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Research Article

Potency of Cocoa Honey, Byproduct of Cocoa Bean Processing as Probiotic Media: Optimization of *Saccharomyces boulardii* Growth and Its Antimicrobial Activity

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Abstract
Cocoa honey, a byproduct of cocoa bean processing, contains high sugars and minerals; thus, it has potential as a fermentation medium. This research investigated the potency of cocoa honey as probiotic media for promising yeast probiotic *Saccharomyces boulardii*. The growth of *S. boulardii* in the cocoa honey medium was optimized through response surface methodology employing a central composite rotatable design to obtain maximum cell production and antimicrobial activity. The effects of inoculum size (2–5%), sucrose addition (2.0–3.0%), and fermentation time (5–20 h) were evaluated. The agar diffusion technique assessed the cocoa honey medium's antibacterial activity. Results showed that these independent variables significantly affected the production of *S. boulardii* cells and their microbial activity against *Escherichia coli.* The optimal values generated by the model with a desirability value of 0.908 were 20 h of fermentation time, 2% of sucrose addition, and 2% of inoculum size. The maximum yeast count ($\log_{10}$ 8.07 cells/mL) and antibacterial activity (4.88 mm) were reached under these optimized conditions, which agreed with the predicted value of the RSM model. This study provided valuable information on the potency of cocoa honey as a fermentative medium for applying *S boulardii* to develop a probiotic drink.

Keywords: antimicrobial activity, cocoa honey, probiotic, RSM, *Saccharomyces boulardii*

1. INTRODUCTION

Much waste is created during coca processing, primarily during fruit breakage and in liquid from the pulp, known as cocoa honey. As the natural byproduct of processing cocoa beans, cocoa honey has high levels of nutrients such as sugar and minerals, which are beneficial bioactive compounds, placing it as a potential fermentative substrate. Cocoa honey is an excellent substrate for fermentation since it contains a total soluble solid of about 16-17◦brix [1], much fructose (3.25 ± 0.03% w/v), and glucose (4.58 ± 0.12% w/v) [2][3]. Other nutrients that are present in considerable quantity include vitamin C, pyridoxine, niacin, K, Mg, and Ca [4]. Cocoa honey contains flavonoids, phenolics, and polyphenolic components with antioxidant potential [1]. These compounds are reported for their antibacterial activity [5]. Other than flavonoid compounds, organic acid is another component of cocoa honey that exhibits antimicrobial properties. Organic acids have antibacterial activities because they can pass through cell membranes and change the cytoplasm's amounts of protons and related anions [6]. The cocoa honey composition was found to contain organic acids, specifically ascorbic, citric, and malic acids (18.3±7.5, 9.1±0.6, and 3.6±0.5 mg mL^{-1}, respectively) according to Vásquez et al. [7]. Due to a high titratable acidity content, cocoa honey has a comparatively low pH of around 3.5 [2]. Thus, cocoa honey is a suitable growth medium for acidic probiotics, such as *Saccharomyces boulardii*. This yeast is reported as a probiotic that can endure acidic conditions and grow at pH levels of 2.5 [8].

The clinical effectiveness of probiotic *S. boulardii* has been studied. Its probiotic activity has been connected through various pathways, such as improved gut barrier function, pathogen competitive exclusion, generation of antimicrobial peptides, immunological modulation, and trophic effects [9]. *S. boulardii* is a unique probiotic and biotherapeutic yeast that can survive in gastric acid. It is neither negatively influenced or hindered by antibiotics nor alters or negatively affects the usual

microbiota [10]. *S. boulardii* has been used as a treatment option for related illnesses, such as the prevention of antibiotic-associated diarrhea [11] acute diarrhea, and other pathogenic enterobacteria [12]. McFarland [13] and Hossain et al. [8] stated that *S. boulardii* is a thermotolerant yeast with a 37 ºC optimal development temperature that can withstand bile acids.

