Enzyme pre-treatment of soybean meal: effects on non-starch carbohydrates, protein, phytic acid, and saponin biotransformation and digestibility in mink (*Neovison vison*).

Hans Jákup Jacobsen*[a,b] , Katerina Kousoulaki[c], Ann-Sofie Sandberg[d], Nils-Gunnar Carlsson[d], Øystein Ahlstrøm[e], Åge Oterhals[c].

[a]Alltech Norway, Horness, 6809 Førde, Norway.

[b]University of Bergen, Department of Biology, Thormøhlensgate 53B, 5020 Bergen, Norway.

[c]Nofima, P.O. Box 1425 Oasen, 5828 Bergen, Norway.

[d]Chalmers University of Technology, Department of Biology and Biological Engineering, 412 96 Gothenburg, Sweden.

[e]Norwegian University of Life Sciences, Faculty of Biosciences, Department of Animal and Aquacultural Sciences, P.O. Box 5003, 1433 Ås, Norway.

*Corresponding author. Tel: +47 97410564. Email address: hjacobsen@alltech.com (H. J. Jacobsen).

Abstract

The study was conducted to examine if a crude enzyme complex (EC) produced from *Aspergillus niger* by solid state fermentation could bio-transform carbohydrates, proteins, inositol phosphate (InsP) and saponins in soybean meal (SBM) under low moisture conditions. Two experiments were performed to determine the effect of mixing, moisture content, treatment time and EC levels during enzyme hydrolysis. 1) A single replicate 2^3 full factorial screening design was used to determine the main effect of the independent continuous variables time (30 and 70 min), moisture (350 and 450 g kg^{-1} DM), and the category variable mixing (i.e. effect of static conditions *vs.* active mixing (X3, S/M)) during the enzyme hydrolysis. 2) A three factorial central composite design (CCD) was used to study the effects of moisture content (316-484 g kg^{-1} DM), time of hydrolysis (16.4-83.6 min), and EC content (0.32-3.68 g kg^{-1} SBM dry matter (DM)). The first experiment demonstrated that hydrolysis of $InsP_6$ was significantly affected by time ($P<0.01$), moisture content ($P<0.0001$) and static vs. mixing ($P<0.01$). The hydrolysis of Bb-DDMP was also significantly affected by static vs. mixing ($P<0.05$). The second experiment demonstrated that the hydrolysis of $InsP_6$ was significantly affected by time ($P<0.001$), moisture ($P<0.001$) and enzyme content ($P<0.01$). The $InsP_6$ content was reduced 84% after 70 min, at a moisture content of 450 g kg^{-1} DM with the addition of 3 g enzyme kg^{-1} SBM DM, the $InsP_5$-$InsP_3$ were not accumulated at these parameter settings. The hydrolysis of the saponin group Bb was explained by the enzyme square regressor ($P<0.001$) and the interaction between moisture and enzyme ($P<0.001$) reaching a maximum by the addition of 2.5 g enzymes kg^{-1} SBM DM at 484 g kg^{-1} moisture content. No significant effects on soluble protein and dietary fibre were observed.

Mink were fed three diet containing 50% of the protein from SBM, SBM treated without enzymes or treated with enzymes. There was a trend of improved ash apparent digestibility in mink by the enzyme treatment ($P=0.07$), but not for phosphorous. The apparent digestibility of protein and amino acids (AA) in mink was improved by the heat treatment used to inactivate the enzymes ($P<0.001$), however, not by the enzyme treatment itself.

Keywords: Enzyme pre-processing; Soybean meal; Response surface methodology; Anti-nutritional factors; Protein; Mink.

Abbreviations

DM, dry matter; AA, amino acids; EC, enzyme complex; ANFs, anti-nutritional factors; InsP, inositol phosphates; CCD, central composite design; ADC, apparent digestibility coefficient; SBM, De-hulled solvent extracted soybean meal; Soy 40, SBM treated in accordance to the center point conditions (400 g kg^{-1} DM moisture, 50 min), however, without the addition of enzyme.

1. Introduction

Soybean dominates the oilseed market with a global annual production of 315.1 million metric tons (Soystats, 2015). Only 2% of the soybean protein is consumed by humans, the rest being used in feed for domestic animals and fish (OECD, 2015a; b). De-hulled solvent extracted and toasted soybean meal (SBM) is widely used in animal feed production because of its high protein content (approx. 48%) and relatively well balanced amino acid profile (Gatlin et al., 2007). However, SBM contains a number of heat-labile and heat-stabile anti-nutritional factors (ANFs). ANFs are defined as substances that, by themselves or through their metabolic products in living systems, interfere with food/feed utilization and affect the health and production performance of animals (Makkar, 1993). The heat-labile ANFs, including protease inhibitor and lectins, can be eliminated or reduced by heat treatment during the processing of SBM (Smith, 1977; Arndt et al., 1999). The heat-stable ANFs include non-starch polysaccharides (NSPs), oligosaccharides (raffinose, stachyose and verbascose), saponins, phytate, phytoestrogens and protein antigens, some of which can be eliminated by aqueous or solvent extraction, fractionation, or digestion by exogenous enzymes (Rumsey et al., 1994; Kaushik et al., 1995; Refstie et al., 2005; Denstadli et al., 2006b; Knudsen et al., 2006).

Addition of exogenous enzymes to improve the utilization of pelletize pig and poultry diets has been successful (Omogbenigun et al., 2004; Woyengo et al., 2010). The use of enzymes in feed to warm water fish, has demonstrated to be successful if added to pelleted feed or by coating (Jackson et al., 1996; Debnath et al., 2005; Liebert and Portz, 2005; Yoo et al., 2005). However, the application in feed for cold water species is less attractive due to lower enzyme activity. In addition, the applied extrusion process during feed manufacturing gives high heat treatment (120-140°C) and mechanical forces that denatures the enzymes. To overcome these limitations, enzymatic pre-processing of the ingredient can be a feasible solution to remove ANFs in plant ingredients and improve nutrients digestibility. Typically peak enzyme activities are obtained between 40-55°C (Denstadli et al., 2006a; Jacobsen et al., 2017). Feeding Atlantic salmon with diets containing phytase pre-treated wheat and soy protein concentrate (SPC) has shown to improved ash and phosphorous digestibility (Denstadli et

al., 2007), whereas the use of carbohydrases to pre-treat a mixed-vegetable dietary blend, did not improve the growth and feed conversion ratio of Atlantic salmon (Denstadli et al., 2011). SBM is a less expensive ingredient compare to alcohol extracted SPC and has a higher potential for improvements and economical incentives based on enzymatic pre-treatment.

High solid enzymatic hydrolysed ingredients might be use directly in the extrusion line for production of compound fish feed (Denstadli et al., 2006b) as long as the ingredient can be mixed homogenously into the feed mix and that the total moisture content in the mix do not surpass approximately 30% in the extruder (Rockey, 2000), because it is difficult to achieve sufficient friction in the extruder at higher moisture content, hence effect the physical quality of the feed.. Steam is added to the mash in the pre-conditioner to increase the temperature (80-90°C) and moisture (25-30%) (Rokey, 1994). The use of high moisture during an enzymatic hydrolysis of an ingredient, an extra drying step is required which increases the energy costs and may negatively affect the nutritional value of the ingredient if the drying temperature is not lenient (Finley, 1989).

The objectives of this study were: 1) To investigate the ability of an *Aspergillus niger* enzyme complex to hydrolyze proteins and reduce the ANFs in SBM at different reaction conditions (mixing, time, moisture and enzyme addition), and 2) to study the nutrient digestibility of the most promising pre-treated SBM against a non-treated SMB in mink.

The enzyme complex used has been described in a previous study (Jacobsen et al., 2017). Total tract apparent amino acid digestibility values in mink (*Neovison vison*) is highly correlates with the apparent amino acid in salmon and chicken, and with ileal digestibility in pigs (Skrede et al., 1998) and dogs (Tjernsbekk et al., 2014).

2. Materials and methods

2.1 *Materials*

De-hulled and solvent extracted SBM was obtained from DSM (Kaiseraugst, Switzerland). The ingredients were ground in a hammer mill (Jesma type 650/315, Jesma-Matodor AS, Vejle, Denmark)

with a 1 mm screen and sifted in a Tumbler Screener (TSM 1200, Allgaier werke GmbH, Uhingen, Germany) with a 0.8 mm aperture at the Feed Technology Center of Nofima in Bergen, Norway. The SBM was homogenized in a horizontal mixer (Amandus Kahl Nache, Hamburg-Reinbek, Germany) for 30 min before packing in 25 kg bags.

2.2 *Enzyme assay*

The EC used in our study was produced by *Aspergillus niger* using solid state fermentation. The crude enzyme product was mixed with 20 mL 100 mM sodium acetate buffer (pH 5.5) and shaken at 220 rpm for 1 h. The mixture was then centrifuged at 5000 rpm for 10 min and the supernatant collected as the crude enzyme extract.

Phytase activity was determined according Engelen et al. (1994) at pH 5.5 and at 45°C. The protease activity was determined by the method of Jones et al. (1998) with some modifications. The reaction mixture consisted of 2 mL crude enzyme extract and 3 mL 1% gelatin (Sigma, G-1890, USA). The solution was incubated at 45°C for 30 min. The reaction was stopped by pipetting 3.75 mL of the reaction mixture into 5 mL of isopropanol and incubating in an ice-water bath for 15 min. The cooled reaction solution was centrifuged at 10000 rpm for 10 min, the supernatants were carefully removed with a Pasteur pipette and the absorbance measured at 280 nm with a UV-VIS spectrophotometer. One unit of enzyme activity (AU) was defined as the amount of enzyme that causes an increase of 0.001 absorbance unit.

