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Cleavage of macromolecule (protein/polysaccharide)-phenolic bond in soybean cell wall through *Lactobacillus casei* and *Lactobacillus helviticus* mixed culture solid-state fermentation for chlorogenic acid extraction

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A R T I C L E I N F O

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A B S T R A C T

Out of the available dietary sources use for chlorogenic acid extraction, soybean is new and cheap that could offer cost-effective chlorogenic acid production under solid-state fermentation (SSF). Consequently, the study aimed at investigating the cleavage of protein/carbohydrate-phenolic bond in heilong48 soybean (HS) variety via solid-state fermentation by *Lactobacillus casei* and *Lactobacillus helviticus* mixed culture for chlorogenic acid extraction with high yield and increased biological activity. A simplistic two-pot bioproduct optimization approach was used; first, screening (Plackett-Burman design) of SSF conditions and second, optimizing (Box-Behnken design) the significant SSF conditions obtained from the former design. The liquid-solid (liquid:solid) ratio of 32:68 (0.47), incubation time of 48 h, pH of 5, and temperature of 50 °C were the best-optimized SSF conditions obtained from the chronological combinatory screening and optimization methods. These conditions gave a satisfactory SSF model, and correspondingly acceptable chlorogenic acid yield (9.20 ± 0.17 mg/g), fermentation efficiency (37.26 ± 0.73%), and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (DPPH) (65.21 ± 2.05 μmol AA eq/g dry sample) results. The *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample (under optimized SSF conditions) had high ($p < 0.05$) chlorogenic acid yield and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity than the raw heilong48 soybean flour (RHSF) sample. The effectiveness of the optimized solid-state fermentation conditions on the structural modifications of heilong48 soybean sample was confirmed by scanning electron microscopy, atomic force microscopy, and Fourier transform infrared results.

1. Introduction

Chlorogenic acid, an ester of caffeic and quinic acids present in numerous plant species (Wang et al., 2022), is a biologically important naturally occurring phenolic compound. Today, much is known from its various biological activities including antioxidative activity, HIV-1 integrase inhibition, antispasmodic activity, and carcinogenic compounds mutagenicity inhibition (anti-cancer activity), making it very important plant secondary metabolite (Gil & Wianowska, 2017). Chlorogenic acid, though unproven, is reported to be beneficial in obesity prevention and glucose-6-phosphatase modification in glucose metabolism, leading to research revival on chlorogenic acid properties and natural occurrence, as well as more new applications in cosmetics, food additives/foodstuffs, and pharmaceuticals. Great consideration is also given to efficient chlorogenic acid extraction from plants and plant products owing to the frequent use of 5-*O*-caffeoylquinic acid (main chlorogenic acid representative in nature) as a quality marker in many natural products control. Furthermore, chlorogenic acid forms bond

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(ester bond) with macromolecules (proteins and/or polysaccharides) which needs to be broken to release more into solution for extraction with high yield. According to Arancibia-Díaz et al. (2023), majority of vegetal matrix phenolic compounds are conjugated with fatty acids, proteins, or sugars; thus, there is a need to develop chemical, physical, and biological extraction processes for their recovery and bioactivity preservation.

The most common technique for extracting chlorogenic acid from plants is using a traditional, long-lasting organic solvent extraction technique carried out at higher temperatures (Gil & Wianowska, 2017). The challenges of traditional extraction methods have prompted researchers to look for novel, alternative extraction methods that could boost the quality of extracts and yield. The latter is especially crucial because of the uses of chlorogenic acid and the ease with which this substance can change or degrade into other substances. It has been reported that 5-*O*-caffeoylquinic acid isomerizes and transforms when heated conventionally in the presence of water (Gil & Wianowska, 2017). The results demonstrate that 5-*O*-caffeoylquinic acid undergoes further changes, including esterification and interactions with water, in addition to isomerizing to 3- and 4-*O*-caffeoylquinic acid. Thus, solid-state fermentation (novel bioprocess) extraction has lately been investigated and used in an effort to enhance the extraction process of chlorogenic acid and make it more efficient and ecologically friendly (Akpabli-Tsigbe et al., 2021a; 2021b). Exoenzymes are produced in solid-state fermentation that hydrolyze parts of plant tissues to extract bioactive compounds (Arancibia-Díaz et al., 2023). The activity of these hydrolytic enzymes (β-glucosidase, cinnamoyl esterase, and chlorogenate hydrolase [CHase]) produced through cell growth during solid-state fermentation, releases phenolic compounds bound in the cell wall, increasing extraction yield. To produce bioactive substances with high yield, solid-state (microbial) fermentation extraction, is currently being utilized. The production of phenolic compounds through microbial fermentation is an intriguing alternative method. With no any of the toxicity associated with organic solvents, this method produces high-yields (via microbial enzymatic hydrolysis/cleavage of protein/polysaccharide-phenolic ester bond) of high-quality extracts (Abdel-Aty et al., 2019).

Solid-state fermentation (SSF), an old culture technique is utilized still for secondary metabolites and enzymes production. It is the growing of microorganisns at the interior and/or on the surface of solid substrate with no any free-flowing water (El-Sayed et al., 2020). The use of solid-state fermentation in the industry (especially that of food) stands consequently significant for phenolic compounds, organic acids, flavor, and pigments production (Soccol et al., 2017). Solid-state fermentation improves the phenolic content of plant extracts via ester bonds cleavage between macromolecules and phenolics in plant cell walls (Abdel-Aty et al., 2019; Palmieri et al., 2018; Santos da Silveira et al., 2019), increasing their concentration and functional characteristics. Solid-state fermentation is used in soybean products for food bio-color, isoflavones (Handa et al., 2019), and enzymes (Li et al., 2017) production. It is also used to increase the content of hydrolyzed amino acids and proteins, reduce verbascose, trypsin inhibitors, stachyose, raffinose, and phytic acid levels (Hassaan et al., 2015), and enhance antioxidant activity.

Solid-state fermentation's effectiveness is influenced by factors such as the microorganism being used, working conditions, and ecological factors. The nature of substrate, pH, aeration, nutrient concentrations, moisture content, and temperature all play important roles in determining the success of solid-state fermentation (Farinas, 2015). Parameters like pH, temperature, and moisture content along with their interactions are important in solid-state fermentation (Farinas, 2015). Response surface methodology (RSM) and factorial designs are used to optimize these parameters and identify significant variables (best conditions) and their interactions (Thomas et al., 2013). Conventional techniques of altering one factor are time-consuming and may overlook interactions as well as laborious for operating conditions and culture medium optimization. Additionally, when dealing with a large number of factors, determining the "optimum" values without considering their interactions can lead to misinterpretation of the results (Buenrostro-Figueroa et al., 2017). Statistical designs like Plackett-Burman design (PBD) and Box-Behnken design (BBD) help identify important variables and construct a second-degree polynomial model (Brzezińska et al., 2023). This model assists in achieving specific targets and optimizing the solid-state fermentation process. By using statistical designs and polynomial models, solid-state fermentation can be effectively optimized for commercial applications.

Fungi and yeasts are mostly the favorite microorganisms utilized in solid-state fermentation due to the similarity of culture conditions to their native environment (Santos da Silveira et al., 2019). However, many other microorganisms including lactic acid bacteria (LAB), and *Bacillus* are currently used in solid-state fermentation (Wu et al., 2019). Lactic acid bacteria utilization as inoculum for fruit and vegetable products fermentation is common in the food industry, owing to their ease of cultivation and adaptability to different environments. Lactic acid bacteria are also very accepting of high phenolic compounds levels (Fritsch et al., 2017; Septembre-Malaterre et al., 2018). ***Lactobacillus*** genus which shows great diversity in nature (living on spoilt food, soil, animal and human intestinal tract, water and plants, and its materials), is the most important and investigated lactic acid bacteria (Zalán et al., 2010). ***Lactobacilli*** degrade carbohydrates to produce organic acids (valeric, formic butyric, caproic, acetic, propionic, and lactic acids), which display antimicrobial activity causing preservative effect (Zalán et al., 2010). Diacetyl, carbon dioxide (CO_2), hydrogen peroxide (H_2O_2), and bacteriocins are also produced by *Lactobacilli* (Zalán et al., 2010).

Today, an increasing number of *in-vivo* studies on chlorogenic acid absorption and its potential health benefits have been published (Santos et al., 2018). Likewise, many studies on the clarification of phenolic acids conversion pathways by microorganisms are done (Filannino et al., 2015). However, at present, industrial-scale production of chlorogenic acid is not cost-effective because production cost is high due to the raw material (coffee – expensive cash crop) and methods (extraction by chemical methods) used. Thus, the development of a low cost relatively feasible process as well as utilization of cheap raw material is needed for cost-effective chlorogenic acid production. Application of solid-state fermentation method (bioprocess) for extraction of chlorogenic acid from soybean (cheap crop – available throughout the year) could be the perfect feasible process to solve this problem. To our best of knowledge, no obtainable literature exists on the usage of solid-state fermentation to produce chlorogenic acid from soybean with a mixed culture of lactic acid bacteria. Recent studies done focus on using pure (single) lactic acid bacteria strains (Akpabli-Tsigbe et al., 2021a; 2021b). This study, a continuation of our previous studies (Akpabli-Tsigbe et al., 2021a; 2021b), therefore, aimed to investigate the effect of solid-state fermentation by *Lactobacillus casei* and *Lactobacillus helviticus* mixed culture on chlorogenic acid extraction from green (new) soybean variety – heilong48. A Plackett-Burman design followed by a Box-Behnken design was used to screen and optimize the best solid-state fermentation conditions for high chlorogenic acid extraction yield with enhanced biological (antioxidant) activity from the heilong48 soybean (HS) variety.

2. Materials and methods

2.1. Materials and chemicals

Tianxia Agricultural and Sideline Products and Distribution Department (China) provided the soybean (heilong48 variety) used in the study. The lactic acid bacteria (*L. casei* LC-122 and *L. helviticus* LH-43) were purchased from Taiwan Synbio Technology Incorporation (China) and stored at 4 °C till use. All the chemicals (2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity, standard chlorogenic acid, methanol, Folin–Ciocalteu phenol reagent, Na_2CO_3, gold powder, saline phosphate, KBr, and deMan Rogosa Sharpe) utilized in the entire experiment were of analytical grade only and bought from Sinopharm Chemical Reagent Co., Limited (China).

2.2. Preparation of heilong48 soybean flour

Heilong48 soybean flour was prepared as described in our previous works (Akpabli-Tsigbe et al., 2021a; 2021b). Briefly, heilong48 soybean variety was milled with a hammer crusher (FC160, Shanghai traditional Chinese medicine machinery factory, China) and further sieved into fine flour of particle size 0.25 mm. The final flour (in weights of 150 g) was packed in zip-lock rubber and stored (−20 °C) for further studies.

2.3. Preparation of inoculum

Both microorganisms (*L. casei* LC-122 and *L. helviticus* LH-43) were separately activated using the methods outlined by Akpabli-Tsigbe et al. (2021a; 2021b). Briefly, *L. casei* LC-122 was revived in deMan Rogosa Sharpe (MRS) broth at 37 °C for 24 h and *L. helviticus* LH-43 was activated by subculturing twice in deMan Rogosa Sharpe broth at 37 °C for 24 h. The cultures were centrifuged using Ruijiang RJ-TDL- 50A centrifuge (Ruijiang Analytical Instrument Co., Ltd., China) at 4000×*g* for 10 min. The supernatant was discarded and the bacterium cells rinsed in 0.1% NaCl solution (sterile). The concentration of the inoculum was estimated and corrected to 10^9 CFU/ml with 0.1% sterile NaCl solution using an XB-K-250 hemocytometer (Jianling Medical Device Co., China). The obtained suspensions were used as starter cultures (inoculum) for solid-state fermentation.

2.4. Solid-state fermentation of heilong48 soybean variety

The solid-state fermentation of the heilong48 soybean variety was done as described in our earlier works (Akpabli-Tsigbe et al., 2021a; 2021b). Briefly, 10 g heilong48 soybean flour (on a dry matter basis) was mixed (hydrated) with distilled water to moisture contents of 20, 30, and 40% in separate 250 ml conical flasks, mixed well (thoroughly) and sterilized at 121 °C for 15 min. The mixtures (after cooling to 25 ± 2 °C) were inoculated with *L. casei* and *L. helviticus* cultures sequentially at 1, 3, and 5% containing cell population of 10^9 CFU/g, mixed well and then cultured at 30, 40, and 50 °C in an incubator (SPX-250, Jintanshizhongdayiqichang, China) for 0, 24 and 48 h under static aerobic conditions. The pH of the solid-state fermentation of the heilong48 soybean variety was adjusted through the addition of 1 N NaOH or 1 N HCl to the culturing medium. All fermented heilong48 soybean samples were stored at −20 °C for further studies.

