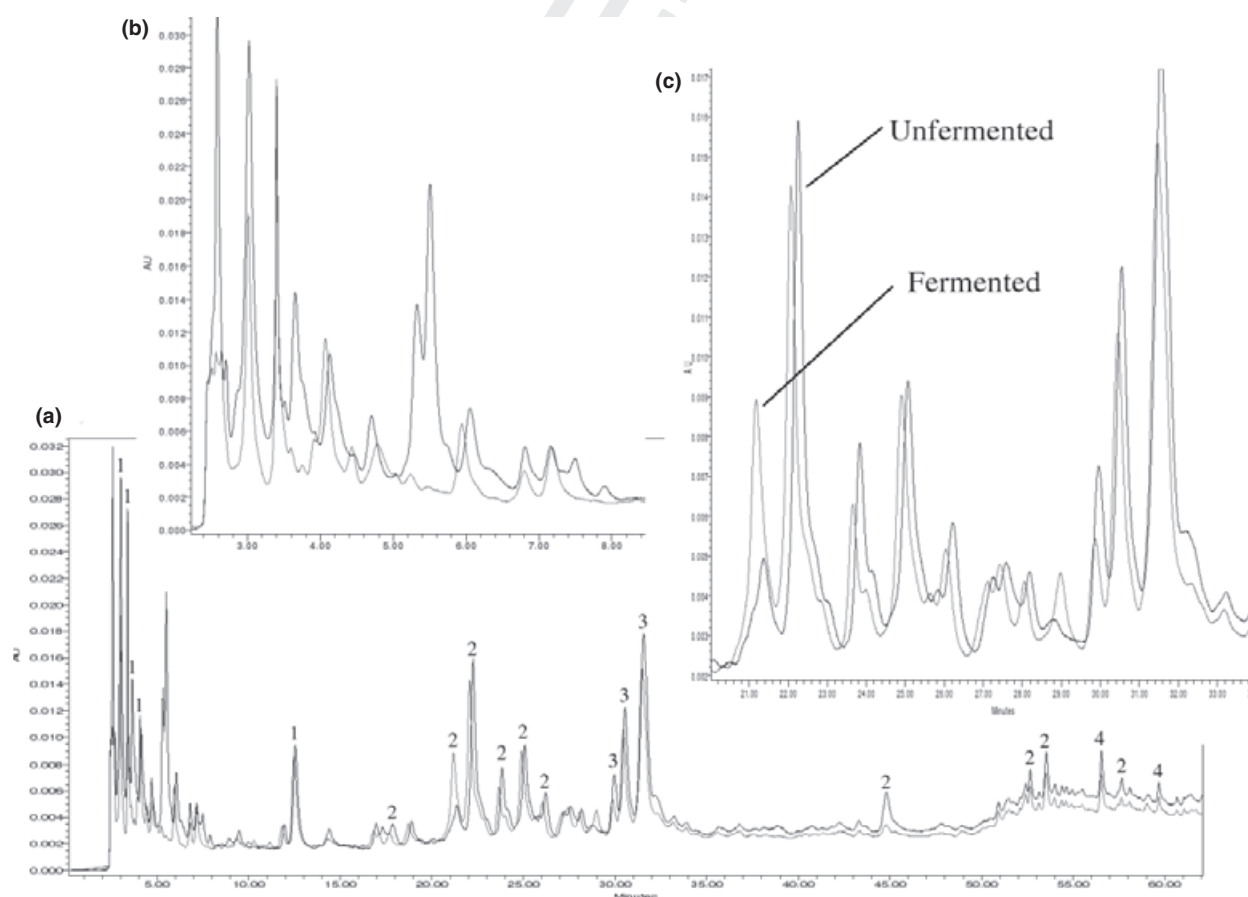
**Figure 2** Response surface plots representing the effect of solid/liquid ratio (w/v), fermentation time (h) and agitation rate (rpm) on overall desirability (high bacterial growth, lactic acid production, TPC, TFC, DPPH scavenging capacity and FRAP). Agitation is constant at 100 rpm.

Finally, for their validation, duplicate confirmatory experiments were conducted using the optimised parameters. Experimentally obtained values of bacterial growth, LA production, TPC, TFC, DPPH scavenging capacity and FRAP were  $10.31 \log \text{cfu mL}^{-1}$ ,  $5.21 \text{ mg mL}^{-1}$ ,  $209.5 \mu\text{g GAE mL}^{-1}$ ,  $180.7 \mu\text{g QE mL}^{-1}$ ,  $232.3 \mu\text{g AscE mL}^{-1}$ ,  $8.96 \mu\text{g TE mL}^{-1}$ , respectively. There was  $\approx 5 \log \text{cfu mL}^{-1}$  increment in bacterial growth after fermentation. The growth of LAB in the YCB was comparable with the results obtained from previous workers in cabbage juice and other vegetables. Yoon *et al.* (2006) reported a maximum growth of *L. plantarum* of  $10^9 \text{ CFU mL}^{-1}$  in cabbage juice. Gardner *et al.* (2001) reported  $10^{10} \text{ cfu mL}^{-1}$  growth of mixed LAB in carrot, cabbage, beet and onion vegetable mixtures after 72 h of fermentation. The results are closely related with the data obtained from optimisation analysis, resulting in a very good agreement. The difference between the experimental and model predicted values was  $< 5\%$  for all the six responses. This

affirms that the models developed are adequate for predicting the responses. Therefore, BBD along with the desirability function could be effectively used to optimise the S/L ratio, fermentation time and agitation rate for maximising the targeted responses.

