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Bioactive-Enriched Spirulina Protein Isolate: Scale-Up for Symbiotic Beetroot Drinks

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Abstract

Spirulina platensis (Arthrospira platensis), a GRAS-approved superfood providing 60-70% high-quality protein and bioactives like phycocyanin, β-carotene, and polysaccharides, addresses India's protein malnutrition crisis (20% child prevalence per NFHS-5) but faces sensory and solubility challenges for beverage use. This study optimized spirulina protein isolate (SPI, 68.2% purity) production using response surface methodology (RSM) with high-pressure homogenization pretreatment, alkaline extraction (pH 11.38, 5.2:1 biomass:water, 35 min), and freeze-drying from NBRI 1023 strain cultivated in modified Zarrouk's/Paoletti's media yielding 2.58-2.71 g/L biomass (0.129-0.136 g/L/day). Biomass harvested via centrifugation (96.2% recovery) produced SPI with superior functionality: 65.1% solubility at pH 7, emulsifying activity index 52.3 m^2/g, foaming capacity 142% (75% 30-min stability), water/oil holding capacities 2.84/1.92 g/g, thermal stability (TGA onset 223°C, DSC peak 285°C), and native structure (FTIR amide I/II 1652/1541 cm^{-1}). Bioactives enriched 2.8-fold: DPPH IC_{50} 92 µg/mL, TPC 22.4 mg GAE/g, phycocyanin 142.3 mg/g (purity ratio 2.84). RSM model (R^2=0.9674, desirability 0.89) and 20 L photobioreactor scale-up (98.5% efficiency, 2.61 g/L/day) validate industrial SPI production for fortifying beetroot probiotic drinks, targeting 10^9 CFU/mL viability, 89.6% digestibility, and sensory scores 7.9 while leveraging Haryana's 2.1 Mt/year beetroot supply.

1. Introduction

Spirulina (Arthrospira platensis), recognized as a nutrient powerhouse and GRAS-approved superfood, provides 60-70% high-quality protein complete with essential amino acids, plus potent bioactives like phycocyanin (antioxidant), β-carotene (provitamin A), and polysaccharides (prebiotic) that deliver anti-inflammatory and immunomodulatory effects.

With rising demand for sustainable plant-based proteins amid India's protein malnutrition crisis (affecting 20% children per NFHS-5), spirulina addresses global food security challenges, yet sensory issues (fishy/earthy notes) and technical hurdles (low solubility at pI 4-5, pigment instability) limit beverage applications.

Beetroot (Beta vulgaris L.) synergizes perfectly as a fermentation matrix, supplying betacyanins for natural pink hue masking algal off-flavors, phenols (28.4 mg GAE/100 mL), dietary nitrates, and Haryana's abundant supply (2.1 Mt/year). Probiotic co-fermentation with Lactiplantibacillus plantarum MTCC 1325 and Lacticaseibacillus rhamnosus MTCC 5462 (1:1) boosts protein digestibility to 89.6%, liberates bioactive peptides via proteolysis (2.1 U/mL), and enhances texture through exopolysaccharides. This research applies response surface methodology to optimize a novel SPI (68.2% purity)-fortified beetroot probiotic drink targeting ≥10^9 CFU/mL viability, 0.8-1.2% acidity, and ≥7.9 sensory scores—bridging gaps for scalable synbiotics in emerging markets.

2. Methodology

2.1 Culture Medium Preparation

Zarrouk's medium was constituted as the standard formulation with modifications for enhanced biomass productivity. The medium was prepared in 4 L Erlenmeyer flasks with a working volume of 3 L using borosilicate glass vessels and analytical grade chemicals dissolved in Milli-Q water (Richa et al., 2011; Raoof et al., 2006).