2.5. Plackett-Burman design for solid-state fermentation conditions screening

Due to the benefits of Plackett-Burman's design, its utilization is extensive in numerous scientific fields. It saves time and in a single experiment, more than one variable can be studied with "hidden projection properties" (Sha et al., 2019). Plackett-Burman design, two-level full, and fractional factorial designs are the most extensively used screening designs in factors selection (Zhou et al., 2018). However, the Plackett-Burman design detects relatively principal factors using a small number of experiments. Hence, an essential screening tool for checking the effect of process parameters on yield (Karlapudi et al., 2018). In addition, it decreases significantly, the number of repeating experiments to be performed in a further optimization study, using response surface methodology (Ekpenyong et al., 2017). Plackett-Burman design was thus selected as the technique of screening design in this study. Five (5) factors: temperature (A), pH (B), incubation time (C), inoculation size (D), and liquid-solid ratio (E) were screened with Plackett-Burman design to obtain the important ones that influence the solid-state fermentation process for extraction of chlorogenic acid with high yield and enhanced antioxidative activity. Thirteen runs with different factors (A – E) combinations obtained from the Plackett-Burman design were experimented with at three levels: "–" (low), "0" (middle), and "+" (high). Plackett-Burman design constituted on a first-order polynomial model as follows was applied:

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i \tag{1}$$

Where; Y = experimental response variable, β_0 = regression coefficient for intercept, β_i = regression coefficients for linear expressions and X_i = independent variable (factor).

2.6. Box-Behnken design for optimization of significant solid-state fermentation conditions

The four (4) important factors [temperature (X_1), pH (X_2), incubation time (X_3), and liquid-solid ratio (X_4)] obtained from the Plackett-Burman design were optimized further using Box-Behnken design. Box-Behnken design determines the design points in a way to avoid extreme design conditions where all the design parameters are at high/low levels simultaneously (Zhang et al., 2018). Fig. 1 shows the geometry of Box-Behnken design with three design parameters (X_1, X_2, and X_3). A four-factor (X_1, X_2, X_3, and X_4) three-level (X_1; 30, 40 and 50 °C, X_2; 5, 6 and 7, X_3; 0, 24 and 48 h and X_4; 20:80 [0.25], 31.60:68.40 [0.46] and 40:60 [0.67]) Box-Behnken design was used. A second-degree polynomial model was used to find the relationship of each factor to the response. The equation used was:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \times \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \tag{2}$$

Where; Y = response, β_0 = intercept (constant), $\beta_{i,}$ = linear regression

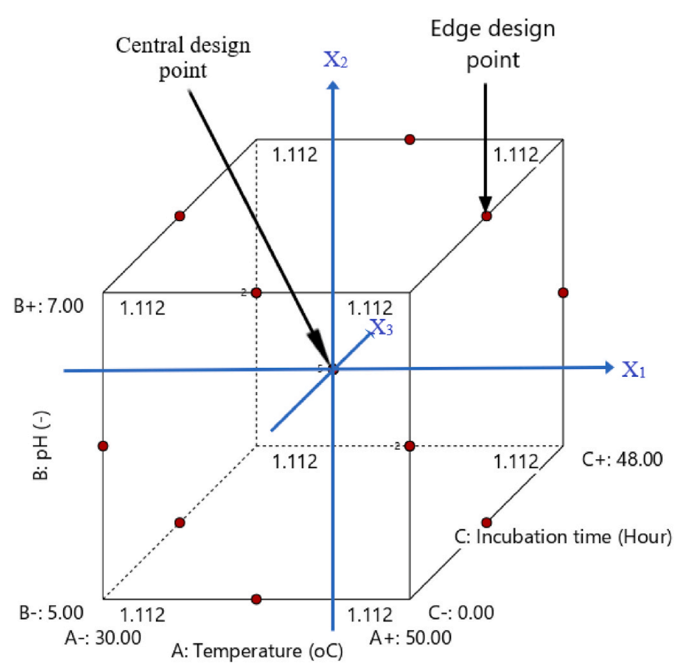

Fig. 1. Demonstration of design point determination using Box-Behnken design: geometry of design points with three design parameters.

coefficient term, β_{ii}, = quadratic regression coefficient term and β_{ij} = interaction regression coefficient term. X_i and X_j = factors. The optimized parameters were selected from the equation below using overall desirability index (DI):

$$DI = \left[\prod_{i=1}^{3} d_i(y_i)\right]^{1/3} \quad (3)$$

Where d_i = desirability index of the response variable (0–1), y_i = responses.

2.7. Model validation and verification

Average absolute deviation (AAD) (Adebo et al., 2018), percentage of average absolute deviation (Shim et al., 2018), bias factor (B_f), accuracy factor (A_f) (Bover-Cid et al., 2012), relative error (RE), and absolute residual standard error (RSE) (Akpabli-Tsigbe et al., 2021b) [Equations (4)–(9) respectively], as well as the coefficient of determination (R^2), were the different statistical parameters used in validating and verifying the adequacy of the models generated.

$$Aerage\ absolute\ deviation = \frac{\left[\sum_{i=1}^{N}\left(\frac{|Y_{i,exp} - Y_{i,cal}|}{Y_{i,exp}}\right)\right]}{N} \quad (4)$$

$$Percentage\ of\ aerage\ absolute\ deviation = \left[\frac{\sum_{i=1}^{N}\left(\frac{|Y_{i,exp} - Y_{i,cal}|}{Y_{i,exp}}\right)}{N}\right] \times 100\% \quad (5)$$

where $Y_{i,exp}$ = experimental response, $Y_{i,cal}$ = calculated response, and N = number of experimental runs.

$$Bias\ factor = 10^{\frac{\sum_{i=1}^{N} \log\left(\frac{Y_{predicted}}{Y_{observed}}\right)}{N}} \quad (6)$$

$$Accuracy\ factor = 10^{\frac{\sum_{i=1}^{N} \left|\log\left(\frac{Y_{predicted}}{Y_{observed}}\right)\right|}{N}} \quad (7)$$

where $Y_{observed}$ = observed or experimental response, $Y_{predicted}$ = predicted response, and N = number of experimental runs. A predictive model would ideally have accuracy factor = bias factor = 1, indicating the exact match between experimental observations and model predictions.

$$Relative\ error = \frac{Experimental\ value - Predicted\ value}{Predicted\ Value} \quad (8)$$

$$Residual\ standard\ error = \left|\frac{Predicted\ value - Experimental\ value}{Experimental\ value}\right| \times 100\% \quad (9)$$

2.8. Preparation of standard chlorogenic acid solution

The standard chlorogenic acid solution was prepared using the method reported by Akpabli-Tsigbe et al. (2021a). Briefly, 1000 mg was dissolved in distilled water (1 L) to make a stock chlorogenic acid solution (standard). The stock solution was thoroughly mixed using a magnetic stirrer (C-MAG HS7S025, IKA, Germany) in the dark. Series of standard solutions (5, 10, 15, 20, 25, and 30 mg/L) were made from the standard solution for chlorogenic acid in distilled water. All measurements were done within 10 min after preparation and absorbance of each series of standard chlorogenic acid solutions were measured immediately. The method was validated against Beer-Lambert's law with the series of standard chlorogenic acid solutions prepared.

2.9. Determination of antioxidative activity

The 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (DPPH) method reported in the literature (Akpabli-Tsigbe et al., 2021a; 2021b) was used to determine the antioxidative activity of the two samples: raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS). Briefly, 1 ml of the raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) extracts was added to 1 mM methanolic dilution of 2,2-diphenyl-1-picrylhydrazyl (2 ml) in separate test tubes. The mixtures after vortexing were incubated in the dark at 37 °C for 3 min and absorbance was measured at 517 nm against a blank using a UV-1600 spectrophotometer (Beijing Rayleigh analytical instrument, China). The results were expressed in μmol AA eq/g dry sample.

2.10. Determination of total phenolic acids (TPA)

Folin–Ciocalteu phenol reagent method (Akpabli-Tsigbe et al., 2021a; 2021b) was used for the total phenolic acids determination. Briefly, 1 ml of the raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) extracts was added to distilled water (9 ml) in separate test tubes. Folin–Ciocalteu phenol reagent (1 ml) was added to each test tube and mixed thoroughly with a vortex. Seven percent (7%) Na_2CO_3 (10 ml) was added after 5 min. Distilled water (4 ml) was added next, and the mixture was adjusted to a final volume of 25 ml. The reaction mixture was incubated at 25 ± 2 °C for 90 min, and absorbance was measured at 750 nm using a UV-1600 spectrophotometer (Beijing Rayleigh analytical instrument, China). The results were expressed in milligrams of gallic acid equivalents per gram of sample.

2.11. Determination of chlorogenic acid

The procedure described by Akpabli-Tsigbe et al. (2021a) was used to determine the chlorogenic acid. Briefly, 40 mg amount of the raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples were weighed and dissolved in distilled water (30 ml) in separate beakers (100 ml). The solutions were vortexed for 30 min using a magnetic stirrer (C-MAG HS7S025, IKA, Germany), heated at 40 °C to increase the solubility of chlorogenic acid in the solution. The solutions were filtered through double-loop qualitative filter paper (NO. 1568, Ge Biotechnology Co., Ltd., China) to get rid of particles from the solutions. The filtrates containing chlorogenic acid were collected and measured to obtain the volume of the sample extracts. The absorbance of the measured raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) extracts was measured using a UV-1601 spectrophotometer (Beijing Rayleigh Analytical Instrument Co. Ltd., China) within wavelength ranges of 190–1100 nm. The chlorogenic acid concentration was computed against the stock solution using Beer Lambert's Law at the maximum wavelength (λ_{max} = 325 nm). The chlorogenic acid contents in raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples were calculated using Equation (10). The percentage of chlorogenic acid content in the raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples was computed using Equation (11).

$$\text{Chlorogenic acid (mg)} = \frac{[\text{chlorogenic acid conc (mg/L)}] \times [\text{total sample volume (ml)}]^2}{[\text{measured sample volume (ml)}] \times 1000} \tag{10}$$

$$\text{Percentage of Chlorogenic acid (w / w \%)} = \frac{[\text{calculated mass of chlorogenic acid (mg)}]}{[\text{mass of sample measured (mg)}]} \times 100 \tag{11}$$

2.12. Microstructure analysis

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to determine the structure of raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples based on the procedure described by Akpabli-Tsigbe et al. (2021a). Briefly, for scanning electron microscopy analysis, freeze-dried raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples were placed on a copper sample-holder with double-sided adhesive tapes and coated with a conductive layer of gold powder (about 10 nm) by using vacuum coating apparatus. Their structure was examined with Hitachi S–3400N (Hitachi High Technologies, Japan) at 15 kV acceleration voltage.

For atomic force microscopy analysis, the freeze-dried raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples were dissolved in 0.01 M saline phosphate buffer (pH 8.0) to prepare a 10 μg/ml final concentration. The solution was heated in a thermostatic water bath (50 °C) for 10 min and centrifuged (4000 rpm, 10 min). Five microliters (5 μL) aliquots of the supernatant were rapidly pipetted onto a newly cleaved mica substrates and dried in an incubator (25 °C) for 12 h in Petri dishes. A multimode microscope (Bruker, Santa Barbara, CA) was used to generate the atomic force microscopy images of the samples. The lens was used in Peak Force^{QNM} mode with Bruker ScanAsyst needle at a typical spring and resonance frequency of 25.1 N/m and 300 kHz respectively.

2.13. Fourier transform infrared (FTIR) spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy was used to determine the functional groups (chemical structure) of raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples based on the procedure described by Akpabli-Tsigbe et al. (2021a). Briefly, 1 mg of freeze-dried raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples was thoroughly mixed and ground with 200 mg of dried spectroscopic grade KBr (at 105 °C for 24 h) powder separately in a mortar with a pestle (both made of agate). The resulting mixture was compacted with a hydraulic machine (15 t) into see-through (transparent) glass-like pellets of thickness, 1–2 mm. The pellets were scanned in the wavenumber ranging from 4000 to 400 cm^{-1} with 128 scans using model Nicolet IS50 device (Thermo Nicolet Corporation, USA) at a resolution of 4 cm^{-1}. The blank (KBr pellet without test samples) used under setting parameters was reported as reference spectra.

2.14. Statistical analysis

Design-Expert software version 11.0.5.0 (STAT-EASE Inc., USA) was used for the experimental design, optimization, and statistical analysis. The MINITAB software version 18.1 (Minitab Inc., USA) was used to screen variables. P-test, coefficient of determination (R^2), coefficient of variation (CV), and lack of fit test at $p < 0.05$, 0.01, and 0.001 were used to assess the accuracy of the model. All experimental analyses were performed in triplicates and data processed using Microsoft Excel version 2016 (Microsoft Corporation, USA) and reported as mean ± standard deviation. OriginPro software version 2018 (OriginLab Corporation, USA) was used to construct all graphs. Tukey's test was applied to compare the averages/means at $p < 0.05$.