Xylanase activity was determined using the method of Bailey and Poutanen (1989) at pH 5.5 and at 45°C. Cellulase activity was determined by the method of Miller et al. (1960) at pH 5.5 and at 45°C. The enzyme activities measured at pH 5.5 and 45°C were: 1253 SPU g^{-1} phytase, 0.196 U g^{-1} protease, 512 XU g^{-1} xylanase and 104 CMCU g^{-1} cellulase.

2.3 *Enzymatic treatment of soybean meal*

155 The SBM was incubated with the *Aspergillus niger* EC in a modified 10 L Varimixer (Metos AR-10,
156 Wodschow & Co A/S, Brøndby, Denmark). A heating jacket connected to a water bath (Julabo TP-12,
157 Julabo labortechnik GmbH, Seelback, Germany) was used to control the temperature based on a
158 temperature sensor in direct contact with the reaction mixture. The bowl was closed with a lid
159 containing a cooling element to condense evaporated water which drained back into the mixture. A
160 temperature logger (EBI 10, Ebroelectronic GmbH & Co. kg, Peringerstraβe, Germany) was used to
161 monitor the temperature during the hydrolysis.
162 The Varimixer was pre-conditioned for one hour, before adding SBM (1000 g DM) and moisture
163 according to the design (Table 1). Moisture was added on a dry matter basis and the pH of the SBM
164 was adjusted to 5.5 by inclusion of 80.15 ml 2 M HCl in the added water. The moistened SBM was
165 conditioned by mixing at 26 rpm for 30 min at 45°C. The EC was added to 100 ml of water (45°C) and
166 conditioned under continuous mixing for 10 min. The hydrolysis commenced when the enzyme
167 mixture was added to the SBM at 39 rpm for 1 min and 26 rpm for the rest of the reaction time
168 (Table 1). The enzyme hydrolysis was stopped by rapidly heating the mixture to above 90°C in a
169 microwave oven (Menumaster commercial DEC18E2, ACP inc., USA) and kept above 80°C for 10 min
170 to terminate the enzyme activity. The hydrolyzed SBMs were divided into two parts: 100 g DM were
171 frozen and then freeze-dried before chemical analysis, and 900 g DM were used in the mink diets.
172 To assess any separate effect of the pH reduction, wet mixing and heat treatment, a SBM sample was
173 treated in accordance to the center point conditions (400 g kg^{-1} DM moisture, 50 min; Table 1),
174 however, without the addition of enzyme (Soy 40).
175 To test the main effect of mixing during the enzymatic hydrolysis the above protocol was repeated at
176 static conditions (Supplementary Table 1). After addition and mixing of the enzymes into the SBM at
177 39 rpm for one minute the Varimixer bowl was placed into an incubator at 45°C to maintain constant
178 temperature.
179
180 2.4 *Animals and diets*

181 The mink digestibility trial was carried out at the Center of Animal Research of the Norwegian
182 University of Life Sciences in Aas, Norway, in accordance with laws and regulations for ethical live
183 animal research (Animal Protection Ordinance concerning animal experiments 15th January, 1996).
184 Four healthy standard brown male mink were assigned for each one of the 3 experimental diets. The
185 animals were kept in individual cages equipped for controlled feeding and quantitative faecal
186 collection, separating feces and urine as described by Jørgensen and Hansen (1973). The experiment
187 was conducted in a ventilated room with controlled temperature and light. The digestibility
188 experiment lasted for seven days, of which three days were for adaptation to the feed and the
189 following four days for faecal collection (Skrede, 1979; Szymeczko and Skrede, 1990). The feed intake
190 and faecal production was recorded once, daily. Faeces from each animal were pooled over four days
191 and freeze-dried, ground and sifted to remove hair prior to analyses of dry matter, ash, crude
192 protein, amino acids, crude fat, energy, starch and phosphorus.
193 To ensure sufficient feed intake, 50% of the dietary protein in the experimental mink diets originated
194 from fishmeal (Norse-LT 94, Norsildmel AS, Bergen, Norway) and 50% from the SBM. The protein and
195 amino acid content, and digestibility of the fishmeal had been determined in a previous study. The
196 chemical analysis of the protein ingredients used in the mink diets is given in Table 2. The diet
197 composition is given in Table 3: Diet 1, untreated SBM (Untreated), diet 2, processed according to
198 ENo 8 in Table 1 but without the enzyme addition (÷Enzyme) and diet 3, processed according to ENo
199 8 in Table 1 (Enzyme).
200
201 2.5 *Chemical analyses*
202 Crude protein was determined by the Kjeldahl method (N x 6.25: ISO 5983-2). The water-soluble
203 fraction of the SBM was extracted with boiling water, filtered using paper filter, and the crude
204 protein content in the water phase determined by the Kjeldahl method. Moisture was determined
205 gravimetrically after drying at 105°C for 16 h (ISO 6496) and ash after 12 h at 500°C (ISO 5984). Total
206 lipid in the SBM was quantified by the Soxhlet method (AOCS Ba 3-38). All samples were analysed at

Nofima BioLab, Bergen, Norway. The dietary fiber was quantified by the AOAC 991.43 method at Eurofins, Rotterdam, Netherlands. Saponins were analyzed by use of HPLC-DAD and quantified according to Knudsen et al. (2006) at Skretting ARC, Stavanger, Norway. Samples for phytic acid analysis were prepared as described by Sandberg and Ahderinne (1986) with some modifications prior to inositol phosphate analysis by HPLC. Duplicate samples of 0.5 g were treated with 10 ml 0.5 M HCl using a magnetic stirrer at 250 rpm and 20°C for 3 h and centrifuged, 5 ml of the supernatant extract was evaporated to dryness and dissolved in 10 ml of water. The inositol phosphates were separated from the extract by ion-exchange chromatography using plastic columns with porous filters containing 2.5 ml resin (AG 1-X8, 200-400 mesh). The extract was washed with 2×5 ml of water and the inositol phosphates were removed from the resin with 4×5 ml aliquots of 2 M HCl. The eluent was evaporated to dryness, diluted with 1 ml of 0.025 M HCl and centrifuged through an ultra-centrifugal filter device (Microcon YM-30, Millipore, Bedford, MA). The samples were analyzed on HPLC using hydrolyzed samples of sodium phytate for identification of inositol phosphate isomers as described by Carlsson et al. (2001). Quantification of the inositol phosphates was done according to Skoglund et al. (1997). All SBM samples were analyzed for inositol-6-phsophate ($InsP_6$), whereas the cube points were also analyzed for the inositol 5 to 3 phosphates ($InsP_5$-$InsP_3$). The InsP analysis where performed at Chalmers University of Technology, Gothenburg, Sweden.

In the digestibility study, meals and freeze dried samples of diets and faeces were analyzed for dry matter (ISO 6496 1999) and ash (ISO 5984 2002). Crude protein was determined as Kjeldahl-N x 6.25 (AOAC International 2002, method 2002.11) and fat content was determined after petroleum and ether and acetone extraction in an Accelerated Solvent Extractor (ASE 200, Dionex, Sunnyvale, CA, USA), starch was analyzed according to McCleary et al. (1994). The non starch carbohydrate content was estimated as the difference between the total wet weight and the sum of the other analyzed components (DM-(crude protein+starch+fat+ash). Amino acids, except tryptophan, were analyzed according to ISO 13903 (2005). Phosphorous was determined according to method described in

232 Endres and Rude (2001) applying Maxmat spectrophotometer (Montpellier, France). The analyses
233 where performed at the Norwegian University of Life Sciences, Ås, Norway.
234 The measurements were analyzed in duplicates except for saponins, which were analyzed in
235 triplicate and dietary fiber which were determined in single samples.
236
237 2.6 *Experimental design and Statistical analysis*
238 *2.6.1* Screening design
239 A single replicate 2^3 full factorial screening design was used to determine the effect of the
240 independent continuous variables time of hydrolysis (X1, minutes) and moisture (X1, g kg^{-1} DM), and
241 the category variable mixing (i.e. effect of static conditions *vs.* active mixing (X3, S/M)) during the
242 enzyme hydrolysis. The determination of the effects of the single replicate design was performed as
243 described previously (Daniel, 1959; Montegomery, 2001; Myers, 2002). The estimate of the effects
244 was plotted on a normal probability plot, negligible effects are normally distributed on the plot,
245 whereas the significant effects are far from the line. The non significant effects are pooled to form
246 the error term in the analysis of variance. Significant effects from the screening design will be tested
247 further in a central composite design. The moisture content was 350 and 450 g kg^{-1} DM and the time
248 was 30 and 70 min (Supplementary Table 1). The EC addition was kept constant at 3.0 g kg^{-1} SBM DM.
249 Main effect analysis of variance was used to test the significance (P>0.05) of the variables. The
250 response variables were dietary fiber, water soluble protein, inositol-6-phosphate ($InsP_6$) and
251 saponins (Supplementary Table 2).
252 *2.6.2 Optimization design*
253 Evaluation and optimization of the SBM enzymatic hydrolysis parameters was carried out by use of a
254 three factor CCD, comprising 17 settings, including 6 axial points (α) and 3 central points. The
255 distance from the axial points to the center points was calculated by the equation $\alpha = (2^k)^{1/4}$, where k
256 is the number of independent variables.