The use of *S. boulardii* as a probiotic yeast starter has been previously introduced in functional beverages from diverse juices such as mixed vegetable juice of radish, red beet, and black carrot juices [14], fruit juice such as coconut water [15], and cashew juice [16]. These studies demonstrate challenges in adding *S. boulardii* probiotics to various juices from nutrition, processing, nutraceuticals, or health enhancement perspectives. However, such experimentation has yet to be conducted with cocoa honey, the natural byproduct of processing cocoa beans. The nutritional and biological properties of cocoa honey juice are believed to influence the characteristics of the final fermentation product.

It has been reported that the factors influencing the growth of microbes in fermentation are incubation time, inoculum levels, and the sugar content of media fermentation. There needs to be an understand of how cocoa honey media affects the growth of *S. boulardii* or how it influences the properties of fermentation. Moreover, there are no reports of the antibacterial activity of *S. boulardii* in cocoa honey mediums. Thus, this study aimed to optimize the *S. boulardii* cell growth and microbial activity in cocoa honey as the substrate medium. Optimization was done using a central composite design (CCD) and response surface methodology (RSM) to determine the optimum fermentation time, sugar addition, and inoculum size. The surface plots of the response produced by this design were analyzed using the corresponding second-order polynomial models. Ultimately, the model's applicability was assessed using an analysis of variance (ANOVA), and the optimized variables were validated through an experiment. Cocoa honey as a medium for *S. boulardii*, a promising probiotic yeast, is rarely covered in studies. Studying with *S. boulardii* in a cocoa honey medium for probiotic yeast distribution presents new challenges and opportunities. This research investigated the potency of cocoa honey as probiotic media for promising yeast probiotic of *S. boulardii*.

2. MATERIALS AND METHODS

2.1. Materials

Cocoa honey was from Tanggamus, Lampung Province of Indonesia. *S. boulardii* was collected from a commercial dietary supplement capsule acquired from a marketplace. *Escherichia coli* isolate was from the Microbiology Laboratory of Universitas Lampung. The chemicals used were chloramphenicol (Sanbe), streptomycin (Oxoid), potato dextrose agar (PDA), potato dextrose broth, nutrient agar, and nutrient broth (Oxoid). Meanwhile, phosphate buffer, phosphoric acid, ethanol, and 2,2-diphenyl-1-picrylhy-drazyl (DPPH) were obtained from Merck. Germany.

2.2. Methods

2.2.1. Preparation of S. boulardii as a Working Starter

S. boulardii supplement capsules were taken aseptically under laminar air flow, spread over PDA media, and then incubated for 48 h at 25 °C. A sterile loop was used to pick up the single colony and to transfer it to a tube containing sterilized PDA. After incubation overnight at 25 °C, the cell was put in a 150 mL PDB and incubated for 24 h. This inoculum had around 10^9 CFU/mL and was ready as a working starter.

2.2.2. Inoculation and Incubation of S. boulardii in Cocoa Honey Media

Cocoa honey media was filtered, pasteurized in a water bath for 10 min at 80 ºC, and allowed to reach room temperature. The working starter *S. boulardii* was added aseptically to the cocoa pulp medium at 1–6% (according to running RSM). The cocoa honey medium was added with 1.6–3.3% sterile sucrose and incubated for (0–22 h) according to the time specified in the RSM in an orbital shaker at 75 rpm. Observations on the samples during incubation were carried out on the number of yeast cells, total soluble solids, and antimicrobial activity test. These data were used to determine the optimized sample using Design Expert software version 12 (STAT-EASE Inc., Minneapolis, USA). The optimized

Table 1. Set of Experiments.

Variables	Coding	Unit	Levels				
			-α	-1	0	1	α
Fermentation time	A	h	0	5	12.5	20	25.1134
Inoculum	B	%	1.0	2	3.5	5	6.02
Sucrose	C	%	1.669	2	2.5	3	3.340

sample was then subjected to antioxidant activity, acetic acid, pH, and titrable acidity (TA) analysis.

2.2.3. Viable Cell of S. boulardii

The cell concentration during the fermentation was regularly monitored to study and track yeast growth. Combining a suitably diluted cell suspension with an equal amount of lactophenol blue solution (0.1%), viable cells (log10 viable cells mL^{-1}) were calculated. An optical microscope (Nikon Eclipse E100) was used to monitor the viable cells in a Neubauer Hemocytometry chamber.