3. Results and discussion

3.1. Evaluation of growth ability of L. casei and L. helviticus mixed culture in heilong48 soybean substrate under solid-state fermentation

The growth ability of *L. casei* and *L. helviticus* mixed culture in the heilong48 soybean substrate was assessed by plate counting on deMan Rogosa Sharpe agar immediately after inoculation (T_0) and after 48 h of solid-state fermentation (T_{48}) at 37 °C. An increase of 10^1 CFU/g in the *L. casei* and *L. helviticus* mixed culture concentration was obtained, probably due to a synergistic effect of the strains. This is in agreement with the study performed by Hadj Saadoun et al. (2021). Interactions of *L. casei*–*L. helviticus* mixed culture during the solid-state fermentation process in heilong48 soybean has never been studied before. However, the knowledge acquired from the food industry shows that metabolic interactions among bacteria can be useful for modifying substrates. Utilization of co-cultures is advantageous compared to single or pure cultures owing to the synergistic action of the metabolic pathways of the strains involved, resulting in increased substrates degradation, which leads to an increase in peptides and amino acids, organic acids, and volatile compounds production (Hadj Saadoun et al., 2021).

3.2. Screening of solid-state fermentation conditions for extraction of chlorogenic acid from heilong48 soybean variety using Plackett-Burman design

Based on previous reports and preliminary experiments on solid-state fermentation (Akpabli-Tsigbe et al., 2021a; 2021b; Farinas, 2015), five (5) parameters namely temperature, pH, incubation time, inoculation size, and liquid-solid ratio were screened using Plackett-Burman design for chlorogenic acid extraction from heilong48 soybean variety. The design matrix with chlorogenic acid yield (mg/g), fermentation efficiency (%), total phenolic acids (mg GAE/g), and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (μmol AA eq/g dry sample) as responses, regression analysis and analysis of variance (ANOVA) are shown in Tables 1 and 2. Thirteen experiments given by the Plackett-Burman design were conducted to determine the effect of the variables on chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. From the Plackett-Burman design results on the modeling of the solid-state fermentation conditions, four (4) out of five (5) solid-state fermentation conditions showed a significant effect on chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of heilong48 soybean variety using *L. casei* and *L. helviticus* mixed culture. This indicates that the contribution of the non-selected solid-state fermentation condition to the responses examined at the selected confidence level was not significant ($p > 0.05$) (Table 2). Likewise, three (3) out of four (4) responses were highly significant for measuring the effects of the solid-state fermentation

Table 1
Plackett-Burman design showing the values of variables and experimental data for chlorogenic acid yield, fermentation efficiency, total phenolic acids, and 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity (DPPH) of heilong48 soybean variety.

Test	Factor					Response[c]			
	A	B	C	D	E	Chlorogenic acid yield (mg/g)	Fermentation efficiency (%)	TPA (mg GAE/g)	DPPH (μmol AA eq/g dry sample)
1	30(−)	5(−)	0(−)	5(+)	0.67(+)	22.136 ± 0.003	20.444 ± 0.002	2.233 ± 0.001	87.533 ± 0.000
2	30(−)	5(−)	0(−)	1(−)	0.25(−)	28.060 ± 0.001	23.795 ± 0.003	2.222 ± 0.000	86.033 ± 0.000
3	30(−)	7(+)	0(−)	1(−)	0.25(−)	13.766 ± 0.002	11.676 ± 0.001	2.230 ± 0.000	85.786 ± 0.000
4	50(+)	5(−)	0(−)	1(−)	0.67(+)	21.995 ± 0.003	15.834 ± 0.001	2.018 ± 0.000	87.333 ± 0.001
5	50(+)	7(+)	0(−)	5(+)	0.67(+)	7.195 ± 0.003	6.103 ± 0.001	2.254 ± 0.005	86.034 ± 0.001
6	50(+)	7(+)	0(−)	5(+)	0.25(−)	5.063 ± 0.003	4.292 ± 0.001	2.377 ± 0.001	85.972 ± 0.005
7	50(+)	5(−)	48(+)	5(+)	0.25(−)	30.197 ± 0.002	25.607 ± 0.002	2.213 ± 0.001	86.683 ± 0.001
8	50(+)	5(−)	48(+)	1(−)	0.25(−)	33.315 ± 0.004	28.256 ± 0.003	2.105 ± 0.001	86.741 ± 0.000
9	50(+)	7(+)	48(+)	1(−)	0.67(+)	35.616 ± 0.003	30.204 ± 0.004	2.222 ± 0.000	87.741 ± 0.001
10	30(−)	5(−)	48(+)	5(+)	0.67(+)	26.254 ± 0.004	22.263 ± 0.002	2.287 ± 0.000	86.860 ± 0.001
11	30(−)	7(+)	48(+)	5(+)	0.25(−)	5.223 ± 0.003	4.452 ± 0.0.003	2.230 ± 0.000	85.329 ± 0.001
12	30(−)	7(+)	48(+)	1(−)	0.67(+)	30.034 ± 0.002	25.466 ± 0.004	2.339 ± 0.000	85.741 ± 0.000
13	40(0)	6(0)	24(0)	3(0)	0.46(0)	6.044 ± 0.002	5.125 ± 0.004	2.352 ± 0.001	86.909 ± 0.005

A: Temperature; B: pH; C: incubation time; D: inoculation size; E: liquid-solid ratio.
TPA: Total phenolic acids; DPPH: 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity.
GAE: Gallic acid equivalents; AA: Ascorbic acid; eq: equivalents.
[c] Data were mean values (x3).

Table 2
Analysis of variance and regression analysis of Plackett-Burman design data for significant factors predictions of heilong48 soybean variety using solid-state fermentation by *L. casei* and *L. helviticus* mixed culture.[a].

Source	Regression data[c]											
	Chlorogenic acid yield (mg/g)			Fermentation efficiency (%)			Total phenolic acids (mg GAE/g)			DPPH (μmol AA eq/g dry sample)		
	Effect	F-value	p-value	Effect	F-value	p-value	Effect	F-value	p-value	Effect	F-value	p-value
Model		22.51	<0.0001*		23.66	<0.0001*		2.34	0.063[NS]		17.87	<0.0001*
A	4.83	23.31	<0.0001*	5.02	25.25	<0.0001*	0.83	0.68	0.415[NS]	0.08	0.01	<0.934[NS]
B	2.66	7.10	0.012*	3.18	10.10	0.003*	−0.22	0.05	0.830[NS]	6.64	44.09	<0.0001*
C	2.55	6.51	0.016*	2.71	7.35	0.011*	1.29	1.67	0.205[NS]	−4.18	17.47	<0.0001*
D	−1.60	2.56	0.119[NS]	−1.50	2.26	0.142[NS]	2.52	6.34	0.017*	−0.61	0.37	0.545[NS]
E	−8.55	73.08	<0.0001*	−8.56	73.33	<0.0001*	1.72	2.97	0.094[NS]	−5.23	27.40	<0.0001*

Regression model

Regression model	R^2	Regression model	R^2
Chlorogenic acid yield = 4.49 + 0.4586A + 2.530B + 0.1010C–0.760D – 38.66E	R^2 = 77.33%	Fermentation efficiency = 0.66 + 0.3982A + 2.519B + 0.0895C–0.596D – 32.32E	R^2 = 78.19%
TPA = 2.080 + 0.00120A–0.0031B + 0.000781C + 0.01824D + 0.1189E	R^2 = 26.19%	DPPH = 84.901 + 0.00057A + 0.4539B–0.01191C – 0.0209D – 1.704E	R^2 = 73.03%

[a]: Results were obtained using Minitab 18.0 *: significant at $p < 0.05$ [C]: Data were mean values (x3) [NS]: not significant.
A: Temperature; B: pH; C: incubation time; D: inoculation size; E: liquid-solid ratio; TPA: Total phenolic acids; DPPH: 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity; GAE: Gallic acid equivalents; AA: Ascorbic acid; eq: equivalents.

conditions (Fig. 2). The chlorogenic acid yield ranged from 5.063 ± 0.003 to 35.616 ± 0.003 mg/g, for the solid-state fermentation conditions used (Table 1). Under the maximum yield of chlorogenic acid (35.616 ± 0.003 mg/g), the fermentation efficiency was 30.204 ± 0.004%, total phenolic acids were 2.222 ± 0.000 mg GAE/g, and 2, 2-diphenyl-1-picrylhydrazyl free radical-scavenging activity was 87.741 ± 0.001 μmol AA eq/g dry sample (Table 1).

3.3. Analysis of screening design (data obtained by Plackett-Burman design)

The analysis of variance showed a statistically significant and adequate ($p < 0.05$) regression model for 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity, fermentation efficiency, and chlorogenic acid yield. However, the regression model for total phenolic acids was insignificant ($p > 0.05$), indicating that total phenolic acids were not a good response index for measuring the effects of the solid-state fermentation factors for chlorogenic acid extraction from heilong48 soybean variety. In addition, the R^2 value of total phenolic acids was 26.19%, suggestive that its first-order model equation was unreliable (Table 2) hence not significant. Furthermore, none of the solid-state fermentation parameters were above the Bonferroni limit line (2.733) for total phenolic acids, therefore not used in the optimization study (further experiments). The R^2 values of 77.33% (chlorogenic acid yield), 78.19% (fermentation efficiency) and 73.03% (2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity) (Table 2) indicated adequate and significant regression model (Boateng & Yang, 2021). The R^2 values of chlorogenic acid, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity obtained showed that their Pareto charts and first-order model equations were reliable. Except for inoculation size (IS), the rest of the solid-state fermentation conditions; liquid-solid ratio, incubation time, pH, and temperature significantly influenced chlorogenic acid extraction ($p < 0.05$) from heilong48 soybean variety (Table 2). These results agree with the report of Farinas (2015). Similarly, Li et al. (2023) also reported fermentation temperature, fermentation time and solid-water ratio as significant solid-state fermentation conditions.

Graphical method (Pareto chart) was used to show the effect of solid-state fermentation factors on response variables and their significance statistically with limit lines. The Pareto chart presents the ordered standardized effect of each variable. It identifies the magnitude and importance of each variable through the ordering of different effects by

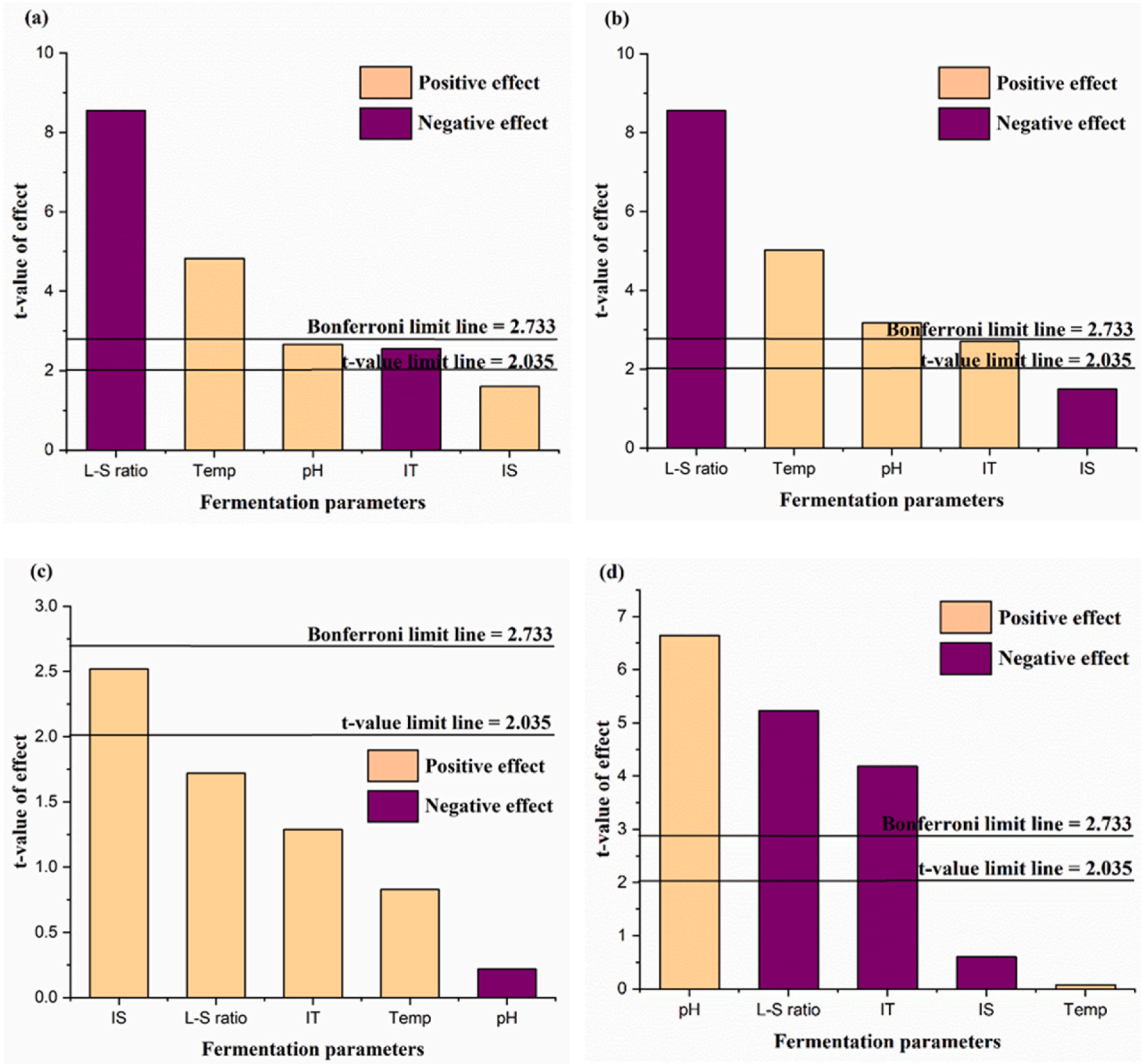

Fig. 2. Pareto chart for chlorogenic acid yield (a), fermentation efficiency (b), total phenolic acids (c), and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (d) of *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample. Variables having *t*-values higher than the critical value (2.035) were considered statistically significant.

magnitude relative to the standardized effect plot against each magnitude of influence (Sha et al., 2019). The length of each bar is proportional to its corresponding effect on the response variables. Also, the standardized effect of each factor is statistically significant if it exceeds a threshold (Bonferroni and *t*-value limit lines). Extremely significant (*t*-value of effect above the Bonferroni limit line), significant (*t*-value of effect above the *t*-value limit line), and insignificant (*t*-value of effect below the *t*-value limit line) coefficients of factors were used to intuitively show the important solid-state fermentation factors (Akpabli-Tsigbe et al., 2021a; 2021b). The most significant influences were ranked in decreasing order based on their significance.