The design had three independent variables: moisture, time of hydrolysis, and EC content. The moisture content varied from 316 to 484 g kg^{-1} DM, the time from 16.4 to 83.6 min and the EC content from 0.32 to 3.68 g kg^{-1} SBM DM (Table 1). The response variables were dietary fiber, water soluble protein, inositol-6-phosphate ($InsP_6$) and saponins (Table 4). The experimental data were analyzed by a second order polynomial according to equation 1:

$$y = \beta_0 + \sum_{i=j}^{3} \beta_i x_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij}\, x_i x_j + \sum_{i=j}^{3} \beta_{ii}\, x_i^2 + \varepsilon \qquad (1)$$

where y is the predicted response, β_0 is the intercept, β_i, β_{ij} and β_{ii} are the measurements of the effects of variables x_i, $x_i x_j$ and x_i^2 respectively. Where x_i represents linear coefficient, the $x_i x_j$ represents the first order interactions between x_i and x_j (i<j), x_i^2 quadratic coefficient and ε is the residual (error).

The best fitted regressors in the model were identified by use of backward elimination of insignificant (*P* remove>0.05) variables. The results obtained from the experiment were submitted to analyses of variance (ANOVA). R^2 values and *F*-test was used to evaluate the quality of the models. Outliers were detected based on normal probability plot of Studentized residuals and removed before final modeling of the respective responses.

2.6.3 Mink digestibility

Statistical analysis for the mink digestibility data was realized by one-way analysis of variance where diet was the class variable, following the model:

$$\gamma_{ij} = \mu + a_i + \varepsilon_{ij} \qquad (2)$$

where γ_{ij} is the response, μ is the overall mean, a_i is the effect of the i treatment (diet) and ε_{ij} is the random error. The significant difference between means (*P*<0.05) was then determined by using Tukey`s multiple range test. Statistical analyses were performed with STATISTICA (v.12.0) from Statsoft (Tulsa, OK, USA).

3. Results

3.1. Screening design

A 2^3 factorial screening design was used to study the effect of the process variables time, moisture and static *vs.* mixing at constant enzyme level (Supplementary Table 1). The results (Supplementary Table 2) show significant negative effect on the $InsP_6$ level by time (F=59.8, P=0.002), moisture (F=379.5, P=0.00004) and static *vs.* mixing (F=39.7, P=0.003) (Supplementary Table 3 and Figure 1). The static *vs.* mixing variable also had a significant negative effect on Bb-DDMP (F=12.5, P=0.02) (Supplemantary Table 4 and Figure 2). No significant effects were observed for the other responses tested.

3.2. Optimization design

A 3-factor rotatable central composite design (CCD) was used to study the effect of the process variables, time (T), moisture (M) and enzyme (EC). Based on the significant effect of mixing in the screening design it was decided to keep this category variable constant. The obtained results (Table 4) were used to generate response surface models describing the effects of hydrolysis on inositol-6-phosphate ($InsP_6$) and saponin group Bb (Bb). No significant effects were observed on the soluble, insoluble and total dietary fiber (sDF, iDF, tDF), soluble protein, and the saponin groups; Ab, Bb-DDMP, Bb-DDMP, Bc and Bc-DDMP.

The main T, M, EC and the EC square effects were significant in the $InsP_6$ model (Table 5). The regression model had a R^2=0.94, however, a significant lack of fit (P=0.04). ENo 15 was removed as an outlier in the saponin modeling together with the analytical parallels 10, 18 and 51 (Supplementary Table 5). The EC square and M×EC interaction were significant for the Bb saponin model. The regression model of Bb saponins was significant with R^2=0.68.

The level of soluble protein, $InsP_6$, Ba-DDMP, Bb-DDMP and Bc-DDMP was higher for the SBM sample compare to all the treated samples, whereas the Bb level of the SBM was lower compare to the

treated samples. The processed Soy 40 (center point without enzyme addition) sample had a lower level of soluble protein and a higher content of $InsP_6$ compare to the rest of the treated samples.

3.3. Mink digestibility

There were no concerns regarding animal health and appetite during the experiment. The chemical composition of the ingredients used, feed formulations and the apparent digestibility in mink is shown in Table 2, 3 and 6, respectively. There were no significant digestibility differences among the diets on starch and non starch carbohydrate digestibility. The Untreated diet had a significant lower digestibility of protein, phosphorous and DM than ÷Enzyme and Enzyme diets (Table 6). The fat digestibility of the ÷Enzyme diet was significantly higher than the other two diets. The digestibility of energy were significant different among the diet where ÷Enzyme > Enzyme > Untreated. The digestibility of ash was close to significant (P=0.07) and showed a trend with Enzyme different to Untreated, with ÷Enzyme in between.

The digestibility of amino acids was significantly different among the diets. Generally, there was a trend that digestibility of AA was lowest for the Untreated diet, but not for all AA. The digestibility of threonine was the only AA that showed the highest values in Enzyme treated. The digestibility of leucine, phenylalanine, arginine and tyrosine was significantly different between all the diets with highest values for ÷Enzyme, intermediate with Enzyme and lowest for Untreated. The methionine digestibility of Enzyme diet was significant higher than ÷Enzyme and the Untreated was in between. The digestibility of alanine was significantly higher for the ÷Enzyme compare to the other two. The digestibility of cysteine was significantly higher for the Enzyme diet compare to the Untreated, with the ÷Enzyme in between.

4. Discussion

4.1. Screening design

330 The 2^3 full factorial design identified that mixing had a significant effect on the hydrolysis of $InsP_6$ and
331 Saponin Bb-DDMP. Mixing is important as it enhance the mass transfer of enzyme reaction. With a
332 high moisture content, molecules and particles are free flowing in the slurry while a reduction in the
333 moisture will increases the viscosity of the slurry as particles start to aggregate. The increased
334 viscosity increases the shear rate, and cellulase has shown to be sensitive to shear force (Gunjikar et
335 al., 2001). Although, high solid (300-400 g kg^{-1}) enzymatic hydrolysis of wheat bran with the use of
336 xylanase during continuous mixing has shown to be efficient (Santala et al., 2011; Santala et al.,
337 2013). The moisture range applied in this experimental design was based on initial trials to secure a
338 free flowing wetted powder during and after the enzymatic treatment. With a higher moisture
339 content, the SBM behaved like a plastic dough, making it difficult to dry, or use further in feed
340 manufacturing operations. No visible free water could be observed in the studied moisture range.
341 The results confirm that mixing of the wetted powder is important by contributing to improved
342 distribution of the EC in the mixture and enhanced mass transfer in the enzymatic reactions.
343 The design temperature of 45°C is below the optimum of 50-55°C for the EC (Jacobsen et al., 2017),
344 however it is a practical compromise to minimize any enzyme inactivation due to higher heat surface
345 temperature in the reactor vessel used. The applied reactor system required the use of a heat
346 exchanger temperature of 58.4°C to maintain a SBM temperature of 50°C. This might give heat
347 inactivation of enzymes in contact with the bowl wall. By reducing the reaction temperature to 45°C,
348 the level could be maintained by a heating temperature of 51.2°C, which is within the optimal
349 window of activity. The critical moisture level for a free flowing wetted powder of SBM was found to
350 be 569 g water kg^{-1} DM at 45°C. When the EC was included at this moisture level, the mixture formed
351 a plasticized and pasty mass after a short reaction time. This could be attributed to the enzymatic
352 hydrolyzes of SBM and is consistent with observations reported by Santala et al. (2011) when
353 hydrolyzing wheat bran with xylanase. The low molecular organic and inorganic (phosphate) reaction
354 products generated may act as additive plasticizers in combination with moisture, as described by
355 Samuelsen and Oterhals (2015) and Oterhals and Samuelsen (2015). This will reduce the glass

transition temperature through the formation of a plasticized material above critical moisture levels. In this experiment, it was important to conserve the flow properties of the SBM to facilitate homogeneous mixing during the enzymatic reaction and a final product possible to mix and dry in post-treatment operations.

4.2. Optimization design

Previous results using an enzyme complex produced by *Aspergillus niger* has shown that these enzymes are able to hydrolyze carbohydrates in soybean flour (Loman et al., 2016; Loman and Ju, 2016). However, these experiments were conducted with a significantly lower solid content (50-250 g L^{-1}), a higher enzyme to soy flour ratio and time of hydrolysis (5-50 h). This indicates that a higher moisture range is required to obtain effects on carbohydrate hydrolysis, however, with a compromise in the powder flow-ability. A higher moisture content above the plastic dough range might have improved the enzymatic activity, however, this was outside the scope of this study. Nevertheless, Denstadli et al. (2011) obtained minor changes in the carbohydrate fraction of a soybean meal using similar moisture and temperature levels as in this study, although with a different enzyme product.

The SBM had a higher soluble protein content compared to the treated samples. The pH of the SBM was 6.3 and prior to the hydrolysis this was reduced to 5.5 by the addition of HCl to comply with the pH optimum (Jacobsen et al., 2017). The major soybean proteins are globulin proteins (glycinin and β-conglycinin), which are insoluble at their isoelectric points (PI). The PI region of soybean proteins is at pH 4-5 (Wolf, 1970). The observed reduction of soluble protein in the treated SBM can be attributed to the pH reduction effect as demonstrated by Lee et al. (2003) and Wolf (1970). Previous results have shown that the type of enzyme, enzyme content, pre-treatment of the soybean meal, and the hydrolysis time has an influence on the solublization of protein (Marsman et al., 1997; Fischer et al., 2001; Lee et al., 2001), although all these experiments have been conducted at high moisture levels (789-900 g kg^{-1}). The Soy 40 (treated without enzyme) had a lower soluble protein

content compared to all the enzyme treated samples in the design. This confirms minor effects of the protease treatment, however, this was counteracted by the negative effect of the pH reduction relative to the starting material.