2.2.4. Measurement of Physicochemical Indicators

A portable analog refractometer (Atc, Jiangsu Victor Instrument Meter Co., Ltd.) was used to measure the TSS content, and the findings were expressed as brix. Orion StarTM A215 pH meter from Thermo Fisher ScientificTM, Waltham, MA, USA, was used to measure the pH. It has previously been calibrated to measure pH 4.0 and 7.0. TA was ascertained by titrating the sample with a sodium hydroxide standard solution until the pH endpoint was 8.20.

2.2.5. Antimicrobial Activity Test

The agar diffusion assay was used to measure the antimicrobial activity of the cocoa honey medium. Initially, cell debris was removed from samples by centrifuging them for 5 min at 10,000 rpm. Sterile supernatant was obtained by bypassing the supernatant through a sterile microfilter with a 0.22 µm pore size on a syringe. A paper disk (Oxoid) was then saturated with a sterile sample and placed on a lawn of *E. coli* seeded on the surface of a nutrient agar medium. Control experiments were carried out under the same conditions with streptomycin disk as a positive control. The plate was incubated at 37 ºC overnight. One paper disk saturated with streptomycin was also prepared as a positive control. A zone of inhibition around the disks was measured after 24 h.

2.2.6. Acetic Acid Analysis

Using a modified method outlined by Jurić et al. [17], acetic acid in the cell-free culture supernatant of the cocoa honey medium was examined using HPLC Shimadzu, detector UV/VIS SPD 20A. Sample preparation was done by diluting 1 mL of the sample into 9 mL of phosphate buffer. The prepared sample was filtered with a 0.45 µm filter cartridge and then was analyzed by reverse phase chromatography using a C18 column and read at a wavelength of 210 nm. Analysis was carried out under isocratic conditions at a temperature of 40 ºC using 50 mM phosphate solution as the mobile phase, and the pH value was adjusted by adding phosphoric acid to pH 2.8 using a pH meter, then adding water to 1 L, then filtered with 0.45 µm Whatman paper. The mobile phase flow rate was set to 0.7 mL/min.

2.2.7. Antioxidant Activity

The initial stage of antioxidant activity was by extracting 5 mL of the samples with 20 mL of ethanol 96% in a shaker for ± 4 h. After centrifuging the extract for 10 min at 3000 rpm; it was filtered using an 8 µm-thick Whatman grade 2 filter paper [1]. The next stage was preparing a DPPH control solution by dissolving approximately 0.0078 g of the DPPH solution in 100 mL of 96% ethanol in a dark room. Then, the absorbance of a DPPH solution was measured in a UV-Vis spectrophotometer (Genesys 840-208100 UV, Thermo Scientific) at 517 nm and was calculated as control absorbance (Ak). As much as 1 mL of each extract was combined with 2 mL of the DPPH solution, mixed thoroughly, and allowed in the dark for half an hour to ascertain the extract samples'

ability to eliminate free radicals. The sample absorbance (As) was then recorded after the absorbance was measured at 517 nm. The absorbance of the extract (As) and DPPH (Ak) were compared to determine the percentage of antioxidant activity. The following formula (% Antioxidant activity = (Ak-As)/Ak x 100%) was used to determine the percentage of antioxidant activity against DPPH radicals at each concentration of the sample solution.

2.2.8. Design of Experiment

CCRD-RSM was used to optimize the growth conditions for *S. boulardii* by determining independent variables that substantially impacted growth. Fermentation time (X1), sucrose addition (X2), and inoculum concentration (X3) were the independent variables. These independent variable levels in the experimental design for response surface analysis were set as seen in Table 1. Viable cells of *S. boulardii* (Y1), total soluble solid (Y2), and antimicrobial activity (Y3) were the dependent variables. The reactions were examined using 20 experiments, with 6 repetitions at the central locations. Design Expert software version 12 was used to fit the equations from the experimental data using regression analysis. The model's validity was evaluated using the correlation coefficient (R^2), and the statistical significance of the equations was evaluated using an analysis of variance (ANOVA).

