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Optimization of Fermentation Conditions for the Utilization of Brewing Waste to Develop a Nutraceutical Rich Liquid Product

Shilpi Gupta

Technological University Dublin, shilpi.gupta@dit.ie

Amit Jaiswal

Dublin Institute of Technology, amit.jaiswal@dit.ie

Nissreen Abu-Ghannam

Dublin Institute of Technology, nissreen.abughannam@dit.ie

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1 **Optimization of fermentation conditions for the utilization of brewing waste to develop**
2 **a nutraceutical rich liquid product**

3 Shilpi Gupta, Amit K. Jaiswal, Nissreen Abu-Ghannam

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7 School of Food Science and Environmental Health,
8 College of Sciences and Health, Dublin Institute of Technology,
9 Cathal Brugha St., Dublin 1, Ireland.

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22 ***Corresponding author: Dr. Nissreen Abu-Ghannam**
23 **Tel: +353-1-402-7570; e mail: nissreen.abughannam@dit.ie**

24 **Abstract**

25 Utilization of brewers' spent grain (BSG), for the development of a fermented liquid product
26 rich in value-added phenolic compounds was investigated. Changes in and liberation of
27 phenolic compounds and antioxidant activity during fermentation of BSG was studied. The
28 effect of various particle size (PS), solid liquid (SL) ratio, fermentation time and rotation
29 speed was optimized using response surface methodology (RSM) for the purpose of
30 improving bacterial growth and the enhancement in the release of polyphenolic compounds.
31 Contour maps generated using the response surface equation showed that the experimental
32 variables significantly affected the response. A production of 10.4 log cfu/ml, 2.95 g/l lactic
33 acid accompanied by a release of 268.6 mg Gallic Acid Equivalent (GAE)/ml of phenolic
34 compounds, 135 mg Quercetin equivalent (QE)/ml of flavonoid compounds, 33.7 mg TE/ml
35 ferric reducing antioxidant power (FRAP) and 75.1% radical scavenging activity (RSA) was
36 obtained with the optimized factors of 19h fermentation time, 0.25 SL ratio, 85rpm and
37 440 μ m PS. Shelf life was monitored over a period of 30 days and the product was shelf stable
38 in terms of bioactive components for 15 days. The cell numbers, total phenol content and
39 acidity (in terms of lactic acid) were maintained till 15 days storage period and a reduction
40 was observed only after that.

41
42 **Keywords:** Brewers' spent grain, fermentation, antioxidant, heat processing, lactic acid, total
43 phenol, nutraceuticals

45 **1. Introduction**

46 Agro-industrial by-product brewers' spent grain (BSG) is a low-value by-product of the
47 brewing process consisting of the barley malt residue after mashing and lautering process.
48 BSG is rich in cellulose (17%) and non-cellulosic polysaccharides (mainly arabinoxylans)

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(39%) (Valverde, 1994). The arabinose may be esterified with phenolic compounds such as hydroxycinnamic acid, monomeric or dimeric ferulic acid and p-coumaric acid (Bartolomé et al., 2002). The content of phenolic compounds in BSG may vary between 0.2-0.4%. The large volume of BSG that is produced from the breweries is mainly utilized as animal feed or in landfills and its utilization for human consumption is relatively small. Because of its high moisture and fermentable sugar content, BSG becomes an environmental problem after a short time (7–10 days) (El-Shafey et al., 2004). There is an increasing pressure to ensure total utilisation of such by-products, so as to address economic and environmental concerns. Due to the presence of polysaccharides and proteins, BSG has been used as a substitute to expensive carbon sources for industrial production of lactic acid. Production of 5.4 g/l lactic acid was produced by *Lactobacillus delbrueckii* using BSG (Mussatto et al., 2007). Recently, interest in the addition of BSG as a means to enhance the quality of food products for human consumption has increased due to its richness in oligosaccharides and phenolic compounds. BSG has been incorporated as a source of dietary fibre in bread, cookies and ready-to-eat products (Ainsworth et al., 2007; Öztürk et al., 2002). However, reference searches indicate that studies on BSG utilization for the development of a functional fermented edible product have not been considered.

Overall performance of the fermentation by microorganisms can be affected by medium composition, presence of oxygen and product concentration. Furthermore, important parameters determining the release of nutrients from the solid substrate into the broth can be the ratio of the solids to liquid media and the particle size (PS) of the substrate. Maaroufi et al., (2000) reported that pea PS was found to have a strong influence on the chemical composition. The smaller the size of the particles, the higher the contents of crude protein and starch and the lower the content of crude fiber and water insoluble cell walls. Moreover, several literature reported work discuss the effects of PS and solid to solvent ratio on the

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74 release of phytochemicals such as phenolics and flavonoids with antioxidant activities during
75 solvent extractions (Franco et al., 2007; Qu et al., 2010).

76 While optimizing a number of parameters to obtain high yields of the desired metabolic
77 products, the ‘one-at-a-time-approach’ is not appropriate. Not only this method is extremely
78 time consuming but also disregards the complex interactions among various physicochemical
79 parameters (Abdel-Fattah et al., 2005). Response surface methodology (RSM) is a collection
80 of mathematical and statistical techniques for searching optimum conditions of factors for
81 desirable responses, and evaluating the relative significance of several affecting factors even
82 in the presence of complex interactions. Box Behnken is a spherical, revolving RSM design
83 that consists of a central point and the middle points of the edges of the cube circumscribed
84 on the sphere. The design leads to the generation of contour plots by linear or quadratic
85 effects of key variables and a model equation is derived that fits the experimental data to
86 calculate the optimal response of the system.

87 This study aimed to utilize BSG for the purposes of development of a fermented liquid
88 product rich in nutraceuticals. These objectives are justified having in mind that the literature
89 lacks information on the fermentation of BSG for food applications. Therefore, a systematic
90 approach was used to optimize the factors, which would facilitate the growth of lactic acid
91 bacteria (LAB) and the release of bioactive components in the broth. Thus, the effects of
92 different SL ratio, rotation speed, PS of BSG and time of fermentation on the growth of LAB,
93 lactic acid production, total phenolic content (TPC), total flavonoid content (TFC) and
94 antioxidant values (in terms of DPPH-RSA and ferric reducing antioxidant power (FRAP))
95 for the development of a fermented liquid product were optimized by Box Behnken designs.
96 Shelf life studies were also undertaken by evaluating the cell viability, lactic acid content, pH
97 and phytochemical constituents. Finally, large scale production of the fermented edible

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98 product was carried out in a 7 L bioreactor under controlled conditions of pH and dissolved
99 oxygen.

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101 **2. Materials and Methods**

102 ***2.1 Raw material and sample preparation***

103 BSG was obtained from a micro distillery plant located at University College Cork, Cork,
104 Ireland and ground in a blender (Moulinex Opti Blend duo grinder). Particles were separated
105 according to size using a sieve shaker (Model VS 1000, Retsch, Germany) with mesh size of
106 0.71, 0.5, 0.355 and 0.18 mm. Ground BSG was placed in the top sieve using the largest
107 mesh and shaken for 5 min at an amplitude setting of 2 mm, disassembled and stirred lightly,
108 then shaken for additional 5 min. The particles that passed from one sieve and were retained
109 on the smaller sieve were then characterized into three different sizes and designated as
110 700µm (passing through 710µm and retained on 500µm), 500µm (passing through 500µm but
111 retained on 355µm) and 350µm (passing through 355µm but retained on 180µm). The sieve
112 sizes were chosen based on the availability in the laboratory.

113

114 ***2.2 Culture and Inoculum preparation***

115 *Lactobacillus plantarum* ATCC 8014 was purchased from Medical Supply Company, Dublin,
116 Ireland. The culture was maintained at -70°C in 20% glycerol stocks and grown in Man
117 Rossa de Sharpe (MRS; (Scharlau Chemie, Barcelona, Spain)) broth at 37°C. Sterile MRS
118 broth (25ml) was inoculated with 1 ml of thawed stock culture and incubated at 37°C for 12-
119 14 h. This was then serially diluted 100 times to obtain working culture containing 6-7 log
120 cfu/ml cells as determined by plate counts.

121

122 ***2.3 Preliminary study***

123 BSG taken for all the experiments was moistened with water in a ratio of 1:1. Moistened
124 BSG (5 gm) was mixed with 50 ml water and autoclaved at 121°C for 15 min. The particle
125 size (PS) and solid liquid (SL) ratio of BSG used was 355µm and 0.1, respectively. The
126 resulting single autoclaved (SA) broth was filtered through a cheese cloth to separate the
127 BSG particles from the water. The filtrate (50 ml) was dispensed in 250 ml Erlenmeyer flasks
128 and autoclaved again to obtain the double autoclaved (DA) broth which was inoculated with
129 5% inoculum and incubated at 37°C. Samples were withdrawn at 0, 12, 24 and 48 h and
130 analyzed for log cfu/ml, acid production and content of phytochemicals.

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132 **2.4 Box–Behnken experimental design**

133 RSM was applied to investigate the influence of fermentation time (X_1), solid liquid (SL)
134 ratio (X_2), speed of agitation (X_3) and particle size ((PS), X_4) on the growth of *L. plantarum*,
135 acid production and the release of phytochemicals into the broth using Design Expert
136 (Version 5.0.9) software (Stat-Ease Corporation, USA). In order to statistically optimize the
137 medium components and evaluate main effects, interaction effects and quadratic effects of
138 the four factors on various responses, a design with four factors and three levels including
139 five replicates at the centre point was used. The non-linear computer-generated quadratic
140 model is given as

$$141 \quad Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{j=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^4 \sum_{j=1}^4 \beta_{ij} X_i X_j \quad (\text{Eq 1})$$

142 where Y is the measured response associated with each factor level combination; β_0 is an
143 intercept; β_i is the regression coefficient computed from the observed experimental values of
144 Y ; and X_i is the coded level of independent variables. The terms X_i , X_j and X_i^2 represent the
145 interaction and quadratic terms, respectively. The independent variables selected are shown
146 in table 1 along with their low, medium, and high levels.

147 **2.5 Fermentation of BSG**

148 To prepare the BSG fermented drinkable product for RSM studies, 5 g BSG of the required
149 PS was mixed with water as per the nutrient illustration (table 2) in order to achieve the
150 required SL ratio. This was then autoclaved at 121°C for 15 min. After cooling, the resulting
151 SA broth was filtered through a cheese cloth to separate the BSG particles from the water.
152 The filtrate (50 ml) was dispensed in 250 ml Erlenmeyer flasks and autoclaved again to
153 obtain the double autoclaved (DA) broth. The DA broth was cooled to room temperature and
154 inoculated with 5% inoculum. The flasks were then incubated at the required agitation as
155 given in table 2. Two flasks of fermented product were withdrawn for sampling as per the
156 time given by the software designed experiments (table 2). The samples were analyzed for
157 pH, viable cell count, lactic acid, TPC, TFC, FRAP and DPPH.