The phytic acid content in the SBM used in this experiment is similar with that reported by Thompson and Erdman (1982). The reduction of the $InsP_6$ in Soy 40 compared to the SBM in our study, might indicate minor endogenous phytase activity still present in the SBM, in accordance with previous studies by Eeckhout and De Paepe (1994). The highest reached hydrolysis of IP6 (84%) in this system at the chosen variable values was at ENo 8 (Table 1 and 4). The response surface plots (Figure 1-3) indicate that total hydrolysis of $InsP_6$ may be achieved at a higher reaction time, moisture and enzyme content. The $InsP_6$ model shows a negative main and positive square effect of the enzyme addition (Table 5), the latter indicating less effect of increased enzyme addition compared to increased moisture and time, both with a negative main effect. Increasing the moisture level will be most efficient, however, formation of a doughy texture with less mixing effect might counteract this effect.

The significance of the square effect of the enzyme indicates that the rate of hydrolysis is limited by the substrate concentration. No accumulation of $InsP_5$-$InsP_3$ was observed, confirming a high efficiency of the EC to completely dephosphorylate the inositol phosphate (Table 7).

Water plays a role in maintaining the secondary and tertiary structure of the enzymes and water molecules can mediate enzymatic catalysis either directly by taking part in the reaction or indirectly by providing a medium for the reactants and products (Simpson et al., 2012). Among the drawbacks of a high solids concentration during hydrolysis, is the reduced enzyme activity due to low moisture levels. The effect appears to be linear as seen in the response surface plot of $InsP_6$ in relation to moisture and time (Figure 1), and is in agreement with the results on saccharification of lingo-cellulose by a mixture of carbohydrases in high solid concentrations (Jørgensen et al., 2007). The low moisture effect has been tested for a variety of substrate materials as described in the review by

Kristensen et al. (2009). The mechanism behind this phenomenon has been reviewed, but the exact cause has not been determined (Modenbach and Nokes, 2013; Chen and Liu, 2016).

The saponin content of the SBM used in this trial differs from the content reported by Knudsen et al. (2008). The DDMP conjugated soya saponins are lower, whereas the corresponding non-DDMP counterparts are higher. It is possible that the SBM used in this experiment has been exposed to a longer or higher heat treatment than that used in Knudsen et al. (2006). The DDMP conjugated soya saponins are the main group B soya saponins present in soybeans and more abundant than the non-DDMP (Kudou et al., 1993). The DDMP soya saponins are unstable and can be converted to the non-DDMP conjugates by an increase of temperature and/or alkaline pH (Zhang et al., 2012). The approximately equal reduction of the DDMP soya saponins in the enzyme treated and the non-enzymatic Soy 40 (Table 4) indicate that the effect is related to the incubation temperature, pH or temperature used to inactivate the enzymes. Reduction of DDMP soya saponins resulted in increased soya saponins Bb and Bc. The results indicate that the EC used could not bio-transform soya saponins Ab, Bc, Ba-DDMP, Bb-DDMP and Bc-DDMP at the tested conditions. The response surface plot of Bb soya saponins shows that enzyme and moisture content has an influence on the Bb content (Figure 4). The Bb saponins were increased by an increase in the enzyme content up to 2.5 g whereas the Bb-DDMP was not affected by the enzyme content. It is noteworthy that Zhang et al. (2012) demonstrated that the reduction of DDMP moiety resulted in an increase in the corresponding non-DDMP during fermentation with lactic acid bacteria *L. rhamnosus.* The increase of the Bb saponins might also be caused by other non-analyzed saponins, which are enzymatically hydrolyzed to the Bb moiety. The Bb´ soya saponins and the soya sapogenols where not analyzed in this experiment. The reduction of Bb saponins at higher enzyme concentration than 2.5 g in the mixture could be explained by hydrolyzing the Bb saponins to structural related Bb´ saponins as reported by Hu et al. (2004) with human fecal microorganisms, and/or soyasapogenol B as reported by Amin et al. (2011) with *Aspergillus niger*. Knudsen et al. (2008) reported that the DDMP-conjugated group B and/or group A soyasaponins concentrations could be an explanation of the induced enteritis observed in

433 the distal intestine of Atlantic salmon. The bio-transformed soya saponins in the present study may
434 not induce enteritis in Atlantic salmon, but this remains to be established.
435
436 *4.3.* Mink digestibility
437 The mink digestibility protocol is primarily developed to quantify protein digestibility, however,
438 expanded to include the main nutrients and minerals in this trial.
439 The improved protein digestibility in ÷Enzyme and Enzyme diets compared to the Untreated diet
440 confirms a significant effect of the heat treatment of the SBMs after the enzymatic hydrolysis. The
441 only significant improvement in the Enzyme diet compare to ÷Enzyme was threonine digestibility.
442 This may be attributed to remaining trypsin inhibitors activity in the SBM, which is inactivated by the
443 heat treatment and causes improved digestibility of proteins and amino acids. Mink trypsin has been
444 shown to be strongly inhibited by trypsin inhibitors in SBM (Krogdahl and Holm, 1983; Struthers and
445 MacDonald, 1983). Romarheim et al. (2005) showed that SBM trypsin inhibitors were reduced by
446 extrusion heat treatment and improved the protein and amino acid digestibility in mink.
447 Some of the AA such as lysine and argenine content in the ÷Enzyme and Enzyme was lower than that
448 of the Untreated SBM (Table 2), this may be due to the Maillard reaction in which free amino acids
449 bond to free carbonyl groups on reducing sugars or carbohydrates (Damodaran, 2008) formed during
450 overcooking which agree with (Ljøkjel et al., 2000). The Enzyme treated had a higher lysine content
451 compare to the ÷Enzyme treated indicating that the enzyme treatment did not increase the reducing
452 sugar which would enhance Maillard products. The heat treatment did not reduce the digestibility of
453 cysteine indication that the microwave heating method used did not from disulphide bridges which
454 have show to reduce the digestibility of cysteine (Opstvedt et al., 1984). The fat digestibility in the
455 diets was improved in the ÷Enzyme diet compare to the other two diets. This indicates a possible
456 counteracting effect of heat treatment (positive) and enzyme treatment (negative) on the fat
457 digestibility. However, this cannot be confirmed based on the present research, and new studies are
458 required to unlock these complex interactions.

459 The phosphorus content in SBM and fish meal in this experiment was not analyzed but previous
460 experiments have showed that it is 6.8 g kg^{-1} in defatted SBM (Jahan et al., 2000), the majority of
461 which is present in the non-bioavailable inositol phosphate form, and approximately 26 g kg^{-1} in blue
462 whiting fishmeal (Albrektsen et al., 2009). The use of these published values to calculate the
463 phosphorous content in the diet (~11.2 g kg) corresponds well with the analyzed values. Thus, the
464 majority of the phosphorous in the mink diets was derived from fishmeal (~72%). Phosphorus
465 digestibility in mink was not improved in the Enzyme diet compare to the ÷Enzyme diet even though
466 84% of the $InsP_6$ in SBM had been hydrolyzed. This can be related to the high content of phosphorus
467 in fishmeal which masked any improvement in the bioavailability of phosphorous in treated SBM
468 induced by dephosphorylation of the phytic acid. Interesting though was the increased phosphorous
469 digestibility of ÷Enzyme and Enzyme diet compare to the Untreated diet. This might be attributed to
470 the endogenous excretion of minerals, which consists of bilary and gastrointestinal secretions and
471 sloughed mucosal cells (Sandström et al., 1993), which is increased by a higher content of heat labile
472 ANFs in the Untreated SBM diet.
473 The improved ash digestibility in the Enzyme diet can be related to the hydrolysis of inositol
474 phosphate. Phytic acid can form chelating conjugates with di- and trivalent minerals (Persson et al.,
475 1998). The resulting complexes are insoluble and reduce the bioavailability of minerals (Weaver and
476 Kannan, 2001). Previous results have showed that $InsP_6$ and $InsP_5$ have a strong inhibitory effect on
477 the absorption of zinc whereas isolated $InsP_4$ and $InsP_3$ have no significant effects in suckling rat pups
478 and human subjects (Lönnerdal et al., 1989; Sandström and Sandberg, 1992). The $InsP_6$ and $InsP_5$
479 have an inhibitory effect on iron absorption and $InsP_4$ and $InsP_3$ in processed food and feed
480 contribute to reduce the iron absorption, presumably by binding iron between different inositol
481 phosphates (Sandberg et al., 1999). The binding strength of the lower inositol phosphates $InsP_4$ and
482 $InsP_3$ is lower than for the $InsP_6$ and $InsP_5$ (Persson et al., 1998). The dephosphorylation of phytic acid
483 increases the bioavailability of the divalent and trivalent minerals and can explain the increase in ash
484 digestibility in the enzyme treated soybean meal.