3. RESULTS AND DISCUSSIONS

3.1. Total cell, TSS, and Antimicrobial Activity

Table 2 displays total cell, TSS, and antibacterial activity in response to *S. boulardii* fermentation optimization in cocoa honey media. Design Expert 12 examined the replies, and the findings are shown in Tables 2 and Figures 1-3. The total cells of the 20 experimental fermentation conditions varied between 7.635 and 8.452 log 10 cells/mL. ANOVA result in Table 3 shows that the polynomial

Table 2. Experimental design of fermentation time, sucrose, and inoculum variables with total cell log ^{10}, soluble solid, and antimicrobial activity measured values of responses.

Run	Fermentation time (h)	Sucrose (%)	Inoculum size (%)	Total cell log^{10} (cell/mL)	Soluble Solid (Brix)	Antimicrobial activity (mm)
1	0	2.5	3.5	7.635 ± 0.4	13.3 ± 0.30	1.5 ± 0.10
2	20.0	2.0	2.0	8.083 ± 0.3	13.1 ± 0.50	4.0 ± 0.07
3	12.5	2.5	1.0	8.057 ± 0.8	14.0 ± 0.00	2.5 ± 0.07
4	12.5	3.3	3.5	8.045 ± 0.4	13.8 ± 1.00	1.5 ± 0.07
5	12.5	2.5	3.5	8.190 ± 0.5	13.5 ± 0.70	3.5 ± 0.10
6	20.0	2.0	5.0	8.207 ± 0.4	13.2 ± 0.07	0.3 ± 0.08
7	12.5	2.5	3.5	8.121 ± 0.4	13.6 ± 0.60	2.5 ± 0.10
8	12.5	2.5	3.5	8.124 ± 0.5	13.9 ± 0.10	2.5 ± 0.10
9	20.0	3.0	5.0	8.452 ± 0.6	13.9 ± 0.07	2.75 ± 0.09
10	12.5	2.5	3.5	8.422 ± 0.5	14.0 ± 0.90	2.5 ± 0.00
11	5.0	3.0	5.0	7.688 ± 0.6	14.0 ± 0.20	0.35 ± 0.07
12	12.5	2.5	3.5	8.137 ± 0.4	14.0 ± 0.07	2.5 ± 0.00
13	5.0	2.0	2.0	7.875 ± 0.6	13.5 ± 0.70	2.0 ± 0.05
14	12.5	2.5	3.5	8.100 ± 0.4	14.0 ± 0.30	2.0 ± 0.08
15	20.0	3.0	2.0	8.338 ± 0.6	14.4 ± 0.40	3.0 ± 0.03
16	25.0	2.5	3.5	8.238 ± 0.5	13.9 ± 0.07	1.25 ± 0.08
17	5.0	3.0	2.0	7.957 ± 0.5	14.2 ± 0.20	1.5 ± 0.10
18	5.0	2.0	5.0	7.918 ± 0.3	13.5 ± 0.70	2.5 ± 0.10
19	12.5	2.5	6.0	8.190 ± 0.5	13.8 ± 0.30	4.0 ± 0.07
20	12.5	1.6	3.5	8.290 ± 0.5	13.2 ± 0.40	3.0 ± 0.10

Table 3. ANOVA for the quadratic model of total cell.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.7273	9	0.0808	5.8900	0.0053	significant
A-Fermentation Time	0.5170	1	0.5170	37.670	0.0001	
B-Sucrose	0.0003	1	0.0003	0.0205	0.8890	
C-Inoculum Size	0.0040	1	0.0040	0.2920	0.6008	
AB	0.0527	1	0.0527	3.8400	0.0786	
AC	0.0269	1	0.0269	1.9600	0.1916	
BC	0.0130	1	0.0130	0.9460	0.3537	
A^2	0.1097	1	0.1097	7.9900	0.0179	
B^2	5.133E-06	1	5.133E-06	0.0004	0.9849	
C^2	0.0063	1	0.0063	0.4597	0.5132	
Residual	0.1372	10	0.0137			
Lack of Fit	0.0639	5	0.0128	0.8711	0.5583	not significant
Pure Error	0.0733	5	0.0147			
Cor Total	0.8646	19				