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159 **2.6 Optimization of the factors**

160 After fitting the models and residual analysis of all responses, the multiresponse analysis of
161 response surface design using desirability approach (Xiao et al., 2006) was used to optimize
162 the four factors for achieving the maximal response. The general approach is to first convert
163 each response into an individual desirability function d_i that varies for the range $0 \leq d_i \leq 1$,
164 where if the response is at its goal or target then, $d_i = 1$, and if the response is outside
165 acceptable region then $d_i = 0$. The desired goal was selected by adjusting the weight or
166 importance that might alter the characteristics of a goal. The goal fields for response have
167 five options: none, maximum, minimum, target and within range. For each goal, the
168 importance can be varied from 1 (less importance) to 5 (maximum importance). As the aim
169 was to achieve higher concentration of all the responses the goal was set to 'maximize' with
170 importance '5'. Then the design variables are chosen to maximize the overall desirability:

1 171 $D = (d_1 \times d_2 \times d_3 \times \dots \times d_n)^{\frac{1}{n}}$ (Eq 2)

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3 172 Where n is number of responses.
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8 174 **2.7 Shelf life evaluation**

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10 175 The optimized values of the different factors were then selected to carry out shelf life analysis
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12 176 of the fermented product. Fermentation under optimized conditions were carried out in 100
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14 177 ml Erlenmeyer flasks containing 50 ml product for 19 h which was then refrigerated at 4°C.
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16 178 Two flasks of the fermented product were withdrawn for sampling at regular intervals of 3-4
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18 179 days for 30 days and analyzed for pH, lactic acid, viable cell count and phytochemical
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27 182 **2.8 Kinetics under controlled pH**

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29 183 Seed culture (200 ml) was prepared as mentioned in section 2.2. Cultivation was carried out
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31 184 at 37°C in a 7 L Bioflo 415 (New Brunswick, Mason Technology, Dublin, Ireland) bioreactor
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33 185 containing 4 L of SA broth. The reactor containing the SA broth was sterilized *in situ* at
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35 186 121°C for 20 min, cooled and then inoculated with 5% inoculum (v/v). Culture pH was
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37 187 maintained at 7.0 by automatic addition of 2 N NaOH. Samples were withdrawn at 3-4 h
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39 188 interval and analyzed for log cfu/ml, acid production, phytochemical content and antioxidant
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49 191 **2.9 Analytical methods**

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52 192 **2.9.1 Estimation of viable cell count, residual sugars and organic acids**

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54 193 The pH of fermented BSG product was measured with a pH meter (Orion Model 520A, ATI-
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56 194 Orion Research Inc, Boston, USA). Viable cell counts in the BSG broth (log cfu/ml) were
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195 determined by the standard plate method with MRS medium. The plates were incubated at
196 37°C for 36-48 h for cell enumeration.

197 Each sample of the fermented broth was centrifuged at 10,000 rpm for 10 min at 4°C. The
198 supernatant was subjected to the analyses of organic acids and total sugar. Total sugars in the
199 centrifuged broth were estimated by the phenol-sulphuric acid method (Dubois et al., 1956).

200 The cell-free broth was used for the determination of organic acids and sugars by HPLC. The
201 system consisted of an HPLC column on an Alliance HPLC (Waters, e2695 Separation
202 module) equipped with an auto sampler and controller with dual pump. The detection system
203 consisted of a Waters 486 UV detector (210 nm) and Waters 410 Differential refractometer
204 (RI detector) connected in series. The data acquisition and integration were performed using
205 the Empower software package. A 20 µl of sample was injected into a thermostatically
206 controlled compartment set at 65°C containing Rezex ROA- Organic acid H+ (8%) (350×7.8
207 mm, Phenomenex, U.K.) column fitted with a guard column (50 × 7.8 mm, Phenomenex,
208 U.K.) at a flow rate of 0.6 ml/min using 0.005 M H₂SO₄ (Sigma-Aldrich, Germany) as the
209 mobile phase. Each sample was injected two times. Standards for the organic acids (lactic,
210 acetic, propionic, malic and citric), alcohols (ethanol and methanol) and sugars (glucose,
211 xylose, mannose and arabinose) were used to identify and quantify the components in the
212 samples.

214 *2.9.2 Phytochemical analysis*

215 *2.9.2.1 Total phenolic content (TPC)*

216 The TPC in the BSG liquid product was determined using Folin-Ciocalteu's phenol reagent
217 (Taga et al., 1984). Absorbance of all the sample solutions against reagent blank was
218 determined at 720 nm with a spectrophotometer (Genesys 20, Thermo Spectronic, WI, USA).

219 The TPC was expressed as mg gallic acid equivalents (GAE)/ml.

220 2.9.2.2 HPLC-DAD analysis of polyphenolic compounds

221 The HPLC system consisted of a reversed-phase HPLC column on an Alliance HPLC
222 (Waters, e2695 Separations modules) equipped with an auto sampler and controller with dual
223 pump, a 2998 photodiode array detector (PDA) and the Empower software. HPLC coupled
224 with PDA was used for identification of the peaks. The PDA carried out recording of UV-vis
225 spectrum of each peak of the chromatogram and thus allowed explicit attribution of each
226 chromatographic peak to different class of polyphenols, since each class exhibits a
227 characteristic UV-vis spectrum. An Atlantis C18 column (250 mm × 4.6 mm, 5µm particle
228 size) from Waters (Waters, Milford, MA) was used for polyphenolic separation at 25°C.
229 Solvent system consisted of 6% acetic acid in 2 mM/l sodium acetate (Sigma-Aldrich,
230 Germany) (Solvent A) and acetonitrile (Fischer Scientific, UK) (Solvent B) (Jaiswal et al.,
231 2011). The system was run with a solvent gradient as follows: 0-15% B in 45 min, 15-30% B
232 in 15 min, 30-50% B in 5 min and 50-100% B in 5 min. A flow rate of 1 ml/min was used
233 and total run time for samples was 70 min. Samples and mobile phases were filtered through
234 a 0.22µm Millipore filter (Millipore, Bedford, MA) prior to HPLC injection and 20 µl of
235 sample was injected. The chromatograms were monitored at 280 nm (hydroxybenzoic acid)
236 and 320 nm (hydroxycinnamic acids) and complete spectral data were recorded in the range
237 of 220-600 nm.

239 2.9.2.3 Total flavonoid content (TFC)

240 The TFC was determined by a colorimetric method described by Liu et al., (2009). TFC of
241 the fermented broth was expressed as mg quercetin equivalents (QE)/ml.

243 2.8.2.4 DPPH radical scavenging assay

244 This assay was carried out as described in our earlier studies (Jaiswal et al., 2011). The ability
245 to scavenge the DPPH radical was calculated using the following equation:

$$246 \text{ Scavenging capacity (\%)} = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{sample blank}}}{A_{\text{control}}} \right) \right] \times 100 \quad (\text{Eq 3})$$

247 where, A_{control} is the absorbance of the control (DPPH solution without sample), A_{sample} is the
248 absorbance of the test sample (DPPH solution plus test sample) and $A_{\text{sample blank}}$ is the
249 absorbance of the sample only (sample without any DPPH solution).

251 2.9.2.5 Ferric reducing antioxidant potential (FRAP) assay

252 Total antioxidant power of the fermented broth was measured using FRAP assay according to
253 the method reported in our earlier study (Jaiswal et al., 2011). Trolox (Sigma-Aldrich,
254 Germany) was used as a standard and the results were expressed as mg trolox equivalents
255 (TE)/ml.

257 2.10 Statistical analysis

258 All the experiments were carried out in triplicate and replicated at least twice. Results are
259 expressed as average \pm standard deviation (SD). Data from the Box-Behnken factorial design
260 were subjected to a second-order multiple regression analysis using least-squares regression
261 to obtain the parameter estimated for the mathematical model. The regression analysis and
262 analysis of variance (ANOVA) for Box-Behnken design were carried out using the Design
263 Expert software. Analysis of variance (ANOVA) for other experiments was done using the
264 STATGRAPHICS Centurion XV (StatPoint Technologies, Inc., Warrenton, VA). Values of p
265 < 0.05 were considered as statistically significant.

267 3. Results and Discussion

268 3.1 Preliminary studies

269 Development of a new functional food demands several important factors to be considered;
270 the most important of which are the bioactive components. In case of a fermented BSG based
271 drinkable product, important parameters will be the content of phytochemicals (phenolic
272 compounds and other compounds responsible for antioxidant activity) and the final viable
273 cell population in addition to the amount of lactic acid being produced. Growth of *L.*
274 *plantarum* in wheat and barley based media without the need of additional nutrients has been
275 reported earlier (Patel et al., 2004). Studies are also available wherein industrial waste such as
276 rice straw has been successfully fermented to lactic acid (Qi et al., 2007). However, no
277 literature is available regarding the use of BSG for the development of a fermented drink.
278 Hence, initial trials were carried out to find the applicability of BSG as a medium for the
279 growth of *L. plantarum*. BSG was autoclaved with water in order to allow the release of
280 nutrients in the broth. The resulting SA broth was a homogenous liquid media with non-
281 fermentable BSG particles in suspension. The TPC and TFC in the SA broth was found to be
282 183.9±3.1 mg GAE/ml and 80±1.9 mg QE/ml, respectively. The FRAP value in the SA broth
283 was 24.3±0.4 mg TE/ml whereas the RSA was 90.3% (34.2±0.5 mg AscE/ml). The total
284 sugar content was 3.1±0.5 g/l. In addition, the presence of free xylose (0.04 g/l), arabinose
285 (0.03 g/l) and glucose (0.1 g/l) was also observed. This was encouraging as it was proved that
286 heating does lead to release of nutrients and phytochemicals into the broth. However, the
287 broth in the present state was not suitable for the development of a fermented drinkable
288 product because of the presence of the BSG particles. Hence, a technique was developed
289 wherein the SA broth was filtered through a cheese cloth and the filtrate was subjected to a
290 second heat treatment (121°C for 15 min). An increment of 5.2, 1.7 and 11.8% in TPC, TFC
291 and FRAP values was seen in the DA broth as compared to the SA broth. There was no
292 significant difference in the DPPH values ($P>0.05$). The increase could be due to the

293 breakdown of complexes between the polysaccharides and phenolics which could have
294 broken due to an extra heat treatment.