5. Conclusions

This study showed that the processing of SBM with an enzyme complex from *Aspergillus niger* at a high solid content can hydrolyze the $InsP_6$ and Bb saponins. Reaction time, EC level, moisture content and mixing have significant effect on the hydrolysis. The heat treatment was the main factor improving nutrient digestibility, while additional effect of the enzyme treatment was minor. The increased protein and amino acid digestibility was probably caused by inactivation of residual trypsin inhibitors in the SBM and/or other ANFs. The enzyme treatment did not improve the apparent digestibility of phosphorous, although it was a tendency for increased apparent digestibility of ash, hence increasing the apparent digestibility of some other minerals.

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Figure legends

Figure 1. $InsP_6$ response surface plot based on regression model given in Table 3. Enzyme level constant at 2 g kg^{-1} DM (design center point).

Figure 2. $InsP_6$ response surface plot based on regression model given in Table 3. Initial moisture content constant at 400 g kg^{-1} DM (design center point).

Figure 3. $InsP_6$ response surface plot based on regression model given in Table 3. Hydrolysis time constant for 50 min (design center point).

Figure 4. Saponin Bb response surface plot based on regression model given in Table 3. Hydrolysis time constant for 50 min (design center point).

Supplementary Figure 1. Normal probability plot of effects for the 2^3 full factorial screening design of the IP6 factor.

Supplementary Figure 2. Normal probability plot of effects for the 2^3 full factorial screening design of the Bb-DDMP factor.

721 **Table 1** Coded and actual levels for the experimental design variables time, moisture and enzyme

722 addition during hydrolysis.

ENo	Coded design value			Time	Moisture	Enzyme
	Time	Moisture	Enzyme	(min)	(g kg^{-1} DM)	(g kg^{-1} SBM DM)
1	-1	-1	-1	30	350	1
2	-1	-1	1	30	350	3
3	-1	1	-1	30	450	1
4	-1	1	1	30	450	3
5	1	-1	-1	70	350	1
6	1	-1	1	70	350	3
7	1	1	-1	70	450	1
8	1	1	1	70	450	3
9	-1.68	0	0	16.4	400	2
10	1.68	0	0	83.6	400	2
11	0	-1.68	0	50	316	2
12	0	1.68	0	50	484	2
13	0	0	-1.68	50	400	0.32
14	0	0	1.68	50	400	3.68
15	0	0	0	50	400	2
16	0	0	0	50	400	2
17	0	0	0	50	400	2

723 DM, dry matter; ENo, experiment number.

724 ENo 1-8: Cube points, ENo: 9-14 Axial points, ENo 15-17: Centre points.

725

726 **Table 2.** Dry matter and chemical composition (g kg^{-1} DM) of fish meal, untreated soybean meal

727 (Untreated), treated soybean meal without addition of enzymes (÷Enzyme), and treated soybean

728 meal with the addition of enzyme (Enzyme).

	Fish meal	Soybean meal		
		Untreated	÷Enzyme	Enzyme
Dry matter g kg^{-1} DM	924	890	617	652
Crude protein (N x 6.25)	720	490	509	494
Crude fat	100	13	13.5	8.7
Starch	0	35	29	41
Non starch carbohydrates (diff)	31	392	379	385
Ash	149	71	70	71
Essential amino acids				
Lysine	66.0	37.6	32.2	34.6
Threonine	34.6	23.2	17.3	24.7
Methionine	22.2	9.5	7.5	7.9
Valine	38.4	26.3	22.9	25.6
Isoleucine	34.3	25.8	23.3	24.5
Leucine	62.4	44.5	37.1	42.3
Phenylalanine	31.4	27.8	21.5	27.4
Histidine	16.6	14.3	12.3	14.3
Arginine	53.1	41.1	35.4	37.9
Non-essential Amino Acids				
Aspartic acid	78.4	64.7	54.4	71.1
Serine	35.2	28.4	23.3	29.7
Glutamic acid	116.0	103.7	89.5	101.1
Proline	33.2	28.2	23.2	28.8
Glycine	52.2	26.5	21.6	27.7
Alanine	51.5	26.8	20.2	25.3
Tyrosine	19.0	17.0	12.2	16.0
Cysteine	7.5	7.6	8.3	9.4

729

730

731 **Table 3**. Composition (g kg^{-1} DM) and chemical content (g kg^{-1} DM) of the experimental mink diets.

Formulation[a]	g kg^{-1} DM as feed		
	Untreated	÷Enzyme	Enzyme
Soybean meal[b]	459	443	456
Fishmeal[c]	311	320	312
Corn starch[d]	103	106	104
Soybean oil[e]	124	128	125
Vit/mineral premix[f]	3	3	3
Chemical content (calculated from ingredient analysis)			
Crude protein	449	456	451
Crude fat	160	165	159
Starch	105	106	105
Non starch carbohydrates (diff)	204	191	203
Ash	82	82	82
Phosphorous	11	12	11

732 [a]Untreated, standard soybean meal; ÷Enzyme, pre-treated soybean meal without added enzymes;

733 Enzyme, pre-treated soybean meal with addition of enzymes. The soybean meal accounts for 50% of

734 the protein in the diet.

735 [b]De-hulled solvent extracted soybean meal delivered from DSM, Kaiseraugst, Switzerland.

736 [c]Norse-LT 94, Norsildmel AS, Bergen, Norway.

737 [d]Pregeflo, Roquette Freres, Lestem Cedex, France.

738 [e]Refined commercial soybean oil for human.

739 [f]Per kg diet, IU: Vit. D_3, 3400; mg: α-tocopherol 38.5, vit. K 2.27, ascorbate polyphosphate 22.7,vit. B_1

740 2.27, vit. B_2 4.53, vit. B_6 2.26, vit. B_{12} 0.002, pantothenic acid 9.1, niacin 3.4, biotin 0.04, folic acid

741 1.81, $ZnSO_4$ 34, $MnSO_4$ 11.3, $CuSO_4$ 2.27.

742

743 **Table 4.** Levels of Dietary fiber, soluble protein, Inositol-6-phosphate and saponins (g kg^{-1} DM) in SBM, Soy 40 and after treatment according to the CCD

744 (ENo 1-17).

	Dietary fiber			Protein	Phytic acid	Saponins					
Sample	insoluble	soluble	total	soluble	$InsP_6$	Ab	Ba-DDMP	Bb	Bb-DDMP	Bc	Bc-DDMP
SBM	190.9	16.8	207.7	104.7	12.30	nd	0.08	3.56	1.05	0.72	0.24
Soy 40	186.5	16.5	202.9	42.8	11.64	nd	0.03	4.11	0.79	0.81	0.14
ENo 1	171.6	19.3	190.9	44.0	7.77	0.09	0.03	3.90	0.80	0.84	0.17
ENo 2	173.0	19.5	192.4	45.4	6.05	nd	0.02	3.96	0.74	0.80	0.16
ENo 3	172.7	22.7	195.4	46.4	5.17	0.01	0.02	4.22	0.75	0.80	0.13
ENo 4	183.2	14.0	197.2	47.4	3.44	0.05	nd	4.36	0.67	0.82	0.15
ENo 5	181.2	14.9	196.2	45.8	5.95	0.08	0.03	4.05	0.77	0.80	0.17
ENo 6	171.7	14.0	185.7	46.1	4.93	0.03	0.03	4.03	0.75	0.82	0.17
ENo 7	192.3	12.8	205.2	45.9	3.66	nd	0.01	4.09	0.72	0.76	0.14
ENo 8	186.0	13.1	199.1	43.8	1.94	nd	0.01	4.67	0.61	0.80	0.12
ENo 9	171.7	15.0	186.7	45.1	6.38	nd	0.03	4.16	0.81	0.73	0.19
ENo 10	170.9	11.7	182.7	50.2	3.21	nd	0.02	4.66	0.80	0.76	0.15
ENo 11	182.4	21.5	203.9	44.0	7.79	nd	0.03	3.87	0.68	0.80	0.15
ENo 12	175.0	14.2	189.2	45.9	2.43	nd	0.02	4.96	0.74	0.73	0.16
ENo 13	183.7	14.0	197.8	46.5	7.59	nd	0.03	4.04	0.73	0.82	0.15
ENo 14	174.1	12.0	186.1	49.0	3.46	nd	0.04	4.28	0.77	0.78	0.17
ENo 15	184.5	13.0	197.5	47.7	3.84	0.07	0.04	3.42	0.77	0.75	0.17
ENo 16	171.2	25.7	196.9	47.1	4.03	nd	0.02	4.43	0.79	0.74	0.15
ENo 17	172.8	16.2	189.0	46.4	3.84	0.02	0.03	4.36	0.80	0.77	0.17

745 ENo, experiment number; SBM, soybean meal; Soy 40, SBM treated in the same way as the center point (ENo 15-17) without adding enzymes; nd, not

746 detected. $InsP_6$, Inositol-6-phosphate; Ab, Ba-DDMP Bb, Bb-DDMP, Bc, Bc-DDMP group saponins.

747 **Table 5.** Response surface models describing the effects of hydrolysis time (T), moisture level (M) and enzyme complex content (EC) on Inositol-6-phosphate

748 (InsP6) and saponin group Bb after backward removal of insignificant regressors.