From the solid-state fermentation model, the liquid-solid ratio exhibited a significant effect on chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity; temperature exhibited a significant effect on chlorogenic acid yield and fermentation efficiency; pH was important for fermentation efficiency and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity, and incubation time exhibited significant effect on 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (Fig. 2). Liquid-solid ratio, temperature, pH, and incubation time were extremely significant with respect to the Bonferroni limit line (2.733) (Fig. 2; a – d). The liquid-solid ratio showed a negative effect on chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. This indicates that a higher liquid-solid ratio reduces the chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. High moisture content reduces permeability, with damage of the structure of the particles and interfering in oxygen diffusion inhibiting the growth of microorganisms (Yazid et al., 2017) and their activities. Thus, the decrease in the responses observed was due to inhibition of the growth of the *L. casei* and *L. helviticus* mixed culture used as the liquid-solid ratio increased (Fig. 2; a, b and d). In addition, at a high liquid-solid ratio, degradation of chlorogenic acid occurs causing a reduction in antioxidant activity (Akpabli-Tsigbe et al., 2021a), hence the negative effect obtained for 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. A similar effect of the liquid-solid ratio was reported by Akpabli-Tsigbe et al. (2021a) and Sha et al. (2019).

Temperature exhibited a positive effect on chlorogenic acid yield and fermentation efficiency. This implies that increasing temperature increased the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture used and the chlorogenic acid yield. These results are in agreement with those reported by Akpabli-Tsigbe et al. (2021a) who found that increasing temperature increases chlorogenic acid extraction. Ahmad and Munaim (2017) also reported a high yield of sorbitol (25.68 g/L) at increasing temperature, produced via solid-state fermentation. Temperature is one of the most important parameters for solid-state fermentation and influences the growth of microorganisms, enzyme activity, and metabolite production (Handa et al., 2019). This probably means that the optimum growth temperature for the bacteria (*L. casei* and *L. helviticus* mixed culture) was obtained when the temperature increased, thus acting efficiently upon the substrate for the production of more chlorogenic acid. Incubation time (negative effect) and pH (positive effect) had an opposing influence on 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. The implication is that 2, 2-diphenyl-1-picrylhydrazyl free radical-scavenging activity increases with increasing pH but decreases as incubation time increases. This might be because, at increased incubation time, the bacteria (*L. casei* and *L. helviticus* mixed culture) had reached their lag phase hence their activity reduced at this point of the solid-state fermentation process consequently resulting in a reduction in the release of phenolic compounds leading to a decrease in 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. Ahmad and Munaim (2017) reported a similar result; decreased sorbitol yield at an increased incubation time. Only inoculation size was positively significant for total phenolic acids, thus, had a *t*-value effect above the *t*-value limit line (2.035). The Plackett-Burman design results showed that liquid-solid ratio, pH, temperature, and incubation time were the most important solid-state fermentation factors for extraction of chlorogenic acid from heilong48 soybean variety, hence were used in further experiments.

According to Yang et al. (2021), responses increase and then decrease when the inoculation size increases. Also, Ku et al. (2009) reported that a higher inoculum density causes a decrease of enzyme activity, and the activity decreases to less than 70% when the inoculum density reached 6%. Less seeding culture generates more viable cells with a higher ability to produce the enzyme. The enzymes produced break phenolics-starch and phenolics-protein bonds in the heilong48 soybean to release more chlorogenic acid. Thus, the highest levels of responses are attained when the inoculation size reached a mid-value. The results indicated that a mid-inoculation size was satisfactory for the growth of the *L. casei* and *L. helviticus* mixed culture and their activities. And might help increase the interaction between the heilong48 soybean and the *L. casei* and *L. helviticus* mixed culture in the fermentation vessel by enhancing the responses. Hence, a 3% inoculation size was used in further experiments.

3.3.1. Fitting models for solid-state fermentation conditions for extracting chlorogenic acid from heilong48 soybean variety

The experiments depicted in the design matrix were implemented to obtain the optimum combination of four solid-state fermentation conditions (X_1: temperature, X_2: pH, X_3: incubation time and X_4: liquid-solid ratio) on chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample (Table 3). Analysis of variance was applied to assess the fitting degree of the response surface quadratic polynomial model. The *p*-values of the P-test, coefficient of determination (R^2), coefficient of variation, and lack of fit test were used to assess the significance of the model equations statistically. The results (Table 4) showed low *p*-values (<0.0001) for the models for all dependent variables (responses), suggestive of highly significant regression models (Gu et al., 2020).

The *p*-values (100%, 38.53%, and 55.32% for chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity respectively) of lack-of-fit indicated insignificant lack-of-fit relative to pure error, hence the model equations were

Table 3
Box-Behnken design and results for solid-state fermentation conditions for chlorogenic acid extraction from heilong48 soybean variety using *L. casei* and *L. helviticus* mixed culture.

Run	Fermentation parameters (actual and coded values)				Response[c]		
	Temperature (°C)	pH	IT (h)	L-S ratio	Chlorogenic acid yield (mg/g)	Fermentation efficiency (%)	DPPH (μmol AA eq/g dry sample)
	X_1	X_2	X_3	X_4			
1	50.00 (+1)	5.00 (−1)	24.00 (0)	0.46 (0)	8.30 ± 0.01	37.65 ± 0.04	48.97 ± 2.42
2	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	6.24 ± 0.01	24.73 ± 0.04	47.50 ± 2.72
3	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	5.92 ± 0.03	26.40 ± 0.14	49.22 ± 3.66
4	50.00 (+1)	7.00 (+1)	24.00 (0)	0.46 (0)	6.60 ± 0.03	28.82 ± 0.15	49.03 ± 3.79
5	50.00 (+1)	6.00 (0)	48.00 (+1)	0.46 (0)	7.04 ± 0.02	29.30 ± 0.08	61.35 ± 3.75
6	30.00 (−1)	7.00 (+1)	24.00 (0)	0.46 (0)	8.04 ± 0.04	34.63 ± 0.18	47.29 ± 6.47
7	50.00 (+1)	6.00 (0)	0.00 (−1)	0.46 (0)	7.52 ± 0.13	31.40 ± 0.53	48.25 ± 9.94
8	30.00 (−1)	6.00 (0)	48.00 (+1)	0.46 (0)	7.19 ± 0.01	30.90 ± 0.04	76.57 ± 7.64
9	40.00 (0)	7.00 (+1)	24.00 (0)	0.25 (−1)	6.90 ± 0.07	21.96 ± 0.29	41.29 ± 6.29
10	40.00 (0)	5.00 (−1)	0.00 (−1)	0.46 (0)	6.44 ± 0.07	29.79 ± 0.31	52.39 ± 6.12
11	30.00 (−1)	6.00 (0)	24.00 (0)	0.25 (−1)	6.85 ± 0.08	20.25 ± 0.32	43.86 ± 7.23
12	40.00 (0)	6.00 (0)	0.00 (−1)	0.25 (−1)	5.90 ± 0.07	19.00 ± 0.28	40.00 ± 4.75
13	40.00 (0)	6.00 (0)	0.00 (−1)	0.67 (+1)	7.45 ± 0.01	22.55 ± 0.04	62.80 ± 5.10
14	40.00 (0)	6.00 (0)	48.00 (+1)	0.67 (+1)	6.07 ± 0.01	22.49 ± 0.04	84.16 ± 3.66
15	40.00 (0)	5.00 (−1)	48.00 (+1)	0.46 (0)	7.84 ± 0.04	29.99 ± 0.19	75.29 ± 6.70
16	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	6.32 ± 0.05	25.80 ± 0.23	49.25 ± 7.07
17	40.00 (0)	6.00 (0)	48.00 (+1)	0.25 (−1)	7.18 ± 0.12	21.34 ± 0.51	66.40 ± 4.29
18	30.00 (−1)	6.00 (0)	24.00 (0)	0.67 (+1)	6.67 ± 0.03	29.25 ± 0.15	65.60 ± 8.66
19	40.00 (0)	7.00 (+1)	24.00 (0)	0.67 (+1)	6.78 ± 0.01	24.16 ± 0.04	68.29 ± 9.88
20	50.00 (+1)	6.00 (0)	24.00 (0)	0.67 (+1)	7.35 ± 0.02	24.24 ± 0.08	61.25 ± 5.55
21	50.00 (+1)	6.00 (0)	24.00 (0)	0.25 (−1)	6.74 ± 0.07	28.54 ± 0.29	41.84 ± 8.71
22	40.00 (0)	5.00 (−1)	24.00 (0)	0.25 (−1)	6.64 ± 0.05	23.19 ± 0.23	53.36 ± 6.54
23	40.00 (0)	5.00 (−1)	24.00 (0)	0.67 (+1)	7.16 ± 0.02	25.70 ± 0.07	66.50 ± 9.85
24	30.00 (−1)	5.00 (−1)	24.00 (0)	0.46 (0)	6.45 ± 0.03	28.55 ± 0.11	58.11 ± 5.97
25	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	5.93 ± 0.01	25.69 ± 0.04	48.29 ± 9.74
26	40.00 (0)	7.00 (+1)	0.00 (−1)	0.46 (0)	7.80 ± 0.01	27.45 ± 0.04	49.70 ± 4.61
27	30.00 (−1)	6.00 (0)	0.00 (−1)	0.46 (0)	6.79 ± 0.00	28.50 ± 0.00	40.89 ± 6.39
28	40.00 (0)	6.00 (0)	24.00 (0)	0.46 (0)	6.19 ± 0.01	25.43 ± 0.04	47.59 ± 4.59
29	40.00 (0)	7.00 (+1)	48.00 (+1)	0.46 (0)	6.35 ± 0.03	25.55 ± 0.14	71.20 ± 2.99

X_1 = Temperature; X_2 = pH; X_3 = IT: Incubation time; X_4 = L-S ratio: Liquid-solid ratio.
DPPH = 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity; AA: Ascorbic acid; eq: equivalents; [C]: Data were average values (x3).

Table 4
Analysis of variance for response surface methodology and regression analysis for Box-Behnken design data of chlorogenic acid extraction from heilong48 soybean variety by solid-state fermentation using *L. casei* and *L. helviticus* mixed culture.