295 Fermentation of the DA broth resulted in a generation of 9 ± 0.08 log cfu/ml with the
296 production of 1.8 g/l lactic acid. The production of lactic acid resulted in a drop in the pH of
297 the media from 5.8 ± 0.08 to 3.6 ± 0.02 . A maximum growth of *L. plantarum* of 10.11 log₁₀
298 cfu/ml was reported for malt based media (Charalampopoulos et al., 2002) whereas 8.97-9.16
299 log₁₀ cfu/ml were obtained with media of whole and white oat-based flour (Kedia et al.,
300 2008). The growth of *L. plantarum* in BSG based media was comparable with these results.
301 Production of 1.2g/l and 1.99 g/l lactic acid has been reported upon fermentation of white
302 flour (Kedia et al., 2008) and malt medium (Charalampopoulos et al., 2002) with *L.*
303 *plantarum*. In another study, chemically pre-treated BSG was saccharified and used as a
304 fermentation medium without nutrient supplementation for production of lactic acid by
305 *Lactobacillus delbrueckii* which produced 5.4 g/l lactic acid (Mussatto et al., 2007).

306 However, fermentation resulted in a slight reduction in the phytochemical content of the
307 fermented product but the change was not significant. Earlier studies have reported that
308 strictly controlled fermentation by some isolated strains of lactic bacteria resulted in no
309 change in the antioxidant potency of final sauerkraut compared to fresh vegetable (Tolonen et
310 al., 2004). Little or no change in the TPC for fermented cereals and tea as compared to the
311 unfermented counterpart has been reported in other studies as well (Heong et al., 2011;
312 Oyarekua, 2010). Nonetheless, the results obtained in this study were encouraging as there
313 was a release of phytochemicals from the BSG particles into the broth and the content
314 increased as a result of the process of double autoclaving. Furthermore, the phytochemical
315 content was not destroyed upon fermentation. Besides, the present study utilized a simple
316 hydrothermal processing as a means to break the complex lignocellulosic material to facilitate
317 the release the sugars, antioxidants and phenolic compounds in contrast to acid-base

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5 318 hydrolysis method used in other studies involving non-food based application of BSG
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7 319 (Carvalho et al., 2004).

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10 320 According to these preliminary trials, it was established that BSG which is generally
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12 321 discarded by the brewing industry could be fermented for the development of novel
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14 322 beverages. Thus, further work involved obtaining conditions which would result in maximal
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16 323 release of these phytochemicals in the broth in addition to supporting the growth of LAB.

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18 325 ***3.2 Optimization of parameters by Box-Behnken design***

19 326 Response Surface Methodology was used to establish the relationship between the variables
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21 327 with the obtained responses. The main factors selected for optimization were the PS of BSG,
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23 328 SL ratio, fermentation time and agitation. The 29 experiments proposed (table 2) by the Box-
24
25 329 Behnken design with four factors and three levels including five replicates at the centre point
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27 330 were used for fitting a second-order response surface. The five centre point runs provided a
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29 331 measure of process stability and inherent variability. A comparison of predicted and
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31 332 experimental values for the effect of the four factors on the growth of *L. plantarum*, acid
32
33 333 production and release of phytochemicals is shown in table 2. Analysis of these data allows
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35 334 construction of a ‘sequential model sum of squares’ summary table, ‘lack of fit’ table and
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37 335 ‘model summary statistics’ table for every response, indicating how terms of increasing
38
39 336 complexity contribute to the total model. The software then analyzes each response
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41 337 separately and establishes a range of models (linear, quadratic, cubic) for each response. The
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43 338 model having a large F-value with $p < 0.05$, insignificant lack of fit and minimum predicted
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45 339 residual sum of squares (PRESS) is selected. Investigation of the associated probability
46
47 340 revealed that for all the responses quadratic models resulted in the best fit. The cubic models
48
49 341 were aliased, as expected, because the design matrix provided very few unique design points
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51 342 to determine all the terms in the cubic model.

343 3.2.1 Effect of process variables on cell growth and lactic acid production

344 The model summary statistics showed the quadratic model to have the minimum PRESS
345 value and maximum predicted R^2 value and thus was chosen for fitting the experimental data.

346 Experimental results for the growth of *L. plantarum* and acid production were fitted to a full
347 quadratic second order polynomial model by applying multiple regression analysis. The
348 equation obtained to predict the polynomial model for *L. plantarum* growth (equation 4) and
349 lactic acid production (equation 5) are as given below:

350
$$\text{Log cfu/ml} = +7.07 + 0.15X_1 + 7.25X_2 + 0.00326X_3 + 0.00468X_4 - 0.0039 X_1^2 - 7.93 X_2^2 - 1.18E-$$

351
$$05 X_3^2 - 3.83E-06 X_4^2 - 0.15X_1 X_2 + 1.19E-04X_1 X_3 - 2.94E-05X_1 X_4 - 0.013X_2 X_3 + 0.001 X_2 X_4 -$$

352
$$3.07E-07 X_3 X_4 \quad \text{(Eq 4)}$$

353
$$\text{Lactic acid} = -0.96 + 0.088 X_1 + 1.03 X_2 - 0.0036 X_3 + 0.0024 X_4 - 0.002 X_1^2 + 6.36 X_2^2 + 4.4E-$$

354
$$06 X_3^2 - 2E-07 X_4^2 + 0.68 X_1 X_2 - 6.5E-06 X_1 X_3 - 9.3E-05X_1 X_4 + 0.014 X_2 X_3 - 0.011 X_2 X_4 + 3E-$$

355
$$06 X_3 X_4 \quad \text{(Eq 5)}$$

356 When the values of X_1 – X_4 were substituted in the above equations, the predicted log cfu/ml
357 for *L. plantarum* and the corresponding concentration of lactic acid were obtained. In order to
358 determine the significance of the quadratic model, ANOVA analysis was conducted. The P -
359 values were used as a tool to check the significance of each coefficient, which also indicated
360 the interaction strength of each parameter. The smaller the P -values are, the bigger the
361 significance of the corresponding coefficient. Corresponding P -values (table 3) for log cfu/ml
362 suggested that among the different factors, X_2 (SL), $(X_1)^2$ (time \times time), $(X_3)^2$ (agitation \times
363 agitation) and $X_2 \times X_3$ (SL \times agitation) were the significant model terms with a P -values less
364 than 0.05. Therefore, they can act as limiting nutrients and a small variation in their
365 concentrations will alter growth of *L. plantarum* to a considerable extent. The goodness of fit
366 of the model was examined by F -test and the determination coefficient R^2 . The greater the F -

367 value is from unity, the more certain it is that the factors explain adequately the variation in
368 the data around its mean, and that the estimated factor effects are real. The analysis of
369 variance showed that this regression model was highly significant ($P<0.01$) as is evident from
370 the Fisher, F -test (F_{model} , the ratio of mean square regression to mean square residual is 5.43)
371 and has a very low probability value [$(P_{\text{model}} > F) = 0.0016$]. The value of 0.1851 for lack of
372 fit implies that it is not significant comparing to the pure error and that the model equation
373 was adequate for predicting *L. plantarum* growth. The fitness of the model was further
374 confirmed by a satisfactory value of determination coefficient, which was calculated to be
375 0.8444, indicating that 84.44% of the variability in the response could be predicted by the
376 model. The low coefficient of variation (CV=1.17%) suggested that the model was precise
377 and reliable.

378 For determination the interaction of factors on different responses, the contour plots were
379 studied in detail for all possible combinations by keeping two parameters constant at a time.
380 Figure 1a shows the effect of PS and fermentation time on log cfu/ml. An increase in log
381 cfu/ml was seen with an increase in the fermentation time and PS up to 17 h and 580 μm ,
382 respectively. The response value decreased with a further increase in the PS or fermentation
383 time. Similarly, contour plots (plots not shown) between time and agitation showed a positive
384 effect of both these factors on log cfu/ml up to 16 h and 125rpm, respectively. However, an
385 increase in the SL ratio resulted in a continuous increase in log cfu/ml in contrast to PS
386 wherein the response value increased only up to a PS of 570 μm .

387 P-values in case of lactic acid showed X_1 (time), X_2 (SL ratio), X_4 (PS), $(X_1)^2$ (time \times time), X_1
388 $\times X_2$ (time \times SL) and $X_2 \times X_4$ (SL \times PS) to be significantly affecting the acid production
389 having $P<0.05$ (table 3). The analysis of variance showed that the regression model was
390 significant ($P<0.01$) with a Fisher, F -test value of 53.77. The value of 0.0553 for lack of fit
391 was insignificant and the model equation was adequate for predicting the production of lactic

392 acid. The determination coefficient was calculated to be 0.9817, indicating that 98.17% of the
393 variability in the response could be predicted by the model.

394 Figure 1b shows the contour plot between PS and agitation for lactic acid production. The
395 contour clearly showed that PS and agitation were inversely affecting the response. An
396 increase in agitation and a reduction in PS increased the value of the response. In contrast,
397 contour between time and SL ratio showed an increase in the concentration of lactic acid as
398 both the factors were continuously increased.

399

400 3.2.2 Effect of process variables on total phenol and total flavonoid content

401 The second order polynomial equation obtained to predict the TPC is:

$$\begin{aligned} 402 \text{ TPC} = & +64.72 - 0.73X_1 + 1548.12X_2 - 0.2X_3 - 0.038X_4 + 0.1 X_1^2 - 1522.8 X_2^2 + 4.52E-04 X_3^2 + \\ 403 & 2.1E-05 X_4^2 - 3.86X_1 X_2 - 0.0026E-03X_1 X_3 - 0.0038E-03X_1 X_4 - 0.36X_2 X_3 - 0.35 X_2 X_4 + 4.2E- \\ 404 & 04 X_3 X_4 \end{aligned} \quad (\text{Eq 6})$$

405 The predicted values for TPC can be obtained by substituting the values of X_1 – X_4 in equation
406 6. Corresponding ANOVA analysis suggested that, X_2 (SL), X_4 (PS) and $(X_2)^2$ (SL \times SL) are
407 significant model terms with $P < 0.05$ and thus can act as limiting factors for TPC. The
408 regression model was found to be highly significant ($P < 0.01$) as is evident from the Fisher,
409 F -test (F_{model} , is 87.03 and has a very low probability value [$(P_{\text{model}} > F) < 0.0001$]. The value
410 of 0.1022 for lack of fit implies that it is not significant comparing to the pure error and that
411 the model equation was adequate for predicting the TPC. The fitness of the model was further
412 confirmed by a satisfactory value of determination coefficient, which was calculated to be
413 0.9886, indicating that only 1.14% variability in the response could not be predicted by the
414 model.