Variable	Intercept	T	T×T	M	M×M	EC	EC×EC	T×M	T×EC	M×EC	R^2
$InsP_6$	21.37***	-0.0413***	ns	-0.0286***	ns	-2.4102**	0.3620*	ns	ns	ns	0.940
Bb	3.56***	ns	ns	ns	ns	ns	-0.1593***	ns	ns	0.0018***	0.682

749 ns, not significant

750 *P < 0.05, **P< 0.01, ***P<0.001

751 **Table 6**. Mink apparent digestibility of the feeds where 50% of the protein is replaced with SBM

752 (Untreated), treated soybean meal without addition of enzymes (÷Enzyme) and treated soybean

753 meal with the addition enzyme (Enzyme).

	Untreated	÷ Enzyme	Enzyme	ANOVA (*P**)
Crude protein	0.843±0.007[a]	0.866±0.004[b]	0.869±0.005[b]	2.37E-04
Fat	0.967±0.002[a]	0.978±0.004[b]	0.961±0.002[a]	3.70E-05
Starch	0.912±0.002	0.925±0.039	0.947±0.004	ns**
Non Starch carbohydrates	0.463±0.015	0.492±0.036	0.511±0.033	ns
Ash	0.376±0.023[a]	0.418±0.031[ab]	0.432±0.036[b]	7.02E-02
Phosphorous	0.422±0.021[a]	0.480±0.028[b]	0.489±0.019[b]	1.79E-02
Gross energy	0.786±0.001[a]	0.813±0.009[b]	0.800±0.011[ab]	3.53E-03
DM	0.708±0.003[a]	0.740±0.012[b]	0.734±0.015[b]	5.49E-03
Essential amino acids				
Lysine	0.910±0.005[a]	0.927±0.004[b]	0.922±0.008[b]	6.87E-03
Threonine	0.827±0.009[a]	0.832±0.004[a]	0.853±0.012[b]	7.88E-03
Methionine	0.917±0.004[ab]	0.909±0.008[a]	0.923±0.006[b]	3.11E-02
Valine	0.886±0.006[a]	0.913±0.007[b]	0.902±0.009[b]	2.15E-03
Isoleucine	0.896±0.006[a]	0.927±0.005[b]	0.917±0.009[b]	4.93E-04
Leucine	0.899±0.006[a]	0.931±0.005[b]	0.919±0.009[b]	2.43E-04
Phenylalanine	0.880±0.005[a]	0.923±0.006[c]	0.902±0.010[b]	4.00E-05
Histidine	0.878±0.005[a]	0.905±0.009[b]	0.896±0.011[b]	5.17E-03
Arginine	0.927±0.008[a]	0.956±0.004[b]	0.946±0.006[b]	2.32E-04
Non-essential Amino Acids				
Aspartic acid	0.807±0.007[a]	0.840±0.010[b]	0.843±0.012[b]	9.52E-04
Serine	0.848±0.006[a]	0.879±0.006[b]	0.882±0.008[b]	7.20E-05
Glutamic acid	0.895±0.005[a]	0.925±0.006[b]	0.920±0.008[b]	2.59E-04
Proline	0.859±0.003[a]	0.895±0.009[b]	0.887±0.013[b]	9.70E-04
Glycine	0.847±0.007[a]	0.870±0.010[b]	0.868±0.011[b]	1.67E-02
Alanine	0.890±0.005[a]	0.914±0.009[b]	0.899±0.007[a]	3.31E-03
Tyrosine	0.893±0.005[a]	0.942±0.006[b]	0.907±0.011[a]	2.80E-05
Cysteine	0.730±0.028[a]	0.762±0.017[ab]	0.784±0.021[b]	2.35E-02

754 * Values not sharing common superscript letters are significantly different (P<0.05).

755 **ns, non significant.

756

757 **Table 7.** Inositol 3-5 phosphate levels ($InsP_3$-$InsP_5$) (g kg^{-1} DM) in SBM, Soy 40 and CCD cube points

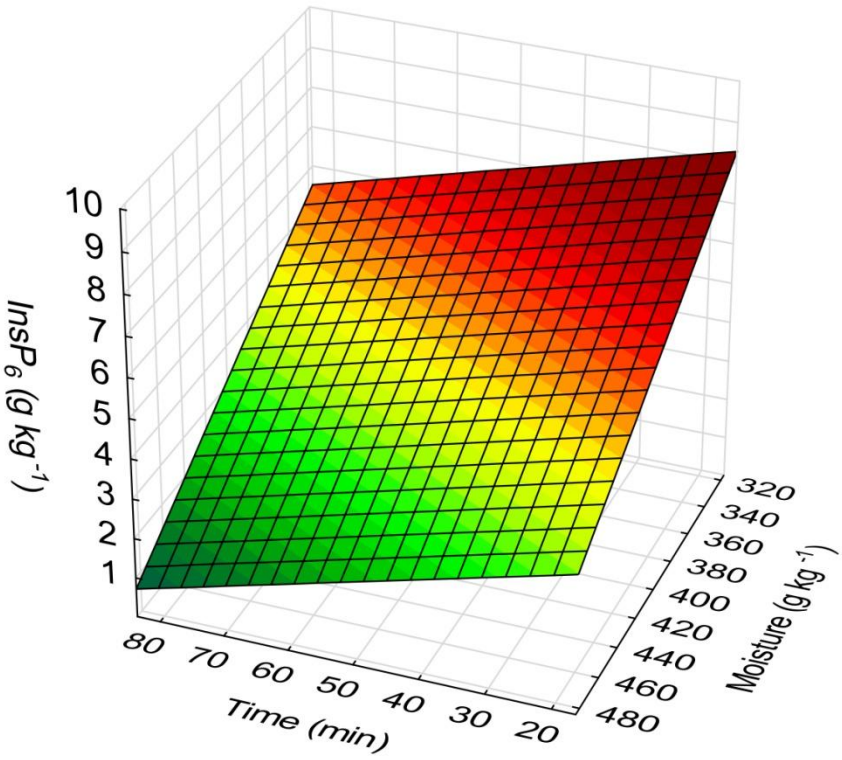
758 (ENo 1-8).

	Inositol phosphate level		
Sample	$InsP_3$	$InsP_4$	$InsP_5$
SBM	0.07	0.54	1.90
Soy 40	0.09	0.60	2.17
ENo 1	0.29	0.36	1.07
ENo 2	0.21	0.21	0.73
ENo 3	0.70	0.54	1.17
ENo 4	0.26	0.15	0.44
ENo 5	0.28	0.24	0.76
ENo 6	0.14	0.18	0.54
ENo 7	0.63	0.22	0.53
ENo 8	0.08	0.07	0.20

759 ENo, experiment number (Table 1); SBM, soybean meal; Soy 40, SBM treated in the same way as the

760 center point (ENo 15-17) without adding enzymes.

761

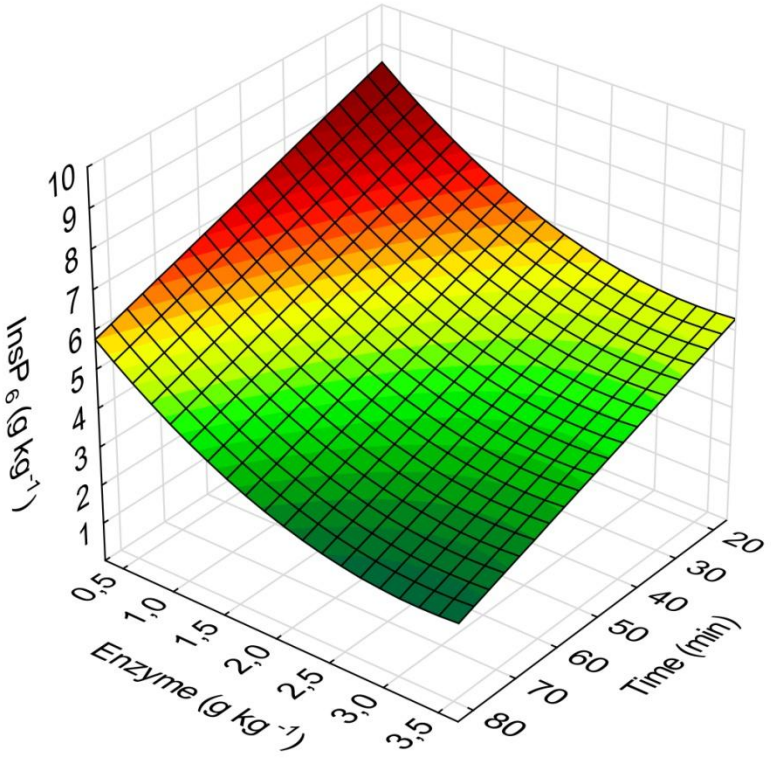


762

763 **Figure 1.** $InsP_6$ response surface plot based on regression model given in Table 3. Enzyme level

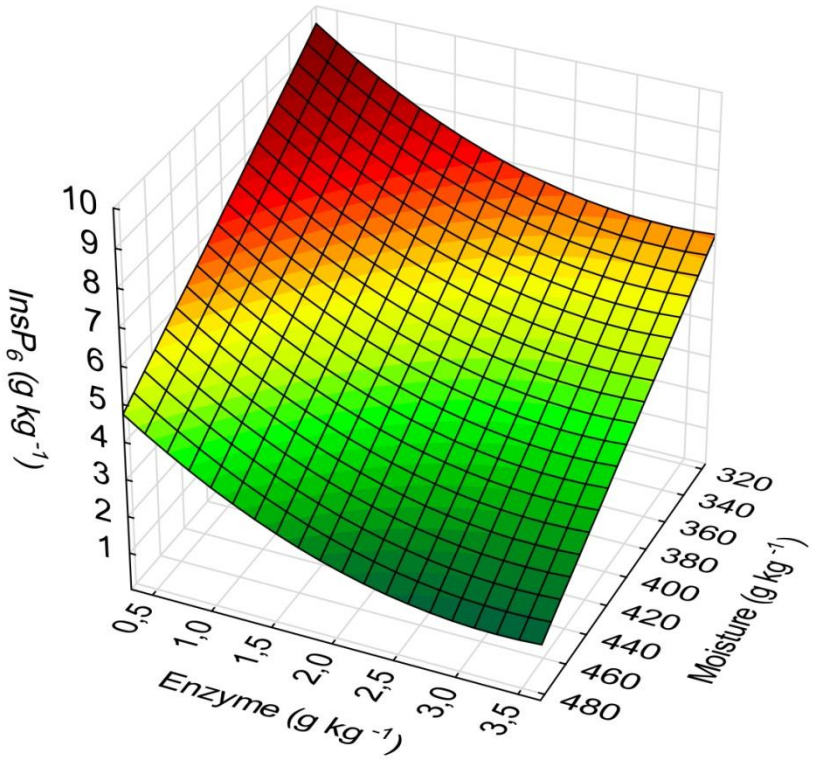
764 constant at 2 g kg^{-1} DM (design center point).