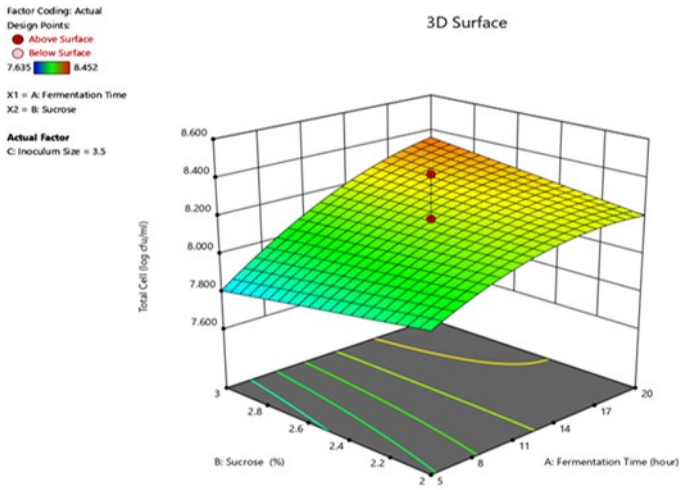

Figure 1. Three-dimensional graph of total cell response.

equation of total cells revealed no interaction effects among fermentation conditions. Furthermore, among the three condition variables, fermentation time was the variable that influenced total yeast (p-value < 0.05). In contrast, the sucrose addition and inoculum size variables had no significant effect (P > 0.05). Based on the D-optimal mixture design analysis, a quadratic model of total cells was created and found to fit the data statistically significantly ($p < 0.05$, $R^2 = 0.8413$).

The response surface 3D graph of the total cell is shown in Figure 1. The polynomial equation for total cells was 7.89 – 0.0067A – 0.0798 B + 0.1473 C + 0.0216AB + 0.0051AC – 0.0537BC – 0.00157A^2 – 0.0023 B^2 – 0.0094C^2. The high total yeast value (log 8.452 cell/mL) was obtained under conditions of fermentation time of 20 h, 3% sucrose addition, and 5% inoculum, while the smallest total yeast value (log 6.35 cell/mL) was obtained at fermentation time of 0 h, 2.5% sucrose addition and 3.5% inoculum. As shown in Table 2, fermentation time significantly affects the number of yeast cells. Longer fermentation time will allow the yeast to utilize sugar for metabolism and growth and multiply the cells. According to González-Figueredo et al. [18], *S. boulardii* can

simultaneously start the fermentation and saccharification processes in the culture. It can hydrolyze sucrose into the appropriate monosaccharide for growth. The increase in *S. boulardii* cells indicated that this yeast can grow well on cocoa honey media with a low pH (pH 3.3). This growth supports the findings, stating that *S. boulardii* can grow at pH above two and withstand acidic conditions [8]. The number of total cells is significant when this media developed as a probiotic drink. The survival of probiotic cells in beverage manufacturing is essential to ensure appropriate health benefits for consumers. The viability of yeast during the fermentation of cocoa honey media could raise 8.45 log cells/mL, fulfilling the minimum criteria established by FAO/WHO (1×10^6 CFU/mL). This value range was comparable to previous studies with other fermentation mediums. The viable cell value of *S. boullardii* ranged from 6.37 to 8.3 log CFU/mL in fruit juice fermentation [16] and 6.56 to 8.98 log CFU/mL in vegetable juice fermentation [14]. Meanwhile, the growth of *S. boulardii* cells was 8.43 log CFU/mL in YPD broth [19].