415 Figure 1c shows the two dimensional contour plot for the effect of agitation and time on the
416 TPC that was released in the broth. An increase in the agitation showed a reduction in the
417 TPC till 80 rpm after which the value started increasing.

418 The quadratic model obtained for TFC was:

$$419 \text{ TFC} = -76.12 + 1.65 X_1 + 611.5 X_2 - 0.002 X_3 + 0.35 X_4 - 0.012 X_1^2 - 1002.7 X_2^2 - 3.2E-04 X_3^2 - 2.9E- \\ 420 04 X_4^2 + 8.2 X_1 X_2 - 0.0024 X_1 X_3 - 0.003 X_1 X_4 + 0.23 X_2 X_3 - 0.17 X_2 X_4 + 1.2E-04 X_3 X_4 \quad (\text{Eq 7})$$

421 Corresponding ANOVA analysis suggested X_2 (SL), $(X_2)^2$ (SL \times SL) and $(X_4)^2$ (PS \times PS) to
422 be significant model terms with P -values of less than 0.05. The regression model (Eq. 7) was
423 found to be highly significant ($P < 0.01$) with a value of 22.84 for F_{model} , a very low
424 probability value [$(P_{\text{model}} > F) < 0.0001$] and a non significant value of 0.2002 for lack of fit.
425 The determination coefficient was 0.9581, indicating a good predictability of the model.

426 Figure 1d shows two-dimensional contour plot for the effect of agitation and PS on TFC.
427 Analyzing the contour showed that TFC content was increased up to a PS of 500 μ m with an
428 agitation of no more than 100 rpm. In a study on extraction of phenolic compounds from
429 grape byproducts, temperature and SL ratio were found to play the most critical role in
430 extraction efficiency (Pinelo et al., 2005). In another study, SL ratio was reported to be most
431 effective for enhancing the yield of total phenolics during extraction from black currants. The
432 content of phenolics increased with ethanol concentration up to a maximum at about 60% and
433 then decreased with further increase in solvent concentration irrespective of the SL ratio
434 (Cacace et al., 2003).

435

436 3.2.3 Effect of process variables on DPPH and FRAP

437 Table 2 shows the predicted and experimental values for DPPH and FRAP. For both the
438 responses the regression model was found to be highly significant ($P < 0.01$) with an

439 insignificant lack of fit (0.8473 for FRAP and 0.3188 for DPPH). A high value of
 440 determination coefficient (0.9759 for FRAP and 0.9360 for DPPH) indicated a good
 441 predictability of the model. X_2 (SL), X_4 (PS), $(X_2)^2$ (SL \times SL) and $(X_4)^2$ (PS \times PS) were found
 442 to be the significant model terms for FRAP and X_2 (SL), $(X_2)^2$ (SL \times SL), $X_1 \times X_2$ (time \times
 443 SL), $X_2 \times X_4$ (SL \times PS) and $X_3 \times X_4$ (agitation \times PS) for DPPH (table 3). The polynomial
 444 equations for FRAP (Eq 8) and DPPH (Eq 9) are:

$$\begin{aligned}
 \text{FRAP} = & -12.05 + 0.7 X_1 + 204.28 X_2 - 0.012 X_3 + 0.056 X_4 - 0.0052 X_1^2 - 323.6 X_2^2 + 6.4 \times 10^{-5} X_3^2 - \\
 & 4.4 \times 10^{-5} X_4^2 - 0.11 X_1 X_2 - 6.3 \times 10^{-4} X_1 X_3 - 7.5 \times 10^{-4} X_1 X_4 - 0.036 X_2 X_3 - 0.042 X_2 X_4 + 2.7 \times 10^{-5} \\
 & X_3 X_4
 \end{aligned}
 \tag{Eq 8}$$

$$\begin{aligned}
 \text{DPPH} = & 21.83 + 2.28 X_1 + 294.3 X_2 - 0.21 X_3 - 0.095 X_4 - 0.0029 X_1^2 + 937.7 X_2^2 + 9.91 \times 10^{-5} X_3^2 \\
 & + 1.5 \times 10^{-4} X_4^2 - 9.2 X_1 X_2 - 2.2 \times 10^{-4} X_1 X_3 - 0.002 X_1 X_4 - 0.15 X_2 X_3 - 0.38 X_2 X_4 + 4.2 \times 10^{-4} X_3 X_4
 \end{aligned}
 \tag{Eq 9}$$

451 Figure 1e and 1f shows the two-dimensional contour plots for FRAP and DPPH, respectively.
 452 In both cases, an increase in the SL ratio resulted in an increase in the respective parameter.
 453 Examining the contour plot between time and PS further confirmed that time did not have an
 454 effect on the FRAP whereas a reduction in the FRAP values was observed after a PS of
 455 500 μ m. Increase in PS has been reported to result in a reduced yield and content of anti-
 456 oxidants from pomegranate marc (Qu et al., 2010).

3.3 Multi response optimization

459 The contour plots clearly showed the effect of different process variables on the responses.
 460 Due to large number responses, the optimization was carried out by Numerical option of the
 461 Design expert software to achieve the best combination of input factors for obtaining
 462 maximal release of phytochemicals accompanied by a good growth of bacteria and acid

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463 production. In the numerical optimization, the desired goal for each factor and response was
464 selected. The desired goal was selected by adjusting the weight or importance that might alter
465 the characteristics of a goal. A weight can be assigned to each goal to adjust the shape of its
466 particular desirability function. The software then converts the goals into an overall
467 desirability function. Desirability function ranges from zero to one for the goals and the
468 program searches to maximize this function. Figure 2 shows a ramp desirability that was
469 generated from 10 optimum points via numerical optimization. The program randomly picks
470 a set of conditions from which it starts the search for desirable results. The ramp display
471 combines the individual graphs for easier interpretation. The dot on each ramp reflects the
472 factor setting or response prediction for that solution. The height of the dot shows how
473 desirable a particular response is. By seeking from 10 starting points in the response surface
474 changes, the best local maximum was found to be 10.4 log cfu/ml, 268.6 mg GAE/ml TPC,
475 135 mg QE/ml TFC, 2.95 g/l lactic acid, 33.7 mg TE/ml and DPPH-RSA of 75.1% with the
476 optimized factors of 19h fermentation time, 0.25 SL, 85rpm and 440 μ m PS. The individual
477 desirability functions (d_i) for each of the responses and the calculated geometric mean as
478 maximum overall desirability ($D=0.882$). Since it is difficult to obtain the exact PS of the
479 BSG as given by the software, PS obtained in the range of 355-500 μ m (passing through
480 500 μ m but retained on 355 μ m) was selected as the optimized one. Duplicate confirmatory
481 experiments were conducted using the optimized parameters for validation. Experimental
482 verification resulted in production of 10.33 \pm 0.12 log cfu/ml and 2.5 \pm 0.22 g/l lactic acid
483 which were in close agreement with the predicted values. The values of TPC (241.4 \pm 1.7 mg
484 GAE/ml), TFC (123.4 \pm 4.7 mg QE/ml), FRAP (31.64 \pm 0.22 mg TE/ml) and DPPH-RSA
485 (69.1%) were also closely related to the data obtained from optimization analysis although
486 exact matching was not obtained due to a difference in the PS optimized by the software and

1 487 that used for the experiment. However, the experiment affirms that the models developed
2 488 could adequately predict the responses.
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6 7 490 ***3.4 HPLC-DAD analysis of the different broths and fermented product***

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9 491 HPLC analysis of phenolics in the SA broth and DA broth obtained from the RSM optimized
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11 492 factors and fermented product was carried out to record the UV-vis spectrum. Explicit
12
13 493 attribution of each chromatographic peak to distinct class of polyphenols was carried out on
14
15 494 the basis of a characteristic UV-vis spectrum for each class. Two different groups of
16
17 495 polyphenols were identified by comparing their UV-vis spectra with spectra of reference
18
19 496 compounds and reported values (Abad-García et al., 2009). The hydroxybenzoic acid (HBA)
20
21 497 derivatives (range 255-280nm) were quantified at 280nm and expressed as gallic acid
22
23 498 equivalents (GAE) and hydroxycinnamic acid (HCA) derivatives (range 310-325 nm) at
24
25 499 320nm and expressed as chlorogenic acid equivalents (CAE). A distinctive HPLC
26
27 500 chromatogram of the polyphenols released in the broth is shown in figure 3. The
28
29 501 chromatogram showed that the broth contained a mixture of more than 20 phenolics in SA
30
31 502 broth and 21 phenolics in DA and fermented broth. Fourteen peaks were identified as HBA
32
33 503 derivatives and six peaks as HCA derivatives in the case of SA broth. This number increased
34
35 504 to 15 (HBA derivatives) and 5 (HCA derivatives) for DA broth. For fermented broth, 15 and
36
37 505 6 peaks were identified as HBA and HCA derivatives, respectively. The HPLC results (table
38
39 506 4) clearly replicated the values obtained in the TPC assay wherein an increase in the content
40
41 507 of phenolic compounds was seen in the DA broth (as compared to the SA broth) which then
42
43 508 reduced slightly upon fermentation. The difference in values of the spectrophotometric
44
45 509 analysis and HPLC analysis could be due to the formation of free hydroxyl group(s) by
46
47 510 linkage cleavages of polyphenol derivatives, for example, flavonoids, as compared to the
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2 511 quantitative content of hydroxybenzoic acid or hydroxycinnamic acids detected by HPLC-
3 512 DAD (Harbaum et al., 2008).

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6 7 514 **3.5 Shelf life analysis**

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9 515 The viable cell count at the end of the 19 h fermentation period was found to be 10.35 log
10 516 cfu/ml. The stability of *L. plantarum* during storage was monitored (figure 4) and a reduction
11
12 517 of 1.34 log cfu/ml was seen at the end of the 30 days storage period. The reduction was found
13
14 518 to be significant (P<0.05). However, there was no significant difference in the log cfu/ml up
15
16 519 to 15 days of storage period and the cell numbers started declining only after that. These
17
18 520 results indicated that *L. plantarum* was capable of surviving for 15 days under high acidic
19
20 521 conditions of BSG based fermented product during storage at 4°C. High survival rates of *L.*
21
22 522 *plantarum* in fermented products during storage under refrigerated conditions have been
23
24 523 reported earlier (Gupta et al., 2010; Yoon et al., 2006). Mårtensson et al., (2002) reported
25
26 524 high survival of *Lactobacillus reuteri* in oat based non-dairy products after 30 days of
27
28 525 storage.