Source	Chlorogenic acid yield (mg/g)		Fermentation efficiency (%)		DPPH (μmol AA eq/g dry sample)	
	F-value	p-value	F-value	p-value	F-value	p-value
Model	83.35	<0.0001***	71.43	<0.0001***	402.54	<0.0001***
Linear						
X_1 = A: Temperature	20.18	0.0005***	10.62	0.0057**	54.77	<0.0001***
X_2 = B: pH	1.07	0.3175 NS	25.94	0.0002***	90.60	<0.0001***
X_3 = C: Incubation time	0.44	0.5185 NS	0.13	0.7210 NS	2325.30	<0.0001***
X_4 = D: Liquid-Solid ratio	13.37	0.0026**	34.14	<0.0001***	1738.05	<0.0001***
Interactions						
AB	269.27	<0.0001***	114.36	<0.0001***	41.57	<0.0001***
AC	19.26	0.0006***	10.42	0.0061**	179.05	<0.0001***
AD	15.53	0.0015**	90.99	<0.0001***	1.91	0.1890 NS
BC	202.06	<0.0001***	2.27	0.1543 NS	0.69	0.4207 NS
BD	10.19	0.0065**	0.049	0.8273 NS	67.46	<0.0001***
CD	176.02	<0.0001***	2.96	0.1072 NS	8.92	0.0098**
Quadratic						
A^2	256.52	<0.0001***	248.55	<0.0001***	10.53	0.0059**
B^2	232.69	<0.0001***	82.86	<0.0001***	126.52	<0.0001***
C^2	95.26	<0.0001***	0.12	0.7359 NS	852.69	<0.0001***
D^2	14.36	0.0020**	253.08	<0.0001***	272.38	<0.0001***
Fitting statistics						
Lack of fit	0.016	1.0000 NS	1.45	0.3853 NS	0.99	0.5532 NS
R^2	0.9881		0.9862		0.9975	
Adjusted R^2	0.9763		0.9724		0.9950	
Predicted R^2	0.9796		0.9330		0.9887	
Adeq. Precision	33.728		36.549		72.178	
C.V. %	0.24		2.61		1.51	
Standard Dev.	0.10		0.70		0.84	

Optimization equations

$$Chlorogenic\ acid\ yield\ (mg/g) = 6.12 + 0.13X_1 - 0.03X_2 - 0.019X_3 + 0.11X_4 - 0.82X_1X_2 - 2.22X_1X_3 + 0.2X_1X_4 - 0.71X_2X_3 - 0.16X_2X_4 - 0.66X_3X_4 + 0.63X_1{}^2 + 0.6X_2{}^2 + 0.38X_3{}^2 + 0.15X_4{}^2$$

$$Fermentation\ efficiency\ (\%) = 25.61 + 0.66X_1 - 1.02X_2 + 0.073X_3 + 1.18X_4 - 3.73X_1X_2 - 1.12X_1X_3 - 3.33X_1X_4 - 0.52X_2X_3 - 0.077X_2X_4 - 0.6X_3X_4 + 4.2X_1{}^2 + 2.49X_2{}^2 + 0.094X_3{}^2 - 4.35X_4{}^2$$

$$DPPH\ (\mu mol\ AA\ eq/g\ dry\ sample) = 48.37 - 1.8X_1 - 2.32X_2 + 11.75X_3 + 10.15X_4 + 2.72X_1X_2 - 5.64X_1X_3 - 0.58X_1X_4 - 0.35X_2X_3 + 3.47X_2X_4 - 1.26X_3X_4 - 1.08X_1{}^2 + 3.73X_2{}^2 + 9.67X_3{}^2 + 5.47X_4{}^2$$

*, ** and *** denote significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively while NS denotes not significant.
DPPH: 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity; AA: Ascorbic acid; eq: equivalents; C.V.: Coefficient of variation; Dev.: Deviation.

satisfactory for the prediction of chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity in the whole experimental run. The goodness of fit of the regression model was evaluated with R^2 values of 98.81%, 98.62%, and 99.75% (chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity respectively). An R^2 value close to one (1) shows a better explanation of the variability of the experimental data by the proposed model; in other words, a better correlation exists between observed and predicted values (Akpabli-Tsigbe et al., 2021a; Handa et al., 2019). This indicates that the experimented values obtained had a better correlation with the predicted values. *Adjusted* R^2 values of 97.63%, 97.24%, and 99.50% (chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity respectively) were close to 100%, representing the satisfactory correlation between predicted and experimental values (Gu et al., 2020).

Coefficient of determination (R^2) values should be at least 80% to have a good fit of the model. The closer the goodness of fit to 100% (or 1), the better the empirical model fits the actual data (Adebo et al., 2018). This demonstrates that the empirical model obtained in this study fits the actual data. Furthermore, the low coefficient of variation values of 0.24, 2.61, and 1.51% (chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity respectively) and the high *Adeq. precision* values of 33.73, 36.55, and 72.18 (chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity respectively) indicates very high reliability between precision and experimental values (Gu et al., 2020). The closeness of the bias factor and accuracy factor to unity (1) and that of average absolute deviation to zero (0) indicates reasonable agreements between the predicted and experimental or observed results (Table 5). The bias factor equal to one (1) obtained for all the models indicates that fail-safe and fail-dangerous predictions were on average, compensated. The bias factors obtained were within the 0.75–1.25 limits (proposed acceptable limits) (Bover-Cid et al., 2012), indicating good predictive performance. Likewise, the accuracy factors of the models were within the acceptable limit, considering that the accuracy factor typically increase by 0.1–0.15 for every variable in the model, introducing 10–15% of variability (Bover-Cid et al., 2012). The analysis of the significance for regression coefficient showed that the *p*-value of the linear coefficients (X_1, X_2, X_3, and X_4) and quadratic term coefficients (X_1^2, X_2^2, X_3^2, and X_4^2) were significant ($p < 0.05$), indicative that the effect of temperature, pH, incubation time and the liquid-solid ratio significantly correlated with chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity.

3.3.1.1. Influence of variables on chlorogenic acid yield of L. casei and L. helviticus mixed culture fermented heilong48 soybean (MCFHS) sample. The quadratic model representing chlorogenic acid yield as a function of temperature (X_1), pH (X_2), incubation time (X_3), and liquid-solid ratio (X_4) was expressed in an equation. The R^2 value (close to 100%) as shown in Table 4 indicates that the proposed mathematical model of the chlorogenic acid yield [Equation (12)] can explain more than 98% of the experimental observations as a function of temperature, pH, incubation time and liquid-solid ratio. Using *p*-values to establish the significance of each coefficient and interaction strength of each parameter, the linear

Table 5
Coefficient of regression for the different mathematical models obtained.

Coefficient	Chlorogenic acid yield (mg/g)	Fermentation efficiency (%)	DPPH (μmol AA eq/g dry sample)
β_o	6.12	25.61	48.37
Linear			
β_1	0.13	0.66	−1.8
β_2	−0.03	−1.02	−2.32
β_3	−0.019	0.073	11.75
β_4	0.11	1.18	10.15
Interactions			
β_{12}	−0.82	−3.73	2.72
β_{13}	−2.22	−1.12	−5.64
β_{14}	0.2	−3.33	−0.58
β_{23}	−0.71	−0.52	−0.35
β_{24}	−0.16	−0.077	3.47
β_{34}	−0.66	−0.6	−1.26
Quadratics			
β_{11}	0.63	4.2	−1.08
β_{22}	0.6	2.49	3.73
β_{33}	0.38	0.094	9.67
β_{44}	0.15	−4.35	5.47
Fitting statistics			
R^2 (%)	98.81	98.62	99.75
Average absolute deviation (%)	0.60	1.53	0.89
Average absolute deviation	0.01	0.02	0.01
Bias factor	1.00	1.00	1.00
Accuracy factor	1.08	1.21	1.12

β represents the coefficients of equations of the different models with β_o representing the constant term, β_1, β_2, β_3, and β_4 the linear effects of temperature, pH, incubation time, and liquid-solid ratio, respectively, β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} their interactions and β_{11}, β_{22}, β_{33}, and β_{44} their quadratic effects.
DPPH: 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity; AA: Ascorbic acid; eq: equivalents.

factors of temperature and liquid-solid ratio had a significant ($p < 0.05$) effect on chlorogenic acid yield of the *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample (Table 4). The polynomial second-order regression equation written in coded variables after removal of insignificant solid-state fermentation conditions was:

$$Chlorogenic\,acid\,yield\,(mg/g) = 6.12 + 0.13X_1 + 0.11X_4 - 0.82X_1X_2 - 2.22X_1X_3 + 0.2X_1X_4 - 0.71X_2X_3 - 0.16X_2X_4 - 0.66X_3X_4 + 0.63X_1{}^2 + 0.6X_2{}^2 + 0.38X_3{}^2 + 0.15X_4{}^2 \quad (12)$$

The solid-state fermentation conditions were optimized with response surface methodology. Both 3-dimensional response surface and 2-dimensional contour plots show graphically the association between two variables when the other variable is fixed at zero level (Gu et al., 2020). Two of the linear terms (temperature and liquid-solid ratio) were in positive significant association with the chlorogenic acid yield of the MCFHS sample, while the effects of the rest were insignificant ($p > 0.05$) though positive. The results also showed that all the interactive and quadratic terms had a positive significant effect on the chlorogenic acid yield of the MCFHS sample (Table 4). Since the positive linear effect of temperature and liquid-solid ratio were significant ($p < 0.05$) on the chlorogenic acid yield, as well as their corresponding quadratic factors, it can be suggested that both variables had a cumulative positive effect on the chlorogenic acid yield of MCFHS sample. From the 3-dimensional response surface charts (Fig. 3), all the solid-state fermentation conditions showed a decreasing effect on chlorogenic acid yield (to some point) as they increased from their lowest value to somewhere mid-range or slightly above, and then increased from this point to the highest value. This was in accordance with the perturbation plot (Electronic Supplementary Fig. 1a). The general decrease of the chlorogenic acid yield with an increase in the solid-state fermentation conditions (from the start of the fermentation process) could be attributed to reduced extractability of the chlorogenic acid due to self-polymerization and/or interaction of phenolic compounds with other macromolecules. These results are in agreement with the report of Adebo et al. (2018).

Increased temperature and incubation time (X_1X_3) resulted in increased chlorogenic acid yield (Fig. 3b). Suggestive that the optimum conditions for adaptation of *L. casei* and *L. helvicus* mixed culture to the fermenting medium were achieved at an increased temperature and a longer incubation time, resulting in proliferation, and in turn degradation of the cell walls of the heilong48 soybean to release a considerable quantity of chlorogenic acid during fermentation (Akpabli-Tsigbe et al., 2021b; Su et al., 2018). This thus, indicates that at increased temperature and sufficiently long incubation time, the chlorogenic acid yield of the MCFHS sample will increase. A similar observation was made for increased temperature and liquid-solid ratio (X_1X_4) (Fig. 3c), and for increased pH and liquid-solid ratio (X_2X_4) (Fig. 3e). This data may be relevant to the industry with respect to the effect or importance of dual-factor combinations on chlorogenic acid yield in solid-state fermentation extraction processes. Hydroxycinnamic acids, mostly exist in linked-form in the cell walls of plants, with the soluble compounds mainly located inside the plant vacuoles (Akpabli-Tsigbe et al., 2021b; Santos da Silveira et al., 2019). Hence, the majority of the bonds/linkages in the cell walls of the heilong48 soybean were broken during the degradation process by the bacteria (*L. casei* and *L. helvicus* mixed culture) during the dual-factor combinations to obtain the increased chlorogenic acid yield observed in this study.

3.3.1.2. Influence of factors on the fermentation efficiency of L. casei and L. helviticus mixed culture in MCFHS sample. A 3-dimensional response surface graph (Fig. 4) was applied to understand the interactions between the solid-state fermentation conditions and their effects on the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture in the MCFHS sample. Each 3-dimensional response surface graph was a function of two variables at a time, with the third and fourth variables kept constant at zero/fixed level. The curvature of 3-dimensional response surface graphs indicates the efficiency of the quadratic terms on the plots (Dabbour et al., 2018). From the results, the predictive equation for explaining the fermentation efficiency of *L. casei* and *L. helviticus* mixed culture in extracting chlorogenic acid with high yield under the optimum solid-state fermentation conditions from heilong48 soybean variety was expressed in coded variables as follows, after removal of non-significant factors:

$$Fermentation\ efficiency\ (\%) = 25.61 + 0.66X_1 - 1.02X_2 + 1.18X_4 - 3.73X_1X_2 - 1.12X_1X_3 - 3.33X_1X_4 + 4.2X_1{}^2 + 2.49X_2{}^2 - 4.35X_4{}^2 \quad (13)$$

Table 4 shows that out of the four linear and quadratic terms, the influences of incubation time (X_3) and its quadratic term (X_3^2) on the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture were not significant, however, positive. Similarly, only the interactive effects of temperature and pH (X_1X_2), temperature and incubation time (X_1X_3), and temperature and liquid-solid ratio (X_1X_4) exhibited significant effects on the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture in the MCFHS sample. The 3-dimensional response surface graph showed that, as the temperature increased, the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture decreased