765



766
767 **Figure 2.** $InsP_6$ response surface plot based on regression model given in Table 3. Initial moisture
768 content constant at 400 g kg^{-1} DM (design center point).

769



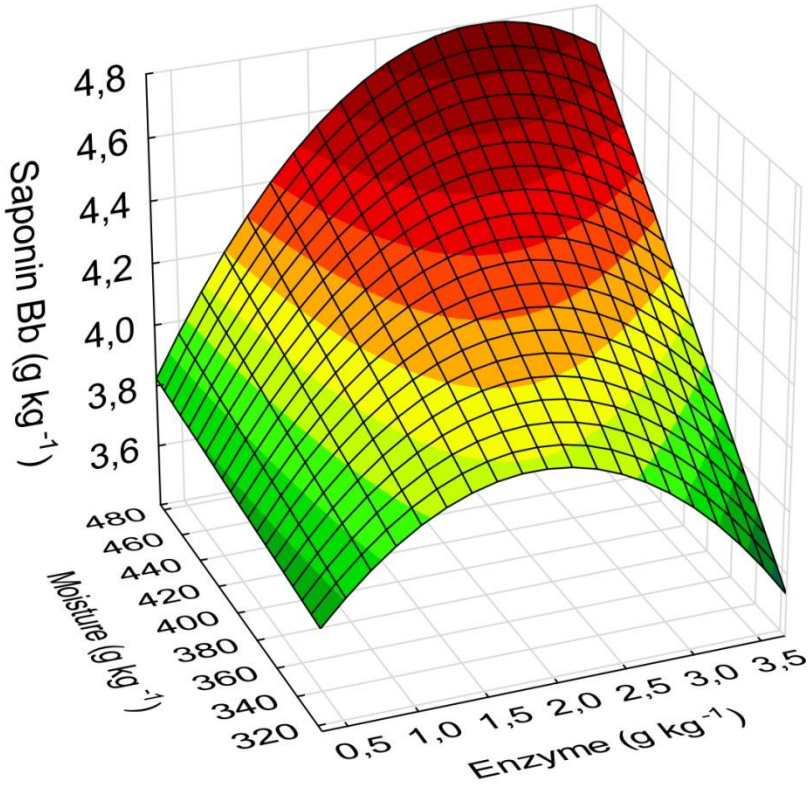
770

771 **Figure 3.** $InsP_6$ response surface plot based on regression model given in Table 3. Hydrolysis time

772 constant for 50 min (design center point).

773

774



775

776

777 **Figure 4.** Saponin Bb response surface plot based on regression model given in Table 3. Hydrolysis

778 time constant for 50 min (design center point).

779

Supplementary Table 1. Coded and actual levels for the continuous variables time (min) and moisture (g kg^{-1} DM), and categorical variable static vs. mixing during hydrolysis in the single replicate 2^3 full factorial design.

ENo	Coded design value			Time	Moisture	Static vs. mixing
	Time	Moisture	Static vs. mixing	(min)	(g kg^{-1} DM)	(S/M)
1	-1	-1	-1	30	350	Static
2	-1	-1	1	30	350	Mixing
3	-1	1	-1	30	450	Static
4	-1	1	1	30	450	Mixing
5	1	-1	-1	70	350	Static
6	1	-1	1	70	350	Mixing
7	1	1	-1	70	450	Static
8	1	1	1	70	450	Mixing

784 **Supplementary Table 2.** Level of dietary fiber, soluble protein, inositol-6-phosphate and saponins in SBM and after treatment according to the single

785 replicate 2^3 full factorial design. Values are in g kg^{-1} dry matter.

	Dietary fiber			Protein	Phytic acid	Saponins					
Sample	insoluble	soluble	total	soluble	$InsP_6$	Ab	Ba-DDMP	Bb	Bb-DDMP	Bc	Bc-DDMP
SBM	190.9	16.8	207.7	104.7	12.30	nd	0.08	3.56	1.05	0.72	0.24
ENo 1	186.2	20.7	206.9	41.4	7.29	nd	0.02	4.11	0.78	0.71	0.17
ENo 2	173.0	19.5	192.4	45.4	6.05	nd	0.02	3.96	0.74	0.80	0.16
ENo 3	186.7	14.5	201.2	42.5	4.00	nd	0.03	3.33	0.81	0.80	0.16
ENo 4	183.2	14.0	197.2	47.4	3.44	0.05	nd	4.36	0.67	0.82	0.15
ENo 5	165.0	17.5	182.5	43.3	6.21	nd	0.03	4.14	0.88	0.78	0.19
ENo 6	171.7	14.0	185.7	46.1	4.93	0.03	0.03	4.03	0.75	0.82	0.17
ENo 7	165.3	19.6	185.0	46.5	2.83	nd	0.03	4.73	0.85	0.80	0.19
ENo 8	186.0	13.1	199.1	43.8	1.94	nd	0.01	4.67	0.61	0.80	0.12

786 ENo, experiment number

787

Supplementary Table 3. Analysis of Variance of the significant effects determined from the Normal Probability Plot (Supplementary Figure 1) of the IP6 factor from the 2^3 full factorial screening design.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_0	*P*-Value
Model	23.75	3	7.92	159.68	0.0001
T	2.96	1	2.96	59.79	0.0015
M	18.82	1	18.82	379.51	< 0.0001
SXM	1.97	1	1.97	39.73	0.0032
Error	0.20	4	0.05		
Total	23.95	7			

Supplementary Table 4. Analysis of variance of the significant effects determined from the normal probability plot (Supplementary Figure 2) of the Bb-DDMP factor from the 2^3 full factorial screening design.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_0	*P*-Value
Model	0.038	3	0.038	12.15	0.0131
SXM	0.038	1	0.038	12.12	0.0131
Error	0.019	6	0.003		
Total	23.95	7			

Supplementary Table 5. The triplicate analytical results of saponin groups of the 17 samples in the CCD. All values are in g kg^{-1} dry matter.

		Saponins						
Sample	Code	Ab	Ba-DDMP	Bb	Bb-DDMP	Bc	Bc-DDMP	Total
1	2T2M2E Par 1	0.11	0.03	3.89	0.80	0.85	0.17	5.86
2	2T2M2E Par 2	0.12	0.03	3.78	0.77	0.84	0.16	5.70
3	2T2M2E Par 3	0.04	0.02	4.02	0.82	0.85	0.17	5.92
4	2T2M4E Par 1	nd	nd	3.83	0.69	0.74	0.15	5.41
5	2T2M4E Par 2	nd	0.03	4.15	0.79	0.85	0.17	6.00
6	2T2M4E Par 3	nd	0.03	3.91	0.74	0.80	0.15	5.63
7	2T4M2E Par 1	nd	nd	4.18	0.74	0.77	0.12	5.81
8	2T4M2E Par 2	nd	0.03	4.26	0.74	0.82	0.12	5.98
9	2T4M2E Par 3	0.04	0.03	4.23	0.76	0.80	0.16	6.02
10	2T4M4E Par 1	0.04	nd	3.83	0.60	0.74	0.13	5.35
11	2T4M4E Par 2	0.05	nd	4.20	0.66	0.78	0.14	5.83
12	2T4M4E Par 3	0.05	nd	4.52	0.69	0.87	0.15	6.28
13	4T2M2E Par 1	0.07	0.03	4.17	0.81	0.81	0.17	6.07
14	4T2M2E Par 2	0.10	0.03	4.07	0.77	0.85	0.17	5.99
15	4T2M2E Par 3	0.07	0.03	3.90	0.74	0.74	0.17	5.65
16	4T2M4E Par 1	0.05	0.02	3.91	0.74	0.80	0.16	5.69
17	4T2M4E Par 2	nd	0.03	4.15	0.75	0.83	0.17	5.94
18	4T2M4E Par 3	0.11	0.03	3.99	0.74	0.71	0.16	5.74
19	4T4M2E Par 1	nd	0.02	4.22	0.75	0.77	0.16	5.92
20	4T4M2E Par 2	nd	0.02	4.07	0.73	0.75	0.15	5.72
21	4T4M2E Par 3	nd	nd	3.97	0.67	0.77	0.12	5.53
22	4T4M4E Par 1	nd	nd	4.49	0.59	0.74	0.13	5.95
23	4T4M4E Par 2	nd	0.02	4.99	0.61	0.88	0.11	6.61
24	4T4M4E Par 3	nd	nd	4.54	0.62	0.79	0.13	6.08
25	1T3M3E Par 1	nd	0.03	4.06	0.85	0.74	0.19	5.87
26	1T3M3E Par 2	nd	0.03	4.28	0.77	0.73	0.17	5.99
27	1T3M3E Par 3	nd	0.02	4.15	0.80	0.73	0.19	5.90
28	5T3M3E Par 1	nd	0.02	4.48	0.83	0.80	0.14	6.27
29	5T3M3E Par 2	nd	0.03	4.86	0.81	0.72	0.14	6.56
30	5T3M3E Par 3	nd	0.02	4.66	0.75	0.77	0.16	6.35
31	3T1M3E Par 1	nd	0.03	3.93	0.69	0.85	0.16	5.65
32	3T1M3E Par 2	nd	0.03	3.94	0.70	0.78	0.15	5.60
33	3T1M3E Par 3	nd	0.03	3.76	0.64	0.77	0.14	5.34
34	3T5M3E Par 1	nd	0.02	4.88	0.75	0.75	0.16	6.57
35	3T5M3E Par 2	nd	0.02	5.25	0.71	0.75	0.15	6.89
36	3T5M3E Par 3	nd	0.02	4.76	0.75	0.68	0.15	6.34
37	3T3M1E Par 1	nd	0.03	4.00	0.76	0.83	0.16	5.78
38	3T3M1E Par 2	nd	0.02	3.94	0.71	0.78	0.15	5.61
39	3T3M1E Par 3	nd	0.03	4.17	0.72	0.86	0.13	5.92
40	3T3M5E Par 1	nd	0.04	4.32	0.76	0.81	0.17	6.10