### Shelf life analysis

There was a complete absence of any *Enterobacteria* or moulds in the fermented broth during the 30 days of storage. The stability of *L. plantarum* during storage was monitored, and a reduction of  $2.14 \pm 0.43 \log \text{cfu mL}^{-1}$  was seen at the end of the 30-day storage. However, there was no significant difference in the  $\log \text{cfu mL}^{-1}$  up to 15 days of storage and the cell numbers started declining only after that. These results indicated that *L. plantarum* was capable of surviving for 15 days under high acidic conditions during storage at  $4^\circ\text{C}$ . The LA content remained constant for the first week after which a slight reduction of  $\approx 5\%$  was



**Figure 3** HPLC–DAD polyphenolic profiles at 280 nm for the fermented and unfermented (Blanched York cabbage) broth; (a) overlay spectra of fermented and unfermented York cabbage, (b) insight showing 2–8 min of chromatogram and (c) insight showing 20–35 min of chromatogram. 1-hydroxybenzoic acid; 2-hydroxycinnamic acids; 3-flavones; 4-methoxylated flavonoid.



observed until 2 weeks. However, storage beyond 2 weeks resulted in a continuous reduction with the content significantly reducing ( $P < 0.05$ ) by 34.4% at the end of 30 days. Initially, the pH was 3.49 and remained almost constant up to 2 weeks. A slight increase in the pH value was seen after third week, which was not significant ( $P > 0.05$ ). Initially, the TPC and TFC value was  $209.5 \mu\text{g GAE mL}^{-1}$ ,  $180.7 \mu\text{g QE mL}^{-1}$ , respectively. There was a slight reduction in the values after 20 days, which were further retained for the next 10 days of storage. No significant ( $P > 0.05$ ) change in the AO content was observed upon the 30 day of storage.

### Effect of fermentation on individual groups of polyphenols

Figure 3 shows the chromatogram for the fermented and unfermented York cabbage. The HBA derivatives was quantified at 280 nm and expressed as gallic acid equivalents (GAE), HCA derivatives at 320 nm and expressed as chlorogenic acid equivalents, and flavones and polymethoxylated flavones at 360 nm and expressed as rutin equivalents (RE). The chromatogram showed that five peaks were identified as HBA derivatives, ten peaks as HCA derivatives, three peaks as flavones and two as polymethoxy flavonoid. From the chromatogram, clearly there was no significant effect of LA fermentation on individual phenolic groups. Prior to fermentation, the concentration of HBA, HCA, flavones and methoxylated flavonoid was  $26.48 \pm 0.39 \mu\text{g GAE mL}^{-1}$ ,  $32.04 \pm 0.46 \mu\text{g QE mL}^{-1}$ ,  $13.94 \pm 0.04 \mu\text{g RE mL}^{-1}$  and  $0.99 \pm 0.06 \mu\text{g RE mL}^{-1}$ , respectively. This value remained almost constant during the fermentation as the concentration of different phenolic groups estimated was  $27.06 \pm 0.66 \mu\text{g GAE mL}^{-1}$ ,  $32.87 \pm 2.06 \mu\text{g QE mL}^{-1}$ ,  $13.75 \pm 1.09 \mu\text{g RE mL}^{-1}$ ,  $0.94 \pm 0.03 \mu\text{g RE mL}^{-1}$  for HBA, HCA, flavones and methoxylated flavonoid, respectively. The sum of all the phenolic groups before and after fermentation was  $73.45 \pm 0.09$  and  $74.63 \pm 2.53$ , respectively.

### Conclusion

The results of the present study indicate that *L. plantarum* grew well in YCB and there was  $\approx 5 \log \text{cfu mL}^{-1}$  increment in bacterial growth after 36 h of fermentation. S/L ratio, agitation rate and fermentation time were optimised by RSM to achieve conditions that result in higher release of phytochemicals and AOC in the fermented broth as well maximum bacterial growth and LA production. Application of RSM in optimisation of LA fermentation helps in cutting down on time and resources for identifying the optimum value for different factors and allows better understanding of the interaction between the variables. HPLC analysis for the polyphenols revealed that fermentation retains all the

polyphenols contents, which is a good indication for the fermented product development.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Response surface plots representing the effect of solid to liquid ratio (w/v), fermentation time (h) and agitation rate (rpm) on (a) bacterial counts (b) lactic acid production (c) total phenolic content (TPC) (d) total flavonoid content (TFC) (e) DPPH scavenging capacity (f) FRAP value. Agitation is constant at 100 rpm. **7**

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