29
30 526 The lactic acid content remained constant up to day 8 after (figure 4) which a slight reduction
31
32 527 of 4% was observed till day 15. However, storage beyond 15 days resulted in a continuous
33
34 528 reduction with the content significantly reducing (P<0.05) by 28% at the end of 30 days
35
36 529 storage period. Results from the shelf life analysis showed that the pH on day 1 was 3.27 and
37
38 530 remained almost constant up to 15 days. A slight increase in the pH value (figure 4) was seen
39
40 531 after 18 days. However, single factor ANOVA analysis showed that the change in pH was not
41
42 532 significant (P>0.05). Similar results were reported by Rozada et al., (2009) during the
43
44 533 fermentation of a malt based beverage by *Bifidobacterium breve*.

45
46 534 The TPC and TFC value on day 1 was 220 ± 2.3 mg GAE/ml and 105.4 ± 5.6 mg QE/ml,
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48 535 respectively. There was a slight reduction in the values after 15 days of storage which were

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536 further retained for the next 15 days storage period. No significant ($P>0.05$) change in the
537 antioxidant content was observed upon the 30 day storage period. HPLC analysis was also
538 carried out on the samples of day 15 and day 30 in order to analyze any change in the peak
539 areas for the phenolic compounds. The content of HBA and HCA had significantly reduced
540 after 15 days storage period. However, there was no significant difference ($P>0.05$) in the
541 HBA and HCA content in samples between 15 and 30 days storage.

542

543 ***3.6 Fermentation in a 7-L bioreactor***

544 In order to study the growth kinetics on a large scale, a 7L lab scale BioFlo (New Brunswick,
545 Mason Technology, Dublin, Ireland) bioreactor was used. Fermentation of BSG with a SL
546 ratio of 0.25 and PS in the range of 355-500 μm was carried out for 19 h at an agitation of
547 100 rpm and under controlled conditions of pH (at 7). The content of TPC, TFC, DPPH and
548 FRAP in the DA broth was 239.6 ± 2.1 mg GAE/ml, 120.8 ± 2.9 mg QE/ml, 30.98 ± 1.1 mg
549 TE/ml and 68.7% RSA, respectively. The change in cell growth and acid production as a
550 result of the growth of *L. plantarum* can be seen in figure 5. The level of phytochemicals
551 (TPC and TFC) and antioxidants (DPPH and FRAP) essentially remained unchanged during
552 the course of fermentation. The lag phase was found to be even less than 2 h as evident from
553 an increase of 0.6 log cfu/ml after 2 h of fermentation. Occurrence of minimal lag phase has
554 been reported in earlier studies on the growth of LAB in vegetable juice (Gupta et al., 2010;
555 Kun et al., 2008) whereas Kedia et al., (2007) reported a lag phase of approximately 2 h upon
556 the inoculation of *L. reuteri* cells into 5% malt suspension. Cell concentration increased from
557 6.4 ± 0.02 log CFU/ml to 10.68 ± 0.03 log CFU/ml after 19 h of fermentation with a rate of
558 production equivalent to 0.39 h^{-1} . Such a cell growth led to the consumption of 2.1 g/l total
559 sugar. The viable cell numbers almost became constant as the stationary phase was achieved.
560 The cell numbers obtained in the bioreactor were higher than those obtained when the

1 561 cultivation was carried out in flasks. The reason for this could be the controlled pH in the
2 562 bioreactor which prevented the reduction in growth rate generally observed in flasks when
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4 563 fermentation is carried out under uncontrolled pH. Studies carried out under controlled pH
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7 564 conditions have indicated that the accumulation of acids during the fermentation is
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9 565 responsible for decrease in growth rate (Desjardins et al., 1990). Volumetric productivities of
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11 566 *L. plantarum* was 2.5×10^{12} cfu/l h. Nazzaro et al., (2008) reported the possibility of
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13 567 producing a functional vegetal beverage based on the growth of *Lactobacillus rhamnosus* and
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15 568 *Lactobacillus bulgaricus* in carrot juice with a growth of 5×10^9 cfu/ml in 48 h, which
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17 569 corresponds to a volumetric productivity of 1.04×10^{11} cfu/l h. However, the results of the
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19 570 present study are better as similar counts were obtained in shorter time resulting in higher
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21 571 productivity.
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26 572 The consumption of sugars during the exponential phase (6–14 h) resulted in the
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28 573 accumulation of lactic acid (2.7 ± 0.7 g/l) and acetic acid (0.4 ± 0.01 g/l). A homofermentative
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30 574 metabolic pattern was observed for *L. plantarum* resulting in a production of mainly lactic
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32 575 acid and small amounts of acetic acid. Peaks for other organic acids such as propionic, malic
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34 576 or citric were not observed further confirming a homofermentative metabolic pathway.
35
36 577 Methanol and ethanol were not detected in the fermentation broth. The accumulation of lactic
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38 578 acid continued during the stationary phase as well although the rate of accumulation had
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40 579 drastically reduced. Once again the amount of lactic acid formed was higher than that
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42 580 obtained for the values obtained in the flasks and can be attributed to the fermentation under
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44 581 controlled pH conditions. However, due to the controlled pH conditions in the bioreactor the
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46 582 product obtained was not acidic in nature and a difference in the aroma between the product
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48 583 fermented in the bioreactor and that fermented in the flasks under uncontrolled pH conditions
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51 584 was detected.
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586 **4. Conclusion**

587 Successful production of a fermented product from an industrial waste was carried out. Heat
588 processing resulted in a release of bioactive compounds into the broth which were then
589 retained upon the process of fermentation. The optimization of the different factors affecting
590 the production of a functional drink established the appropriate solid liquid ratio, particle
591 size, fermentation time and rate of agitation for completing a controlled fermentation. The
592 finished product had high antioxidant capacity and could support the growth of LAB as well.
593 The phytochemical content remained unchanged during the storage period. Shelf life of the
594 fermented liquid product was estimated to be around 15 days under refrigerated conditions.
595 Large-scale fermentation for the production of drink was also successfully carried out. The
596 present study showed a good adaption of LAB in BSG based broth thus opening potentials
597 for the use of probiotic strains as a starter culture for a further improvement in the bioactive
598 property of the finished product. In addition, further studies regarding the sensory attributes
599 are needed in order to obtain a commercially viable product.

600

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604

605 **References**

606 Abad-García, B., Berrueta, L.A., Garmón-Lobato, S., Gallo, B., Vicente, F., 2009. A general
607 analytical strategy for the characterization of phenolic compounds in fruit juices by high-
608 performance liquid chromatography with diode array detection coupled to electrospray
609 ionization and triple quadrupole mass spectrometry. *J. Chromat. A* 1216, 5398-5415.

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56
57
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65
- 610 Abdel-Fattah, Y.R., Saeed, H.M., Gohar, Y.M., El-Baz, M.A., 2005. Improved production of
611 *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical
612 experimental designs. *Process Biochem.* **40**, 1707–1714.
- 613 Ainsworth, P., Ibanoglu, S., Plunkett, A., Ibanoglu, E., Stojceska, V., 2007. Effect of brewers
614 spent grain addition and screw speed on the selected physical and nutritional properties of an
615 extruded snack. *J. Food Eng.* **81**, 702–709.
- 616 Bartolomé, B., Santos, M., Jiménez, J.J., del Nozal, M.J., Gómez-Cordovés, C., 2002.
617 Pentoses and hydroxycinnamic acids in brewer's spent grain. *J Cereal Sc.* **36**, 51-58.
- 618 Cacace, J.E., Mazza, G., 2003. Optimization of extraction of anthocyanins from black
619 currants with aqueous ethanol. *J. Food Sc.* **68**, 240-248.
- 620 Carvalho, F., Esteves, M.P., Parajó, J.C., Pereira, H., Girio, F.M., 2004. Production of
621 oligosaccharides by autohydrolysis of brewery's spent grain. *Bioresource Technol.* **91**, 93-
622 100.
- 623 Charalampopoulos, D., Pandiella, S.S., Webb, C., 2002. Growth studies of potentially
624 probiotic lactic acid bacteria in cereal-based substrates. *J. Appl. Microbiol.* **92**, 851–859.
- 625 Desjardins, M.-L., Roy, D., Toupin, C., 1990. Uncoupling of growth and acids production in
626 *Bifidobacterium spp.* *J. Dairy Sc.* **73**, 1478-1484.
- 627 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method
628 for determination of sugars and related substances. *Anal. Chem.* **28**, 350–356.
- 629 El-Shafey, E.I., Gameiro, M.L.F., Correia, P.F.M., De Carvalho, J.M.R., 2004. Dewatering of
630 brewer's spent grain using a membrane filter press: a pilot plant study. *Separ. Sc. Technol.*
631 **39**, 3237–61.
- 632 Franco, D., Pinelo, M., Sineiro, J., & Núñez, M.J., 2007. Processing of *Rosa rubiginosa*:
633 Extraction of oil and antioxidant substances. *Bioresource Technol.* **98**, 3506-3512.

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634 Gupta, S., Cox, S., Abu-Ghannam, N., 2010. Process optimization for the development of a
635 functional beverage based on lactic acid fermentation of oats. *Biochem. Eng. J.* 52, 199–204.
636 Harbaum, B., Hubbermann, E.M., Zhu, Z., Schwarz, K., 2008. Impact of fermentation on
637 phenolic compounds in leaves of Pak Choi (*Brassica campestris* L. ssp. *chinensis* var.
638 *communis*) and Chinese Leaf Mustard (*Brassica juncea* Coss). *J. Agric. Food Chem.* 56, 148–
639 157.
640 Heong, C.S., Kaur, B., Huda, N., Karim, A.A., Fazilah, A., 2011. Effect of fermentation on
641 the composition of *Centella asiatica* Teas. *Am. J. Food Technol.* 6, 581-593.
642 Jaiswal, A.K., Rajauria, G., Abu-Ghannam, N., Gupta, S., 2012. Effect of different solvents
643 on polyphenolic content, antioxidant capacity and antibacterial activity of Irish York
644 cabbage. *J. Food Biochem.* 36, 344-358.
645 Kedia, G., Vázquez, J.A., Pandiella, S.S., 2008. Fermentability of whole oat flour, PeriTec
646 flour and bran by *Lactobacillus plantarum*. *J. Food Eng.* 89, 246-249.
647 Kedia, G., Wang, R., Patel, H., Pandiella, S.S., 2007. Use of mixed cultures for the
648 fermentation of cereal-based substrates with potential probiotic properties. *Process Biochem.*
649 42, 65-70.
650 Kun, S., Rezessy-Szabó, J.M., Nguyen, Q.D., Hoschke, A., 2008. Changes of microbial
651 population and some components in carrot juice during fermentation with selected
652 *Bifidobacterium* strains. *Process Biochem.* 43, 816-821.
653 Liu, S.C., Lin, J.T., Wang, C.K., Chen, H.Y., Yang, D.J., 2009. Antioxidant properties of
654 various solvent extracts from lychee (*Litchi chinensis* Sonn.) flowers. *Food Chem.* 114,
655 577–581.
656 Maaroufi, C., Melcion, J.P., de Monredon, F., Giboulot, B., Guibert, D., Le Guen, M.P.,
657 2000. Fractionation of pea flour with pilot scale sieving. I. Physical and chemical
658 characteristics of pea seed fractions. *Anim. Feed Sc. Technol.* 85, 61-78.