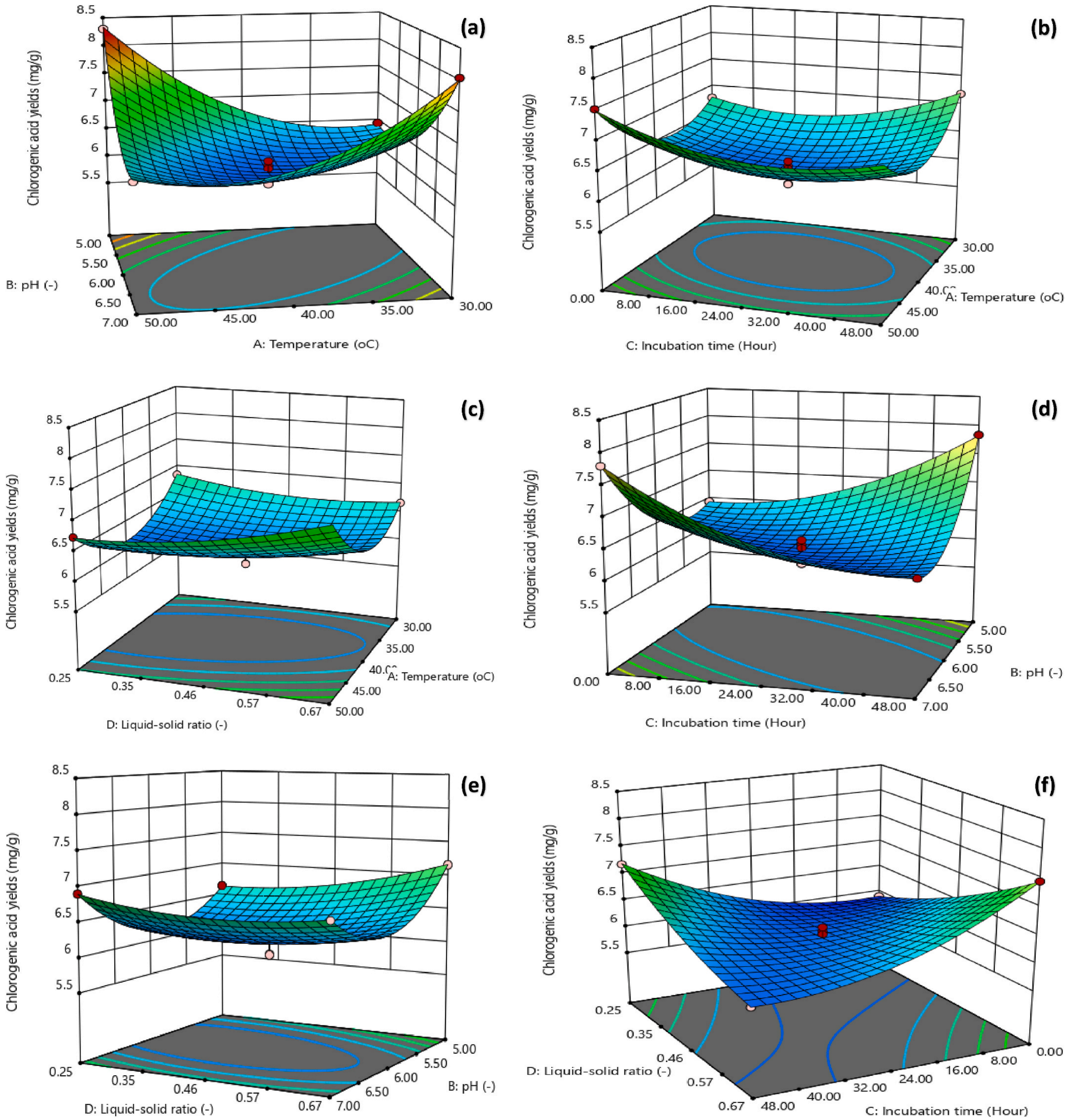

Fig. 3. Response surface and contour plots of the interactive effect of temperature, pH, incubation time, and liquid-solid ratio on the chlorogenic acid yield of *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample.

gradually to some point mid-ranged of temperature value and then increased from this point to the highest value at 50 °C (Fig. 4; a, b and c). *Lactobacillus* species (*L. casei* and *L. helviticus*) possess cinnamoyl esterase enzymes which are temperature specific for hydrolysis of ester bonds (resulting in the release of chlorogenic acid) (Aguirre Santos et al., 2018; Akpabli-Tsigbe et al., 2021a). This indicates that the optimum temperature for the activities of cinnamoyl esterase enzymes produced by the *L. casei* and *L. helviticus* mixed culture was not attained when the temperature was below the threshold level. Nonetheless, as temperature increased beyond the threshold level, the optimum temperature was attained and the activities of the enzymes increased resulting in the high fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture obtained after the threshold level. This result agreed with the perturbation plot shown by the gentle slope (Electronic Supplementary Fig. 1b).

The same observation (Fig. 4; a, d and e) was made for pH. This could be that the conditions for the growth of *L. casei* and *L. helviticus* mixed culture were not favorable when pH was below the threshold level. However, as it increased beyond the threshold level, the optimum conditions for the proliferation of *L. casei* and *L. helviticus* mixed culture

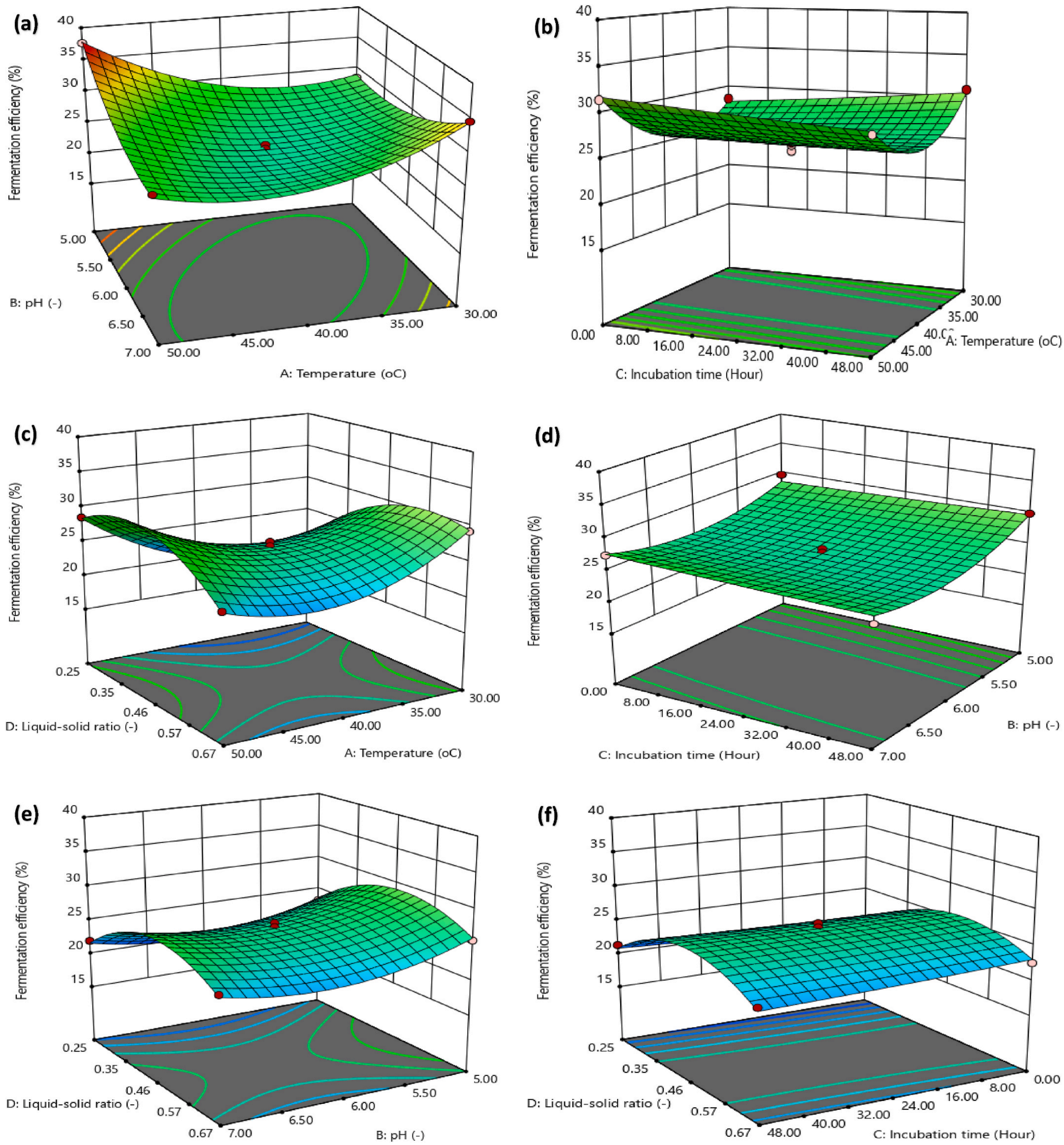

Fig. 4. Response surface and contour plots of the interactive effect of temperature, pH, incubation time, and liquid-solid ratio on the fermentation efficiency of *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample.

were attained and their activity increased leading to the high fermentation efficiency obtained after the threshold level. Though pH enhances the efficiency of solid-state fermentation, it also influences the growth, proliferation, and substrate colonization of microorganisms (Akpabli-Tsigbe et al., 2021a). Hence, optimum pH is required by microorganisms for high fermentation efficiency. Similar results were obtained in our previous work (Akpabli-Tsigbe et al., 2021b). On the contrary, the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture in the MCFHS sample increased gradually to some point mid-ranged of liquid-solid ratio value and then decreased as the liquid-solid ratio increased to the highest value (0.67 [40:60]) (Fig. 4; c, e, and f). The fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture decreased at a high liquid-solid ratio level. High moisture content decreases porosity, with the loss of the structure of the particles and interfering in oxygen diffusion which leads to inhibition of microbial growth and activities (Yazid et al., 2017). Due to this, the *L. casei* and *L.*

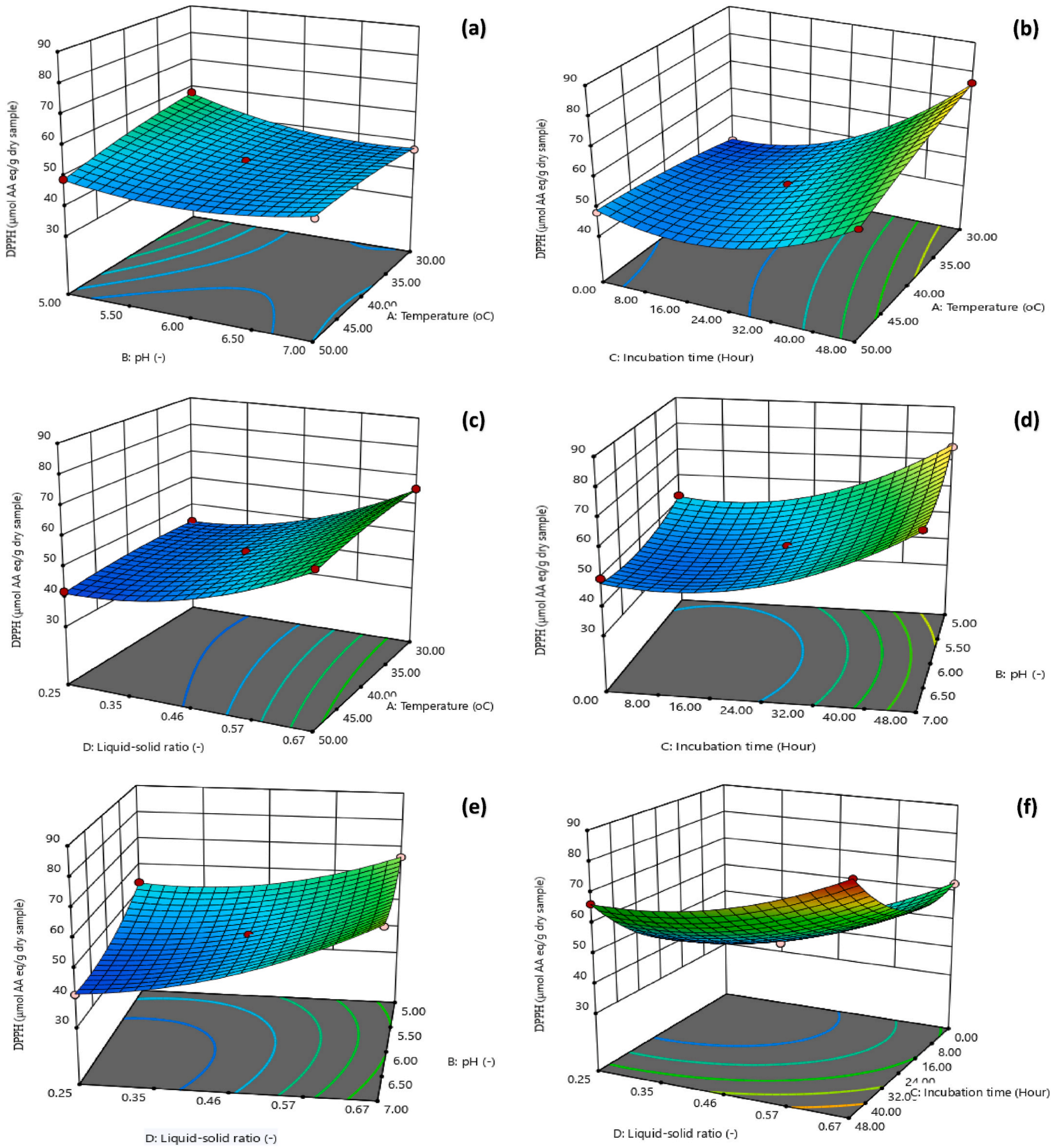

Fig. 5. Response surface and contour plots of the interactive effect of temperature, pH, incubation time, and liquid-solid ratio on the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (DPPH) of *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample.

helviticus mixed culture was therefore not active at a high liquid-solid ratio to act upon the heilong48 soybean substrate, resulting in a decrease in their fermentation efficiency as obtained. However, there were no observable changes in the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture with respect to incubation time, thus the fermentation efficiency of the *L. casei* and *L. helviticus* mixed culture was constant with increased incubation time (Fig. 4; b, d, and f). This might be that the *L. casei* and *L. helviticus* mixed culture was very active and reached a stationary phase within a relatively short incubation time hence their efficiency became constant as the incubation time increased further.