This study's TSS sugar of cocoa honey fermentation ranged from 13.01–14.04 Brix. The ANOVA (Table 4) revealed that the interaction model (2FI) and only the variable of sucrose addition were significant ($p \leq 0.05$). The 2FI model was suggested as the highest-order model with significant terms that could describe TSS's relationship with fermentation time, sucrose addition, and inoculum size. There was no interaction among fermentation time, sucrose, and

Table 4. ANOVA for 2FI model of total soluble solid.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.7100	6	0.2855	3.7700	0.0214	significant
A-Fermentation Time	0.0304	1	0.0304	0.4008	0.5376	
B-Sucrose	1.4000	1	1.4000	18.520	0.0009	
C-Inoculum Size	0.0725	1	0.0725	0.9574	0.3457	
AB	0.1313	1	0.1313	1.730	0.2108	
AC	0.0095	1	0.0095	0.1247	0.7296	
BC	0.0657	1	0.0657	0.8671	0.3687	
Residual	0.9851	13	0.0758			
Lack of Fit	0.7221	8	0.0903	1.7200	0.2861	not significant
Pure Error	0.2630	5	0.0526			
Cor Total	2.7000	19				

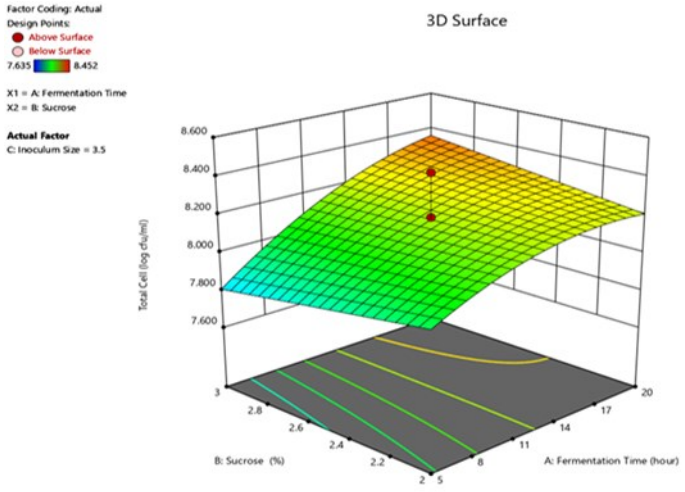

Figure 2. Three-dimensional graph of total soluble solid.

Table 5. ANOVA for a quadratic model of microbial activity.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1113	9	0.0124	3.6000	0.0292	significant
A-Fermentation Time	0.0009	1	0.0009	0.2522	0.6264	
B-Sucrose	0.0186	1	0.0186	5.4100	0.0424	
C-Inoculum Size	0.0030	1	0.0030	0.8595	0.3757	
AB	0.0153	1	0.0153	4.4600	0.0609	
AC	0.0378	1	0.0378	11.010	0.0078	
BC	0.0003	1	0.0003	0.0910	0.7691	
A^2	0.0190	1	0.0190	5.5400	0.0404	
B^2	0.0006	1	0.0006	0.1611	0.6966	
C^2	0.0132	1	0.0132	3.8300	0.0787	
Residual	0.0343	10	0.0034			
Lack of Fit	0.0223	5	0.0045	1.8400	0.2595	not significant
Pure Error	0.0121	5	0.0024			
Cor Total	0.1456	19				

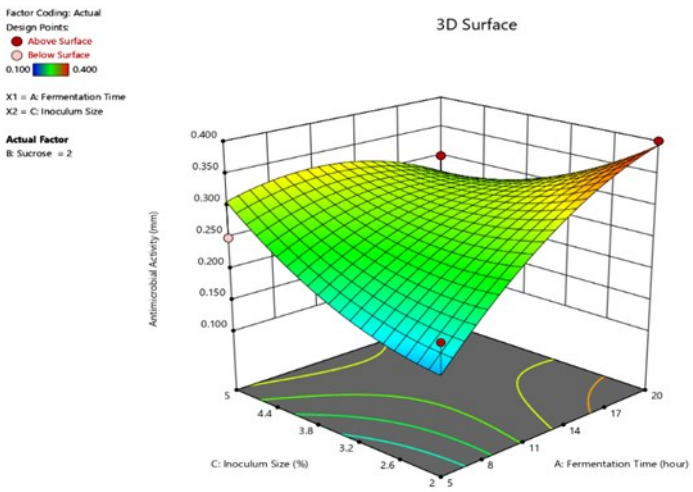

Figure 3. Three-dimensional graph of antimicrobial activity response.

inoculum (Figure 2 & Table 4). The R^2 values were 0.635.