659 Mårtensson, O., Öste, R., Holst, O., 2002. The effect of yoghurt culture on the survival of
1
2
3 660 probiotic bacteria in oat-based, non-dairy products. *Food Res. Int.* 35, 775–84.
4
5 661 Mussatto, S.I., Fernandes, M., Dragone, G., Mancilha, I.M., Roberto, I.C., 2007. Brewer's
6
7 662 spent grain as raw material for lactic acid production by *Lactobacillus delbrueckii*.
8
9 663 *Biotechnol. Lett.* 29, 1973-1976.
10
11 664 Nazzaro, F., Fratianni, F., Sada, A., Orlando, P., 2008. Synbiotic potential of carrot juice
12
13 665 supplemented with *Lactobacillus* spp. and inulin or fructooligosaccharides. *J. Sc. Food Agric.*
14
15 666 88, 2271-2276.
16
17 667 Oyarekua, M.A., 2010. Sensory evaluation, nutritional quality and antinutritional factors of
18
19 668 traditionally co-fermented cereals/cowpea mixtures as infant complementary food. *Agric.*
20
21 669 *Biol. J. North Am.* 1, 950-956.
22
23 670 Öztürk, S., Özboy, O., Cavidoğlu, İ., Köksel, H., 2002. Effects of Brewers' spent grain on the
24
25 671 quality and dietary fiber content of cookies. *J. Inst. Brew.* 108, 23-27.
26
27 672 Patel, H.M., Pandiella, S.S., Wang, R.H., Webb, C., 2004. Influence of malt, wheat and
28
29 673 barley extracts on the bile tolerance of selected strains of lactobacilli. *Food Microbiol.* 21,
30
31 674 83–89.
32
33 675 Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J., Nunez, M.J., 2005. Effect of solvent,
34
35 676 temperature, and solvent-to-solid ratio on the total phenolic content and antiradical activity of
36
37 677 extracts from different components of grape pomace. *J. Agric. Food Chem.* 53, 2111-2117.
38
39 678 Qi, B., Yao, R., 2007. L-lactic acid production from *Lactobacillus casei* by solid state
40
41 679 fermentation using rice straw. *Bioresources* 2, 419-429.
42
43 680 Qu, W., Pan, Z., Ma, H., 2010. Extraction modeling and activities of antioxidants from
44
45 681 pomegranate marc. *J. Food Eng.* 99, 16-23.
46
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4
5 682 Rozada, R., Vázquez, J.A., Charalampopoulos, D., Thomas, K., Pandiella, S.S., 2009. Effect
6
7 683 of storage temperature and media composition on the survivability of *Bifidobacterium breve*
8
9 684 NCIMB 702257 in a malt hydrolysis. Int. J. Food Microbiol. 133, 14-21.
10
11 685 Taga, M.S., Miller, E.E., Pratt, D.E., 1984. Chia seeds as a source of natural lipid
12
13 686 antioxidants. J. Am. Oil Chem. Soc. 61, 928–31.
14
15 687 Tolonen, M., Rajaniemi, S., Pihlava, J.M., Johansson, T., Saris, P.E.J., Ryhanen, E.L., 2004.
16
17 688 Formation of nisin, plant-derived biomolecules and antimicrobial activity in starter culture
18
19 689 fermentations of sauerkraut. Food Microbiol. 21, 167–179.
20
21 690 Valverde, P., 1994. Barley spent grain and its future. Cerveza y Malta 122, 7–26.
22
23 691 Xiao, J.H., Chen, D.X., Wan, W.H., Hu, X.J., Qi, Y., Liang, Z.Q., 2006. Enhanced
24
25 692 simultaneous production of mycelia and intracellular polysaccharide in submerged cultivation
26
27 693 of *Cordyceps jiangxiensis* using desirability functions. Process Biochem. 41, 1887-1893.
28
29 694 Yoon, K.Y., Woodams, E.E., Hang, Y.D., 2006. Production of probiotic cabbage juice by
30
31 695 lactic acid bacteria. Bioresource Technol. 97, 1427–30.
32
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697 **List of figures**

698 **Figure 1:** Contour plot showing the effect of experimental factors on the growth of (a) *L.*
699 *plantarum*, (b) lactic acid, (c) TPC, (d) TFC, (e) FRAP and (f) DPPH

700 **Figure 2:** Multi response optimization showing desirability ramp for numerical optimization

701 **Figure 3:** A comparative HPLC-DAD chromatogram of BSG broth for tentative
702 identification of phenolic groups at 280nm after various processing treatments (3a) SA broth;
703 (3b) DA broth; (3c) fermented broth. Peaks marked: 1-hydroxybenzoic acid derivatives; 2-
704 hydroxycinnamic acid derivatives

705 **Figure 4:** Effect of storage time on the log cfu/ml (\diamond), pH (Δ) and lactic acid (g/l) (\square) of the
706 BSG based liquid product

707 **Figure 5:** Growth kinetics of *L. plantarum* in a 7 L bioflo bioreactor (\diamond : log cfu/ml; \square : Lactic
708 acid (g/l); Δ : total sugar (g/l))

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712 **Table 1:** Level and code of independent variables used for Box-Behnken experimental design

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Independent variables	Coded symbols	Levels		
		-1	0	+1
Fermentation time (h)	X_1	8	16	24
Solid liquid ratio	X_2	0.05	0.15	0.25
Agitation (rpm)	X_3	0	100	200
Particle size (μm)	X_4	350	525	700

Table 2: Predicted and experimental values for *L. plantarum* growth, lactic acid production, total phenolic content, total flavonoid content, DPPH-RSA and FRAP values for fermentation of BSG

		<i>L. plantarum</i>														
X_1	X_2	X_3	X_4	Lactic acid (g/l)		TPC (mg GAE/ml)		TFC (mg QE/ml)		DPPH (%)		FRAP (mg TE/ml)				
				Exp	Pred	Exp	Pred	Exp	Pred	Exp	Pred	Exp	Pred			
1	16	0.05	100	700	10.12	10.12	0.51	0.6	94.2	86.6	45	45.8	47.3	46.1	16.5	15.4
2	8	0.15	200	525	9.94	10.06	0.74	0.82	200.3	208.8	89.6	94.8	44.9	48.9	27.6	28.4
3	8	0.25	100	525	10.48	10.41	1.17	1.15	282.0	269.3	120.9	117.5	82.8	85.6	33.8	32.1
4	16	0.15	0	350	9.96	10.08	1.41	1.54	219.7	217.5	91.6	95.9	57.2	53.7	29.6	28.8
5	8	0.15	100	350	10.02	9.99	0.74	0.68	216.3	211.1	85.3	86.6	51.6	45.9	25.3	25.8
6	16	0.25	0	525	10.56	10.48	2.25	2.23	267.2	263.6	118.1	124.6	73.1	76.9	33.5	33.9
7	8	0.05	100	525	9.87	9.84	0.24	0.36	100.1	101.2	48.1	56	22.6	27.8	16.4	15.9
8	16	0.05	200	525	10.35	10.28	0.53	0.51	104.8	105.1	51.6	48.4	38	34.8	17.6	17.8
9	16	0.15	100	525	10.4	10.45	1.46	1.41	197.8	191.4	105	102	50.7	43.9	27.4	28.1
10	16	0.15	100	525	10.36	10.45	1.33	1.41	190.2	191.4	105.6	102	41.5	43.9	30.8	28.1

11	16	0.15	100	525	10.55	10.45	1.41	1.41	184.6	191.4	100.9	102	43.7	43.9	27.4	28.1
12	24	0.15	0	525	9.99	9.9	1.82	1.82	200.4	200.4	110	105.1	43.1	40.9	30.3	29.4
13	16	0.25	100	350	10.31	10.35	2.73	2.73	263.3	279.4	127.2	126.6	80.2	83.4	32.2	33.1
14	8	0.15	0	525	10.1	10.11	0.68	0.65	193.6	201.2	100.6	92.4	48.3	47.6	26.9	27.5
15	24	0.25	100	525	10	10.15	3.57	3.41	264.5	258.2	151	139.5	71.5	63.8	32.8	32.8
16	16	0.05	100	350	10.05	10.04	0.65	0.45	105.3	105.1	52.2	45.9	22.6	27.1	16.2	15.7
17	16	0.15	200	350	10.2	10.23	1.52	1.6	210.9	206.5	95	90.2	44.6	39.8	28.4	27.8
18	16	0.15	200	700	10.34	10.33	1.64	1.46	193.3	190.3	96.3	88.3	59.5	60.5	26.5	26.9
19	24	0.15	100	700	10.2	10.08	1.57	1.6	173.5	175.4	87.2	89.2	38.3	44.6	24.9	24.9
20	16	0.15	0	700	10.11	10.2	1.32	1.19	172.8	172.2	84.4	85.6	42.5	44.7	25.8	26.1
21	24	0.05	100	525	9.87	10.05	0.46	0.43	94.9	102.2	51.9	51.7	40.8	35.5	15.8	17.1
22	24	0.15	100	350	10.2	10.05	2.05	2.11	221	217	98.4	104.5	39.1	45.4	28.6	28.9
23	8	0.15	100	700	10.18	10.18	0.78	0.69	190.4	191.2	92.2	89.5	63.8	58.2	25.8	26.1
24	24	0.15	200	525	10.21	10.23	1.87	1.98	198.9	199.8	91.3	99.7	39	41.5	29.1	28.3
25	16	0.25	100	700	10.45	10.49	1.81	2.09	227.7	236.3	107.8	114.4	78.5	75.9	29.5	29.8