3.3.1.3. Influence of variables on 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (DPPH) of L. casei and L. helviticus mixed culture fermented heilong48 soybean (MCFHS) sample. Soybeans and soy products are known to be rich in flavonoids, phenolic compounds, and antioxidants and thus considered health-promoting foods. Antioxidants prevent auto-oxidation of food components and neutralize the excess free radicals produced in the human body (Akpabli-Tsigbe et al., 2021a). Many fermented products are very useful in this regard because they have high antioxidant activity. Fermentation of food materials is therefore a valuable technology for the enhancement of the anti-oxidative activity of food products. In line with this, the antioxidant activity of the MCFHS sample was evaluated with the 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity method. A second-order quadratic equation obtained through regression analysis was applied to investigate the relationship between the 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity and solid-state fermentation conditions of the MCFHS sample. After the elimination of the insignificant terms, the regression equation depicting the effects of solid-state fermentation conditions on the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of MCFHS sample in coded terms was:

$$DPPH\ (\mu mol\ AA\ eq\ /\ g\ dry\ sample) = 48.37 - 1.8X_1 - 2.32X_2 + 11.75X_3 + 10.15X_4 + 2.72X_1X_2 - 5.64X_1X_3 + 3.47X_2X_4 - 1.26X_3X_4 - 1.08X_1^2 + 3.73X_2^2 + 9.67X_3^2 + 5.47X_4^2 \quad (14)$$

The results showed that all the linear terms were extremely significant ($p < 0.0001$) (Table 4). Incubation time showed the main effect on 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity. Increased incubation time, increased the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of MCFHS extremely (Fig. 5; b, d, and f). According to Dulf et al. (2016), solid-state fermentation enhances the recovery of valuable hydro- and lipophilic compounds positively, liberating free polyphenols and increasing the antioxidant activity. This means that the increased incubation time gave the carbohydrate degrading enzymes produced by the *L. casei* and *L. helviticus* mixed culture optimum time for enzymatic hydrolysis of phenolic conjugates (bonded to sugar moieties, organic acids, amines, and lipids) to increase the concentration of free phenolics which increased the 2,2-diphenyl-1--picrylhydrazyl free radical-scavenging activity of the MCFHS sample. This result agrees with literature (Adebo et al., 2018; Akpabli-Tsigbe et al., 2021a). Two of the interactive terms, temperature and liquid-solid ratio (X_1X_4), and pH and incubation time (X_2X_3) were not significant, even though had a positive effect (Table 4). The rest of the interactive terms had a positive significant effect on 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. As liquid-solid ratio and incubation time (X_3X_4) increased, 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity decreased gradually to some point mid-ranged of the factors and then increased from this point to the highest value at the highest levels of the factors (Fig. 5f). This indicates that the optimum conditions for the proliferation of *L. casei* and *L. helviticus* mixed culture were attained when liquid-solid ratio and incubation time increased enhancing the activities of the microorganisms to release more chlorogenic acid which in turn increased the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity. However, as temperature increased and incubation time decreased, the 2, 2-diphenyl-1-picrylhydrazyl free radical-scavenging activity decreased (Fig. 5b). According to Handa et al. (2019), the growth of microorganisms, enzyme activity, and metabolite production are influenced by temperature. High temperatures kill microorganisms (inhibiting their activities) while optimum temperatures enhance microbial proliferation and thus their efficiency, leading to the production of desired products (Akpabli-Tsigbe et al., 2021a). This could be that most of the *L. casei* and *L. helviticus* were killed as temperature increased and incubation time decreased, resulting in decreased phenolic compounds production as well as decreased 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (obtained in the present study). The effects of the quadratic terms of pH, liquid-solid ratio, and incubation time were highly significant ($p < 0.0001$), while that of temperature was significant ($p < 0.05$).

The 3-dimensional response surface and planar contour graphs between dual-response variables showing the influences of the solid-state fermentation conditions on the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of MCFHS (Fig. 5) were based on equation (14). The 3-dimensional response surface plots opening up depicted that the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity value decreased with the increasing of each solid-state fermentation condition, and then increased, indicative of a steady model and minimum stationary point (Fig. 5). The influence of incubation time and pH on the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity when the temperature and liquid-solid ratio were kept constant at 40 °C and 31.60:68.40 (0.46) respectively, showed that the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of MCFHS decreased as the incubation time and pH increased from 0 to 24 h and 5 to 6 respectively (Fig. 5d). Subsequently, the 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of the MCFHS sample increased slowly as the incubation time and pH exceeded 24 h and 6 respectively. This could be explained by longer incubation time and higher solid-state fermentation pH, which caused enzymatic hydrolysis of phenolic conjugates to increase the concentration of free phenolics during solid-state fermentation (Dulf et al., 2016), hence increasing the 2, 2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of the MCFHS sample. All the 3-dimensional response surface plots showed a similar trend (Fig. 5; a – f). The 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of the MCFHS sample increased when the temperature increased from 30 to 40 °C and decreased when the temperature exceeded 40 °C, suggestive of a stable model and maximum stationary point (Gu et al., 2020). The perturbation plot affirmed it (Electronic Supplementary Fig. 1c).

3.3.2. Optimization and verification of the predictive model

The optimized solid-state fermentation conditions were determined to obtain maximal 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity, fermentation efficiency, and chlorogenic acid yield. Consistent with the model prediction, the optimum solid-state fermentation conditions producing chlorogenic acid with high yield and increased biological (antioxidative) activity from heilong48 soybean variety were determined as liquid-solid ratio, 0.47 (32:68); incubation time, 48 h pH, 5 and temperature, 50 °C. The maximum predicted 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity, fermentation efficiency, and chlorogenic acid yield at the optimum solid-state fermentation factors were 65.19 μmol AA eq/g dry sample, 37.25% and 9.18 mg/g respectively. To examine and validate the reliability of the results, verification tests were conducted under these conditions. Three parallel (triplicate) experiments were performed. The average values of chlorogenic acid yield, fermentation efficiency, and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity of MCFHS sample obtained from the actual experiments were 9.20 ± 0.17 mg/g, 37.26 ± 0.73%, and 65.21 ± 2.05 μmol AA eq/g dry sample respectively. The residual standard errors of the predicted values and experimental values were found to be less than 5% (Akpabli-Tsigbe et al., 2021b) showing the feasibility and consistency of the model.

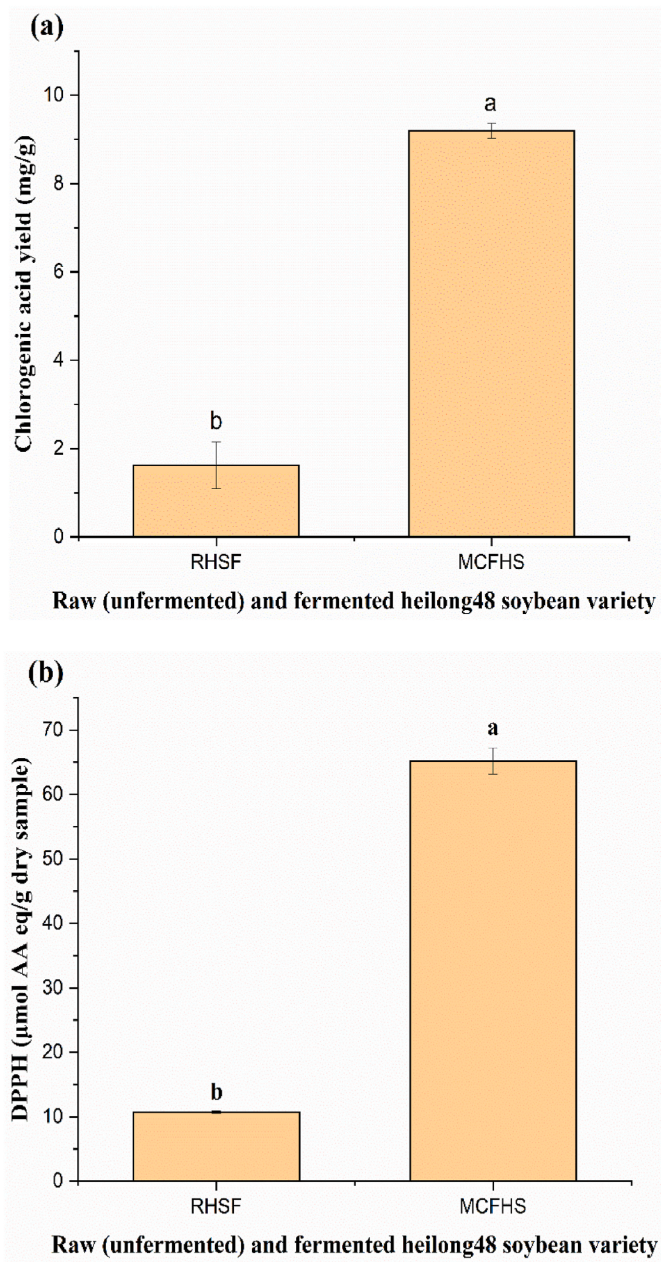

Fig. 6. Chlorogenic acid yield (a) and 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging activity (b) of raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples.

3.4. *Comparison of chlorogenic acid yield and antioxidant activity of raw (unfermented) heilong48 soybean flour (RHSF) and L. casei and L. helviticus mixed culture fermented heilong48 soybean (MCFHS) samples*

A comparison between raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples in terms of their antioxidant activity contents and chlorogenic acid yields was made. From the results (Fig. 6), the *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) sample had chlorogenic acid yield and antioxidant activity of 9.20 ± 0.17 mg/g and 65.21 ± 2.05 μmol AA eq/g dry sample respectively while the unfermented/raw heilong48 soybean flour (RHSF) sample had 1.62 ± 0.53 mg/g and 10.73 ± 0.20 μmol AA eq/g dry sample for chlorogenic acid yield and antioxidant activity respectively. The chlorogenic acid yield and antioxidant activity of the MCFHS sample were significantly ($p < 0.05$) higher than that of the RHSF sample, indicative of tremendous improvement. The results conformed to the report of Li et al. (2020); fermented soy products exhibit higher antioxidative activities. However, the chlorogenic acid yield (9.20 ± 0.17 mg/g) obtained in this study was higher than 8.80 ± 0.08 mg/g obtained using pure or single culture of *L. casei* (Akpabli-Tsigbe et al., 2021b) but less than 11.41 ± 0.27 mg/g obtained using pure or single culture of *L. helvituus* (Akpabli-Tsigbe et al., 2021a) in our previous works. And in contradiction to other works (Abd Razak et al., 2017; Ali et al., 2016; Hadj Saadoun et al., 2021) which reported higher bioactive compounds production using mixed or co-culture. The result of chlorogenic acid yield in the present study can largely be explained by the fact that levels of bioactive compounds can be modified by the metabolic activity of microbes, enzyme hydrolysis, and biochemical metabolism during fermentation. Hölker et al. (2004) reported that metabolic synergisms among microorganisms during fermentation can be exploited during fermentation but the results of investigations are usually difficult to interpret. The enhancement of bioactive compounds content varies for each microorganism used. According to Hadj Saadoun et al. (2021), not all tested strains grow in a substrate due to differences in their growth performances, ascribed to the different adaptability of the strains in stressful matrices, such as heilong48 soybean without pre-treatment and nutrient addition. Ju et al. (2009) also, stated that microbial oxidation, reduction, or degradation of phenolic compounds by fermenting microbes contributes to the decreased phenolic acid content in fermented samples. Furthermore, Arancibia-Díaz et al. (2023) reported that chlorogenate hydrolase (CHase) degrades free-form compounds including chlorogenic acid into caffeic acid, quinic acid, ferulic acid, and isoferulic acid, thus, result in decrease of chlorogenic acid content in fermented samples.