Furthermore, there was no significant lack of fit (p-value < 0.05), suggesting that the model was fit. Table 2 shows that the variation of TSS during fermentation was narrow (13.01–14.04 Brix). The transformation of cocoa honey juice into a functional cocoa honey beverage through fermentation was a biological process that generated many byproducts. There were also complex changes in the chemical composition of the medium contributing to TSS, which are groups of substances that can be dissolved in water, mainly soluble sugar and other soluble substances [3]. Thus, TSS may refer to pigment, soluble fiber, organic acid, other byproducts, microbe cells, and the sugar left over in a product following fermentation. Cell microbes and sugar might contribute the most soluble solid component in this cocoa honey media fermentation. During fermentation, there was an increase in the total cells of yeast following the quadratic model, as shown in Figure 1. As fermentation time increased, the yeast had more time to consume the sugars available, leading to higher cell counts and lower residual sugar. This condition might be the reason for this

study's slight increase in the TSS. The TSS slightly increased after 12.5 h when the cell production was around 8.0 log cell/mL. Among variables affecting the TSS, sucrose significantly influenced the model for TSS. It was found that TSS was increased with an increase in sucrose level irrespective of inoculum level during fermentation.

The antimicrobial activity of the 20 experimental fermentation conditions varied between 0.1 ± 0.09 and 4 ± 0.07 mm. Table 5 of ANOVA shows that among the three fermentation condition variables, only the sucrose variable had an effect (p-value < 0.05). In contrast, fermentation time and inoculum size variables had no significant effect ($p > 0.05$). Furthermore, there was an interaction effect between fermentation time and inoculum size on antimicrobial activity ($p < 0.05$). A quadratic model of antibacterial activity fitted the data with statistical significance ($p < 0.05$, $R^2 = 0.7642$). The polynomial equation for microbial activity was $0.2568 + 0.0080A - 0.0369B + 0.0148C - 0.0438AB - 0.0688\ AC + 0.0063BC - 0.0368A^2 - 0.0061B^2 - 0.0307C^2$. The response surface 3D graph of antimicrobial activity is shown in Figure 3. The individual variable sucrose and the interaction between fermentation time and inoculum size significantly influenced the antimicrobial parameter. Many researchers have revealed that sugar concentration is an essential factor in the production of active antimicrobial substances, and at higher sugar concentrations, there is an increase in antibacterial activity [19][20]. In this study, the highest antimicrobial was found in the 2.0–2.5% sucrose treatment (medium level), with a value of 4 mm obtained in run 2 and run 19.

Aside from biomass, the byproducts of sucrose fermentation, including ethanol and organic acid, are assumed responsible for the pathogens' inhibition [21]. The essential antimicrobial agent of *S. boulardii* is the acetic acid content produced during its growth [22], strongly inhibiting bacterial growth in agar-well diffusion assays. In this study, the acetic acid contained in the sample ranged from 2.45% to 3.98%. Apart from acetic acid, there is probably a role for other secondary metabolites, such as polyphenolic compounds that function as antimicrobial compounds during the fermentation of cocoa honey media by *S. boulardii*. Datta et al. [23] reported that the extracellular portion of the *S. boullardii* culture was discovered to contain a high concentration of polyphenolic metabolites.

The presence of an inhibition zone surrounding the colony of *E. coli* confirms the findings of previous researchers who reported that *S. boulardii* has antimicrobial activity against *E. coli* [8][22]. However, the antimicrobial activity value of our work was significantly lower than those reported by Chelliah et al. [24], which might be due to different strains of *S. boulardii*. The clear zone formed due to antimicrobial activity in the fermented cacao honey media sample was from 1.10 to 4.00 mm, placing the *S. boulardii* fermented cocoa honey medium's antibacterial activity in the weak criterion (< 5 mm). This data indicates that honey cocoa media has antibacterial prospects because the results are from fermentation broth media testing, which means it is only naturally extracted with water and has not been purified. Partial purification or different extraction methods of the cocoa honey medium extract could ascertain the extract samples' ability to eliminate free radicals to increase the antibacterial activity, as demonstrated by Lei et al. [25] and Sharayei et al. [26] in broad-spectrum bacteriocin produced by a Lactobacillus and in ultrasound-assisted extraction of bioactive compounds from pomegranate, respectively.