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26	16	0.05	0	525	9.94	9.88	0.59	0.63	95.4	94.4	53.4	54.4	30.7	30.9	16.7	17.2
27	16	0.15	100	525	10.4	10.45	1.5	1.41	192	191.4	105.3	102	46	43.9	28	28.1
28	16	0.25	200	525	10.44	10.36	2.76	2.78	262.1	259.9	125.3	127.6	74.4	74.9	32.9	33
29	16	0.15	100	525	10.51	10.45	1.35	1.41	192.6	191.4	93.1	102	37.9	43.9	26.8	28.1

716 X₁: Fermentation time; X₂: Solid liquid ratio; X₃: Agitation; X₄: Particle size

717

Table 3: Coefficients of the response function for predicting the different responses by regression analysis and their significance values obtained

by ANOVA

	<i>L. plantarum</i>		Lactic acid		TPC		TFC		DPPH		FRAP	
	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value	t-value	P-value
X ₁	-0.29	0.7737	13.57	<0.0001	-1.03	0.3225	2.01	0.0637	-2.07	0.0579	1.33	0.2050
X ₂	4.94	0.0002	21.92	<0.0001	33.67	<0.0001	17.04	<0.0001	12.62	<0.0001	22.29	<0.0001
X ₃	2.01	0.0639	1.93	0.0742	0.73	0.4749	-0.35	0.7326	0.28	0.7871	-0.17	0.8672
X ₄	1.59	0.1346	-2.86	0.0127	-6.39	<0.0001	-1.40	0.1828	1.69	0.1129	-2.59	0.0214
X ₁ * X ₁	-5.37	<0.0001	-2.3	0.0371	2.01	0.0639	-0.26	0.7969	-0.08	0.9374	-0.69	0.5040
X ₂ * X ₂	-1.69	0.1123	1.09	0.2949	-4.65	0.0004	-3.36	0.0046	4.05	0.0012	-6.67	<0.0001
X ₃ * X ₃	-2.51	0.0249	0.75	0.4664	1.38	0.1890	-1.08	0.2996	0.43	0.6760	1.33	0.2061
X ₄ * X ₄	-2.5	0.0254	-0.11	0.9176	0.20	0.8454	-2.95	0.0106	2.04	0.0603	-2.76	0.0154
X ₁ * X ₂	-2.0	0.0647	7.31	<0.0001	-0.74	0.4707	1.73	0.1052	-2.49	0.0258	-0.15	0.8841
X ₁ * X ₃	1.61	0.1306	-0.07	0.9455	-0.5	0.6278	-0.51	0.6199	-0.06	0.9527	-0.81	0.4306
X ₁ * X ₄	-0.69	0.5011	-1.76	0.1008	-1.3	0.2147	-1.19	0.2526	-1.1	0.2881	-1.71	0.11

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$X_2 * X_3$	-2.2	0.0453	1.91	0.0762	-0.87	0.4004	0.6	0.5603	-0.5	0.6227	-0.58	0.5698
$X_2 * X_4$	0.29	0.7732	-2.61	0.0205	-1.47	0.1644	-0.80	0.4357	-2.24	0.042	-1.2	0.2509
$X_3 * X_4$	-0.09	0.9295	0.71	0.4897	1.75	0.1024	0.56	0.5873	2.51	0.025	0.77	0.4563

720 *X₁: Fermentation time; X₂: Solid liquid ratio; X₃: Agitation; X₄: Particle size

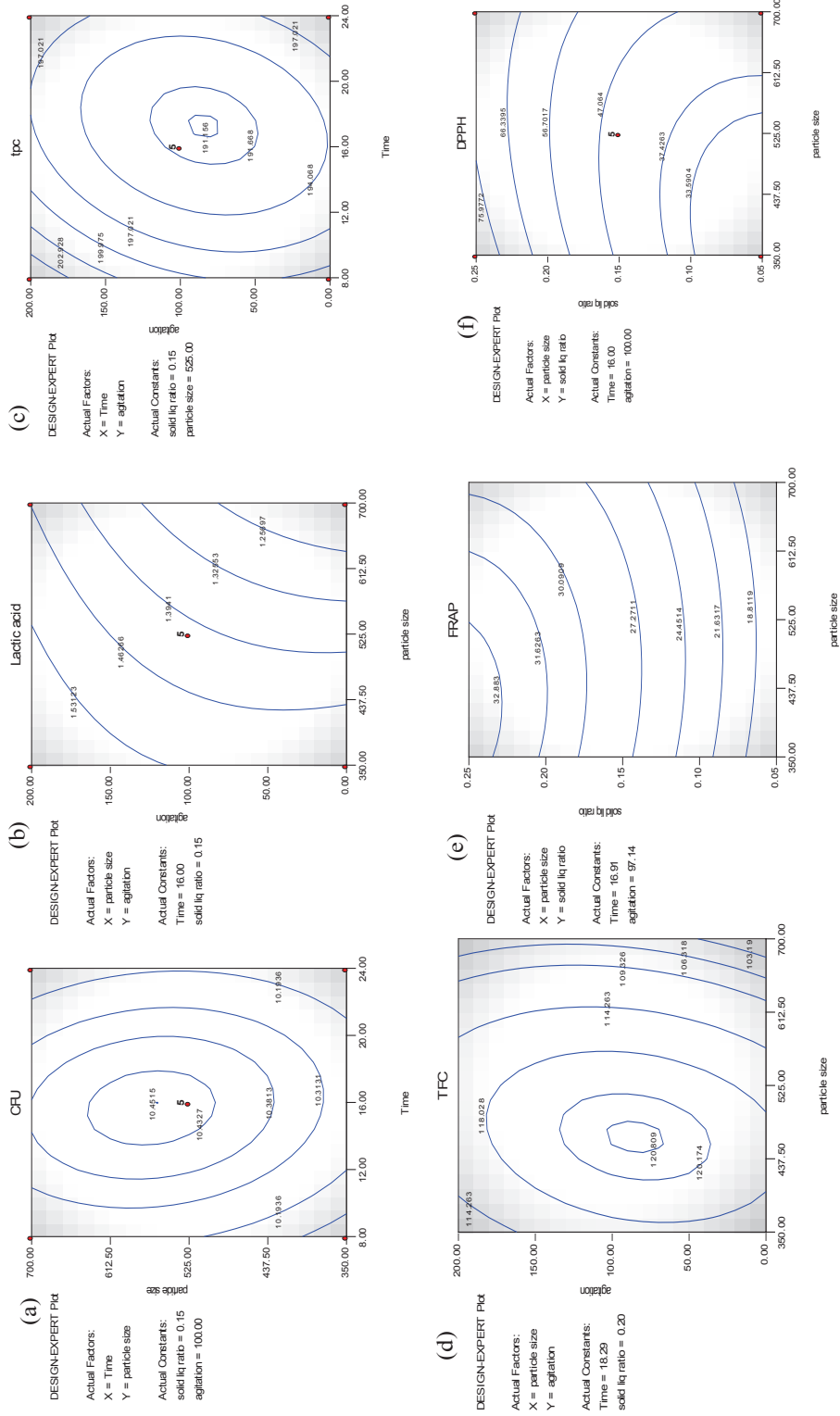
721 **Table 4:** Hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA) contents of broth
 722 at different stages of BSG fermentation

723

	HBA (GAE/ml)	HCA (CAE/ml)	Total
SA broth	50.9±6.4	10.7±0.8	61.68
DA broth	61.9±0.13	8.13±0.74	70.4
Fermented broth	53.7±0.2	9.8±0.22	63.49
Shelf life (day 15)	36.1±0.04	7.65±0.2	43.76
Shelf life (day 30)	35.7±3.2	8.1±0.06	43.8

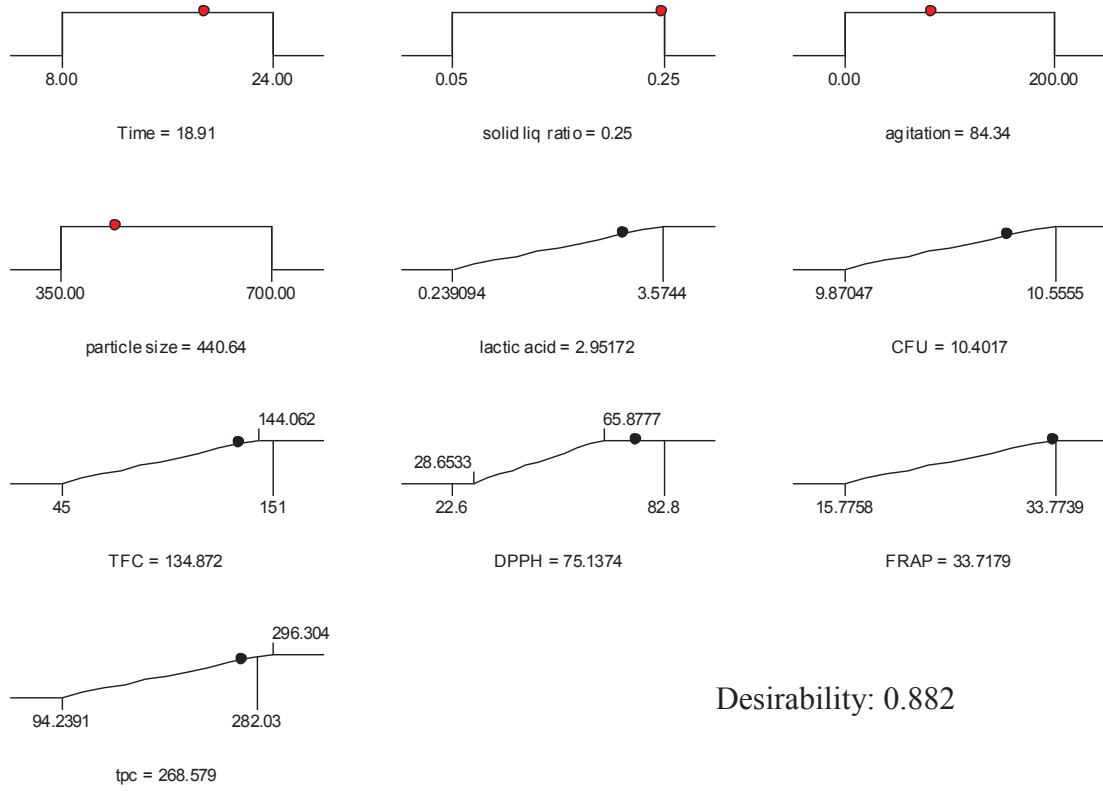
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725 **Figure 1**