41	3T3M5E Par 2	nd	0.03	4.23	0.82	0.77	0.18	6.04
42	3T3M5E Par 3	nd	0.05	4.28	0.72	0.77	0.15	5.97
43	3T3M3E 1 Par 1	0.13	0.04	3.31	0.84	0.82	0.17	5.32
44	3T3M3E 1 Par 2	0.09	0.03	3.32	0.81	0.78	0.17	5.21
45	3T3M3E 1 Par 3	nd	0.03	3.62	0.66	0.65	0.15	5.12
46	3T3M3E 2 Par 1	nd	0.02	4.43	0.82	0.70	0.17	6.14
47	3T3M3E 2 Par 2	nd	0.02	4.27	0.79	0.81	0.13	6.03
48	3T3M3E 2 Par 3	nd	0.02	4.60	0.76	0.72	0.16	6.26
49	3T3M3E 3 Par 1	nd	0.03	4.35	0.80	0.78	0.17	6.14
50	3T3M3E 3 Par 2	0.04	0.02	4.36	0.80	0.77	0.17	6.17
51	3T3M3E 3 Par 3	0.09	0.03	4.39	0.53	0.50	0.12	5.65

803 nd, not detected

804 T, time; M, moisture, E, enzyme

805 1T, 16.4 min; 2T, 30 min; 3T, 50 min; 4T, 70 min; 5T, 83.6 min

806 1M, 316 g kg^{-1} DM; 2M, 350 g kg^{-1} DM; 3M, 400 g kg^{-1} DM; 4M, 450 g kg^{-1} DM; 5M, 484 g kg^{-1} DM

807 1E, 0.32 g kg^{-1} DM; 2E, 1.00 g kg^{-1} DM; 3E, 2.00 g kg^{-1} DM; 4E, 3.00 g kg^{-1} DM; 5E, 3.68 g kg^{-1} DM

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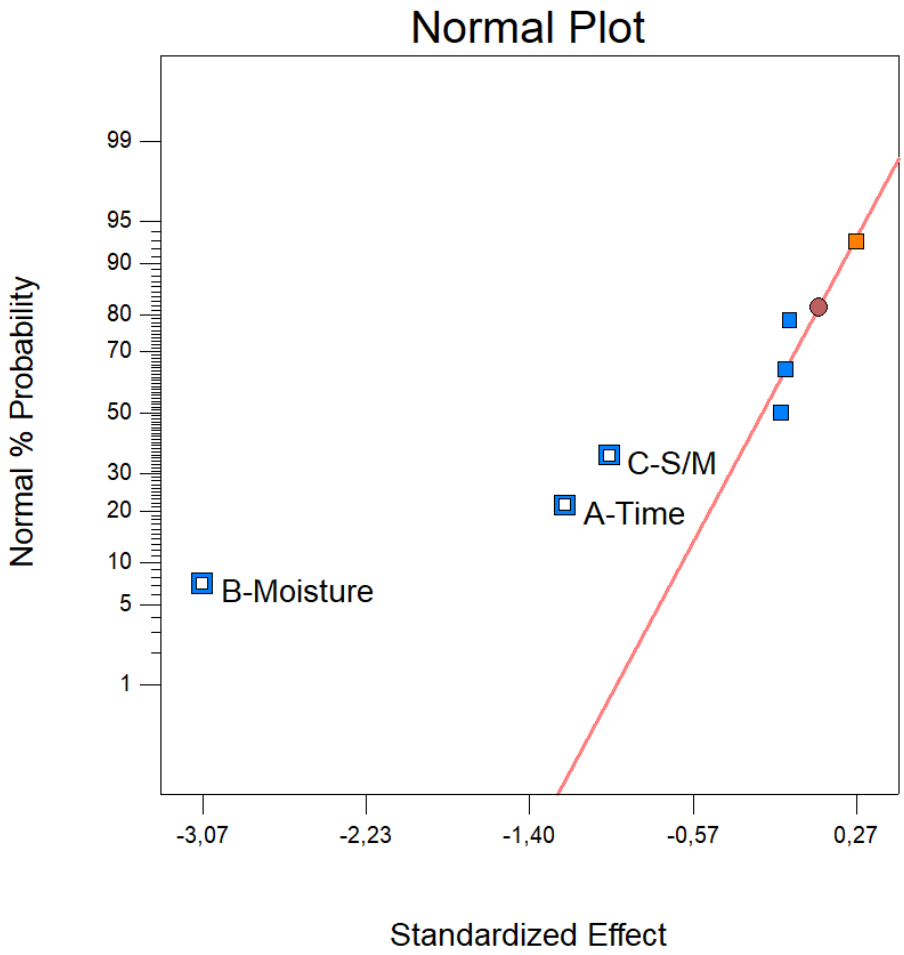

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812 **Supplementary Figure 1.** Normal probability plot of effects for the 2^3 full factorial screening design of
813 the IP6 factor.

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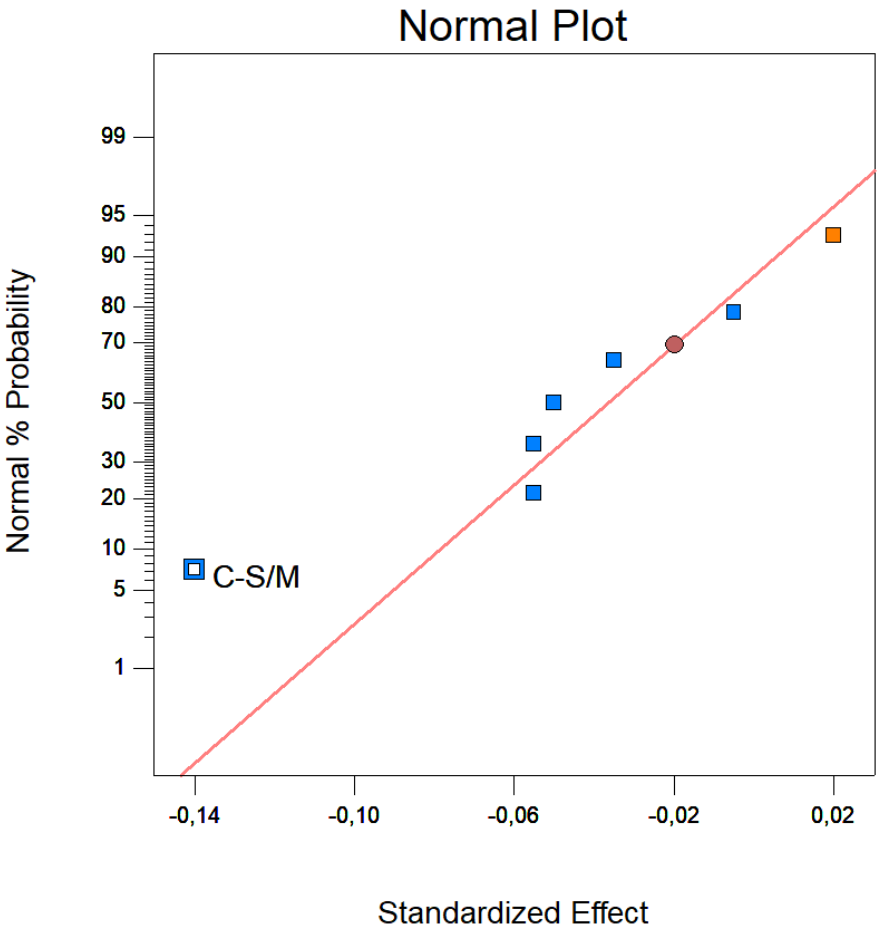

Supplementary Figure 2. Normal probability plot of effects for the 2^3 full factorial screening design of the Bb-DDMP factor.