3.2. Optimization of Fermentation Conditions and Validation of The Model

Numerical optimization of independent variables, fermentation time, sucrose, and inoculum size were optimized using Design Expert Software. The dependent variables of total cell and antimicrobial activity were ranked to maximum

Table 6. Predicted and experimental values of responses under optimum conditions.

Response Variable	Goal	Predicted	Experimental
Total cell (cell/mL)	Maximize	8.07	8.10
TSS	In range	13.10	11.10
Antimicrobial activity (mm)	Maximize	3.93	4.88

Table 7. Microbiological and physicochemical characteristics of an optimized sample.

Characteristics	Value
Total probiotic cell (cell/mL)	8.070 ± 0.0013
Antimicrobial activity on *E. coli* (mm)	4.880 ± 0.0392
TA (%)	1.146 ± 0.0138
pH	3.480 ± 0.0035
Antioxidant activity (%)	83.545 ± 0.0257
Acetic acid (%)	2.450 ± 0.0254

importance (5, +++++) because both variables significantly influence the probiotic characteristic. Meanwhile, the variable of TSS was set in the range. The optimal conditions for *S. boulardii* to ferment cocoa honey were 20 h of fermentation time, 2% sucrose, and 2% inoculum size, with a desirability value of 0.908, as determined by RSM analysis. Antibacterial activity of 3.93 mm, total cell count of 8.07 log cells/mL, and TSS of 13.2 brix were predicted for this optimal condition. The optimal conditions for fermenting cocoa honey were revalidated, and the predicted values of the response variables were validated by comparing the experimental findings with the expected values (Table 6).

The overall verification results are in the range of 95% CI low and 95% CI high. The total cell and antibacterial activity predicted by the RSM model showed a good correlation, falling within the actual values' range. This investigation demonstrated that the chosen regression models were appropriate for explaining the factor-response relationship during the fermentation of cocoa honey with *S. boulardii* as a starter and that the optimal fermentation conditions predicted to produce high levels of probiotic cells and microbial activity are valid.

3.3. Profile of the Best-Selected Sample

The best-selected sample of *S. boulardii* fermentation was from cocoa honey fermented for 20 h, 2% sucrose, and 2% inoculum size. The microbiological and physicochemical profiles of this optimized sample are shown in Table 7. The sample's viable cell strain count was 8.07 log cells/mL, exceeding the probiotic drink's minimum probiotic level of 10^6 CFU/mL. The physicochemical profile revealed a pH range of 3.40–4.90 and a TA value of 1.146 ± 0.0138. These fall within the accepted range of pH and TA for fruit and vegetable juice drinks. The presence of organic acids like acetic acid, which comprised 2.45% of the sample, was one of the contributing factors to its low pH and acidity, as well as antimicrobial activity. This sample's antioxidant activity content demonstrated good promise. Flavonoids and other phenolic and polyphenolic chemicals found in cocoa pulp are thought to have possible antioxidant properties [1][2][4][27][28]. The metabolites of the fermentation starter cultures of *S. boullardii* may be responsible for the antioxidant activity of this sample in addition to its natural substrate. According to Datta et al. [23], the extracellular portion of the *S. boullardii* culture was discovered to contain a high concentration of polyphenolic metabolites.

4. CONCLUSIONS

This study was a ground-breaking endeavor using cocoa honey as media to grow *S. boullardii*, a probiotic yeast starter. The optimal parameters were fermentation time of 20 h, inoculum size of 2%, and sucrose addition of 2%. These selected fermentation parameters yielded the cocoa honey probiotic drink with viable yeast cell value that met probiotic standards (>10^6 CFU/mL), had antimicrobial activity against *E coli*, contained low pH, and had comparable antioxidant activity.

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Conflicts of Interest

The authors declare no conflict of interest.

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