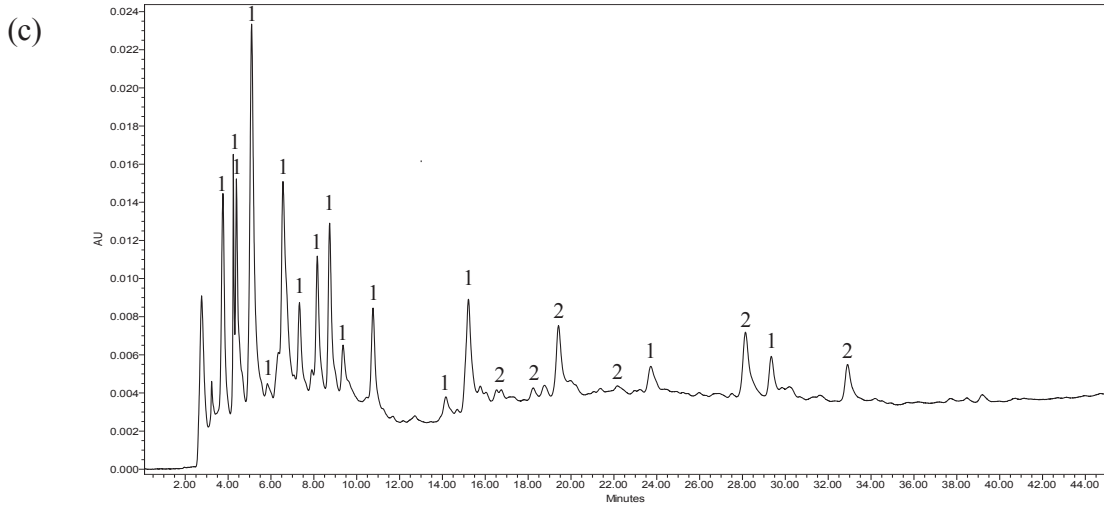
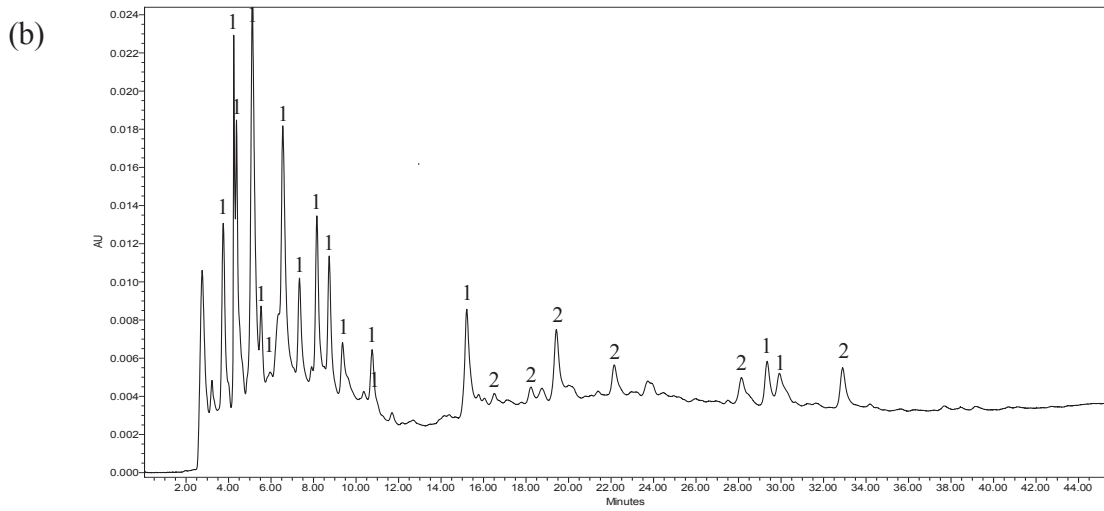
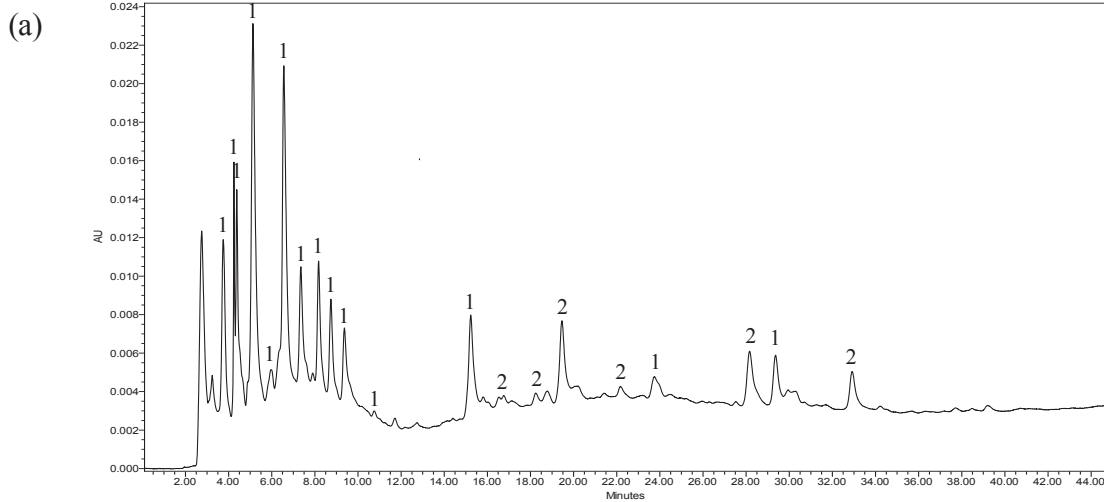
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Figure 2

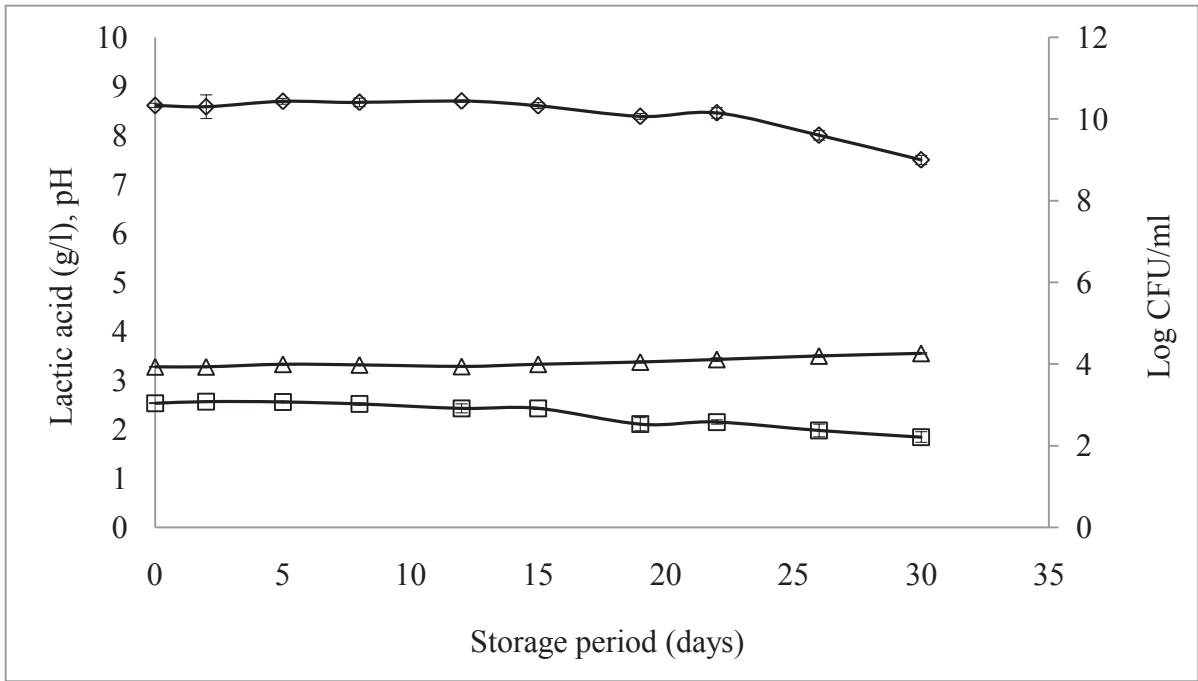


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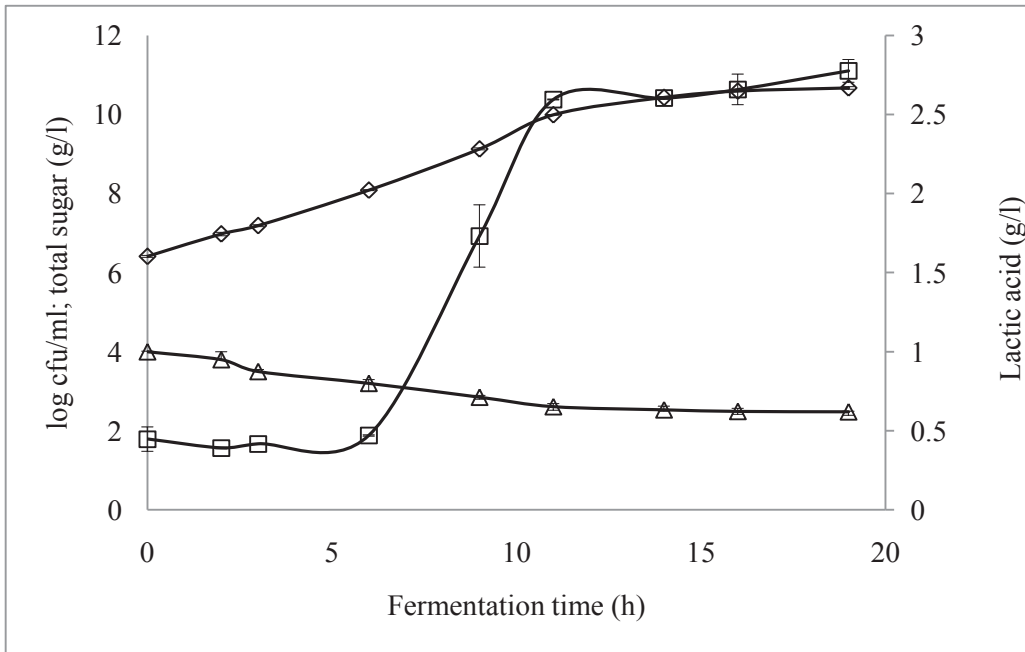
732 **Figure 4**



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736 **Figure 5**



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