Comparing this result to that of our previous works (Akpabli-Tsigbe et al., 2021a; 2021b), it could probably mean that the tested lactic acid bacteria, single *L. helviticus* culture was more effective than *L. casei* and *L. helviticus* mixed culture (which was also more efficient than single *L. casei* culture) in the extraction of chlorogenic acid from heilong48 soybean. Phenolics in plants are usually found in conjugated forms via the hydroxyl group. It is suggested that the increase in phenolic content in fermented substrates can be attributed to hydrolytic enzymes, such as β-glucosidase (Abd Razak et al., 2017), which are produced by lactic acid bacteria. Furthermore, Zheng and Shetty (2000) stated that an improvement in phenolic acids content usually relates to the action of microbial enzymes such as β-glucosidase, α-amylase, and laccase, along with other enzymes, which play essential roles in the immobilization of bioactive phenolic compounds during solid-state fermentation. These enzymes act upon the substrate and increase the availability of free hydroxyl groups on the phenolic structure, thus increasing the content of free phenolics and subsequently increasing the antioxidant activity of the substrate. Jung et al. (2021) reported that the growth and metabolism of *L. casei* mostly cease after 24 h of incubation. On the other hand, *L. helveticus* can adapt to fermentation conditions more easily than most lactobacilli, due to its ability to survive various environmental stresses such as high temperatures or low pH, osmotic pressure, and oxygen (Taverniti & Guglielmetti, 2012). *Lactobacillus helviticus* also possess cell envelope-associated proteinases (CEPs), cinnamoyl esterase, β-glucosidase (Taverniti & Guglielmetti, 2012), and high extracellular proteinase activities and thus release specific bioactive compounds during fermentation (Zhou et al., 2019), thus explaining the high content of chlorogenic acid in single *L. helviticus* and mixed culture fermented heilong48 soybean than *L. casei* fermented heilong48 soybean. However, the high chlorogenic acid yield in *L. helviticus* fermented heilong48 soybean than *L. casei* and *L. helvticus* mixed culture fermented heilong48 soybean could be that the was limited resources and competition between the two microorganisms for growth and survival, resulting in the reduction of their activities as well as the final product (chlorogenic acid). It could also be that the growth and metabolism of *L. casei* ceased after 24 h of incubation as reported by Jung et al. (2021)

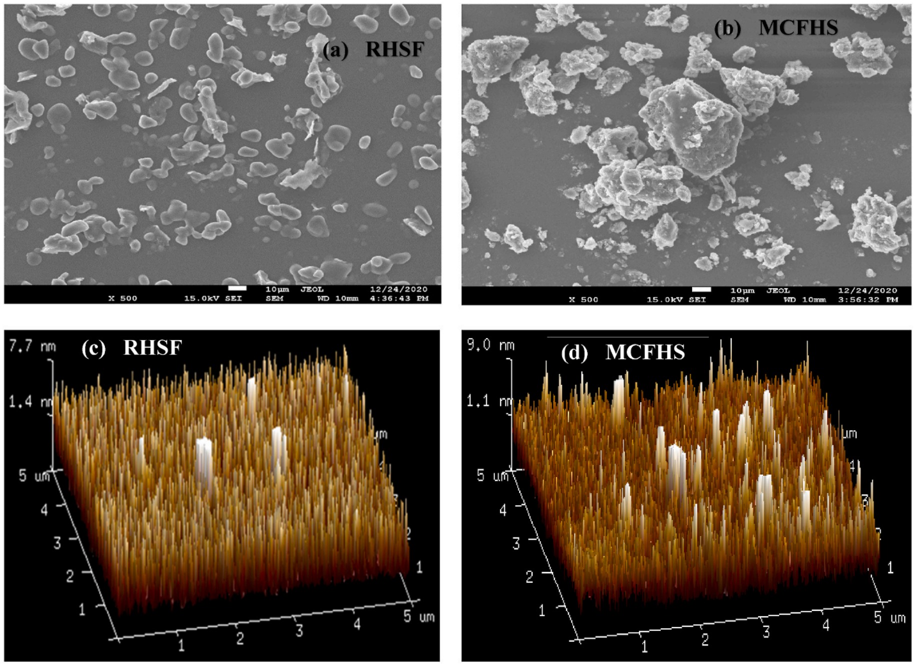

Fig. 7. Scanning electron microscopy and atomic force microscopy micrographs of raw (unfermented) heilong48 soybean flour (RHSF) (a,c) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) (b,d) samples.

(implying that there was no activity of *L. casei* to release chlorogenic acid after 24 h) but competed with *L. helviticus* in the mixed culture for survival, which resulted in limited resources for full growth and metabolism of *L. helviticus* reducing its potential and/or efficiency to fully act upon to the heilong48 soybean substrate to release more chlorogenic acid as reported for a single culture of *L. helviticus* (Akpabli-Tsigbe et al., 2021a). According to Santos da Silveira et al. (2019), solid-state fermentation increases the phenolic content of plant extracts by breaking the ester bonds between the phenolics and the cell wall of plants, increasing their concentration and thus functional characteristics. Consequently, the model obtained for the production of chlorogenic acid with high yield and enhanced biological (antioxidative) activity was satisfactory, accurate, and feasible. This work demonstrates the importance of studying solid-state fermentation parameters to improve bioactive compounds.

3.5. *Influence of solid-state fermentation on the structure of L. casei and L. helviticus mixed culture fermented heilong48 soybean (MCFHS) sample using scanning electron microscopy*

Scanning electron microscopy is an instrument that qualitatively analyzes the surface morphology of polysaccharides (Liu et al., 2016). The scanning electron microscopy images of raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples were compared to analyze the morphological differences between them. From Fig. 7; a and b, the structure of the RHSF sample was comprised of smaller, scattered, oval/irregular, smoother, and compact granular structures relative to the MCFHS sample. An amorphous, loose, agglomerated/clustered and rough-surfaced structural network was observed in the MCFHS sample, indicative that the solid-state fermentation caused changes that have played a role in forming an agglomerated structure (Wang et al., 2023). In addition, the granules of the MCFHS sample appeared bigger with pits. The pits formed in the MCFHS sample were due to the breakdown of proteins (amino acids formation) and starch (sugars formation) (Adebiyi et al., 2016) by enzymes produced by the *L. casei* and *L. helviticus* mixed culture used in the solid-state fermentation, resulting in the release of more chlorogenic acid. This correlated with the increased chlorogenic acid yield with enhanced antioxidant activity obtained for the MCFHS sample, owing to the cleavage of phenolics-starch and phenolics-protein bonds in the heilong48 soybean variety (Akpabli-Tsigbe et al., 2021a; 2021b; Santos da Silveira et al., 2019; Su et al., 2018). These findings conformed to the report of Adebiyi et al. (2016).

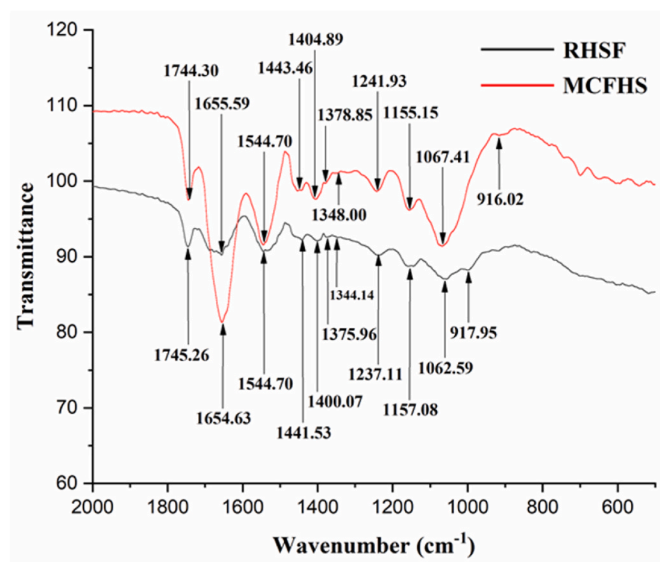

Fig. 8. Fourier transform infrared spectra of raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples.

3.6. *Influence of solid-state fermentation on the structure of L. casei and L. helviticus mixed culture fermented heilong48 soybean (MCFHS) sample using atomic force microscopy*

The atomic force microscopy method fundamentally involves the placement of a probe (a sharp tip) at the free end of a thin raster-scanned microcantilever structure by a piezoelectric actuator above the surface of the sample to be detected and examined (Peñuela et al., 2018). It images virtually any surface type namely ceramics, glass, composites, polymers, and biological samples. Atomic force microscopy application has become more popular and used in many fields of biological, polymer science, and material research. It provides information on topographical/structural modifications and is suitable for endosperm structure and hardness properties investigation (Chichti et al., 2015). Accordingly, atomic force microscopy was applied in this study to examine the microstructure of raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples. Fig. 7; c and d show the morphology of the MCFHS and RHSF samples. The topography of the RHSF sample showed scattered, small with few clustered particles as observed in scanning electron microscopy analysis. On the contrary, the MCFHS sample showed a topography of irregular big-sized particles. The irregular shape shown by the MCFHS sample is an indication that the solid-state fermentation process had caused changes that have played a role in the breakdown of the cell walls and cleavage of phenolics-starch and phenolics-protein bonds between molecules of the heilong48 soybean, releasing more chlorogenic acid. This resulted in the extraction of chlorogenic acid with increased yield and improved antioxidant activity as obtained. Furthermore, the number of MCFHS particles reduced compared to that of the RHSF sample. This was due to the agglomeration of MCFHS particles caused by the solid-state fermentation as reported by Adebiyi et al. (2016) and Liu et al. (2023). Similar findings were obtained in our previous works (Akpabli-Tsigbe et al., 2021a; 2021b). From the results, it was evidenced that the solid-state fermentation exhibited significant structural alterations in the MCFHS sample, which led to the high chlorogenic acid yield with improved antioxidant activity obtained.

3.7. Influence of solid-state fermentation on the functional groups of L. casei and L. helviticus mixed culture fermented heilong48 soybean (MCFHS) sample using Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared spectroscopy shows that molecules have specific frequencies at which they can spin (rotate) or vibrate, which corresponds to vibrational modes (Abid et al., 2018). It also distinguishes between amylose patterns in different granules (Ajayi et al., 2019). The Fourier transform infrared spectra of the raw (unfermented) heilong48 soybean flour (RHSF) and *L. casei* and *L. helviticus* mixed culture fermented heilong48 soybean (MCFHS) samples were determined in regions between 1800 and 900 cm^{-1} (Fig. 8). The β-glycosidic bonds between hemicellulose, cellulose, and glucose sugar polymers (Akpabli-Tsigbe et al., 2021b) for unfermented sample (RHSF) occurred at approximately 916 cm^{-1} absorption peak while that for MCFHS sample changed to approximately 918 cm^{-1}. The differences displayed in the positions of the peaks revealed that the functional group or chemical structure of the cellulose, hemicellulose, and glucose sugar polymers contained in the heilong48 soybean was affected by the solid-state fermentation. This, thus shows that there was a breakdown of starch, cell walls, and cleavage of phenolics-starch bonds in the heilong48 soybean variety caused by the solid-state fermentation process, releasing more chlorogenic acid. The band at approximately 1067 cm^{-1} for the unfermented sample (RHSF) that changed to approximately 1063 cm^{-1} for the MCFHS sample represented stretching of C–O–C vibration bond (Loow & Wu, 2018). The broad peak at approximately 1155 cm^{-1} (RHSF sample) and 1157 cm^{-1} (MCFHS sample) was the asymmetrical stretching vibration C–O–C group of cellulose and hemicellulose (Fakayode et al., 2020). Aryl–alkyl ether C–O–C bond of lignin (Akpabli-Tsigbe et al., 2021a; 2021b) appeared at approximately 1242 and 1237 cm^{-1} for the unfermented and fermented (RHSF and MCFHS) samples respectively. The broad bands at approximately 1348 and 1378 cm^{-1} (RHSF sample) and 1344 and 1376 cm^{-1} (MCFHS sample) respectively were a result of angular vibration of CH_3 symmetrical bond of cellulose and hemicellulose (Akpabli-Tsigbe et al., 2021b). The two broad peaks showed by the spectra at approximately 1405 and 1444 cm^{-1} for unfermented sample (RHSF) shifted to 1400 and 1442 cm^{-1} respectively for MCFHS was confirmed as symmetrical CH_2 bending groups in cellulose (Loow & Wu, 2018). The peak at 1545 cm^{-1} for both samples represented the vibration of the C═C aromatic skeletal bond in the aromatic ring of lignin (Akpabli-Tsigbe et al., 2021a; 2021b). The strong broad peak at approximately 1656 cm^{-1} (RHSF sample) shifted to 1655 cm^{-1} (MCFHS sample) was attributed to aliphatic C–N stretching of amide I peak (Adebiyi et al., 2016). The changes in the amide peak intensities were due to the β-sheet structures embedded in proteins. This indicates that the solid-state fermentation caused a breakdown of proteins, cell walls, and cleavage of phenolics-protein bonds in the heilong48 soybean variety, which led to the release of more chlorogenic acid. The peak obtained at approximately 1744 cm^{-1} for the unfermented sample (RHSF) shifted to 1745 cm^{-1} for the MCFHS sample was attributed to the C═O acetyl group of carbonyl ester in hemicellulose (Akpabli-Tsigbe et al., 2021b). The carbonyl peaks alteration in the MCFHS sample caused by the solid-state fermentation correlated to reduction of total lipids present. This result agreed with the report of Adebiyi et al. (2016). The changes in the positions of the peaks (characteristic of structural changes) in the MCFHS sample showed by the Fourier transform infrared spectroscopy results confirmed the effectiveness of the solid-state fermentation of the heilong48 soybean variety for extraction of chlorogenic acid with high yield and improved antioxidant activity.

4. Conclusion

A satisfactory solid-state fermentation model was developed with integrated statistical designs for extracting chlorogenic acid with high yield (9.20 ± 0.17 mg/g) and enhanced antioxidant activity (65.21 ± 2.05 μmol AA eq/g dry sample) from heilong48 soybean variety using *L. casei* and *L. helviticus* (two lactic acid bacteria strains) mixed culture. The results showed a significant increase of chlorogenic acid compared to the unfermented sample, due to the breakdown of the cell wall of the heilong48 soybean and cleavage of phenolics-starch/protein bonds by the microorganisms and the activities of their enzymes produced respectively. The scanning electron microscopy, atomic force microscopy, and Fourier transform infrared spectroscopy results additionally established the effectiveness of the solid-state fermentation. This study was novel, hence, presented a potential area for further work especially the bioactivity of chlorogenic acid extracted from soybean.

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Credit author statement

Nelson Dzidzorgbe Kwaku Akpabli-Tsigbe: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Kwaku Tano-Debrah, Dr. Benjamin Kumah Mintah, Juliet Osabutey:** Writing – Review and Editing. **Yongkun Ma:** Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102903.

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