Table 1: Culture Medium

Component	**Concentration (g L^{-1})**	**Function**	**Reference**
$NaHCO_3$	16.8	Carbon source	Richa et al., 2011
$NaNO_3$	2.5	Nitrogen source	Raoof et al., 2006

K_2SO_4	1.0	Potassium/sulfur	Madkour et al., 2012
NaCl	1.0	Osmotic balance	Habib et al., 2008
$MgSO_4 \cdot 7H_2O$	0.2	Magnesium/sulfur	Markou et al., 2014
K_2HPO_4	0.5	Phosphorus source	Bezerra et al., 2011
$CaCl_2$	0.04	Calcium source	Lu et al., 2016
$FeSO_4 \cdot 7H_2O$	0.01	Iron source	Devi et al., 2020
EDTA	0.08	Chelating agent	Kumar et al., 2018
Trace elements*	1 mL L^{-1}	Micronutrients	Zarrouk, 1966

*Trace element solution: H_3BO_3 (2.86), $MnCl_2 \cdot 4H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.222), $Na_2MoO_4 \cdot 2H_2O$ (0.39), $CuSO_4 \cdot 5H_2O$ (0.079), $Co(NO_3)_2 \cdot 6H_2O$ (0.0494) g L^{-1}.

The medium was autoclaved at 121°C and 15 psi for 20 minutes, then cooled to 40°C. The pH was adjusted to 9.5 ± 0.2 using 1 N NaOH or HCl with an Elico LI120 pH meter, followed by sterility testing through pour plate method on nutrient agar with 48-hour incubation. Paoletti's medium variant, modified with $NaHCO_3$ at 20 g L^{-1}, was tested for comparative biomass yield assessment.

2.2 Inoculum Development and Cultivation

Spirulina platensis strain (NBRI 1023/FACHB-314) [National Botanical Research Institute (NBRI), Lucknow Culture Collection of Algae (CCALA), Rana Pratap Marg, Lucknow 226001, Uttar Pradesh, India] was maintained on Zarrouk agar slants containing 1.5% agar with characteristic dark green pigmentation at 4°C and subcultured monthly. Primary inoculum develops by scraping 2-3 week slant growth into 100 mL Zarrouk liquid (250 mL flask), incubating 30 ± 1°C, 50 μmol photons m^{-2} s^{-1}, 72 h until OD_{600} 0.4-0.6. (Habib et al., 2008 and Markou et al., 2014)

Secondary inoculum (10% v/v, 300 mL) transfers to 3 L production flasks under controlled conditions: temperature 30 ± 1°C (thermostatic incubator shaker), illumination 140 μmol photons m^{-2} s^{-1} (cool white fluorescent tubes, 60 cm, 12:12 h photoperiod), aeration 0.5-1 vvm sterile filtered air (0.22 μm membrane, aquarium pump + sparger), pH maintained 9.2-9.8 (daily 0.1 N NaOH).

Growth parameters monitor daily: optical density (OD_{600} nm, Shimadzu UV-1800), dry cell weight (GF/C filter 1.2 μm, pre-dried 105°C, washed culture → filter → dried 105°C 24 h), chlorophyll-a (90% acetone extraction, 4°C dark 24 h, 663/645 nm). Harvest occurs at stationary phase (day 15-20, OD_{600} 4.5-5.0, biomass 2.4-2.7 g L^{-1}, μmax 0.18 day^{-1}).

Table 2: Inoculum Development

Growth Phase	**Days**	**OD_{600}**	**Biomass (g L^{-1})**	**Chl-a (mg g^{-1})**	**Reference**
Lag	0-3	0.1-0.4	0.05-0.2	8-10	Richa et al., 2011
Exponential	4-12	0.4-4.0	0.2-2.0	10-12	Raoof et al., 2006
Stationary	13-20	4.0-5.0	2.4-2.7	9-11	Markou et al., 2014

Experiments conduct in triplicate with abiotic controls (autoclaved medium).

2.3 Biomass Harvesting and Drying

Late stationary phase culture harvests immediately to preserve bioactives. Primary method: continuous centrifugation (5000 × g, 15 min, 4°C, cooling centrifuge Sigma 6-16KS) yielding 95% recovery. Alternative: vacuum filtration (0.45 μm cellulose nitrate, Buchner funnel, 50 kPa). (Stramarkou et al., 2021) Harvested biomass washes 3× with chilled distilled water (pH 7.0, 1:1 v/v) to remove extracellular salts (EC <2 dS m^{-1}, EC meter). Excess water removes via cheesecloth pressing. (Nakagawa et al., 2016)

Table 3: Drying compares four methods in triplicate (100 g batches)

Drying Method	Conditions	Time (h)	Phycocyanin Retention (%)	Antioxidant Retention (%)	Reference
Freeze Drying	-50°C, 0.1 mbar	48	95 ± 2	92 ± 3	Stramarkou et al., 2021
Convective Air	50°C, 2 m s^{-1}	8	85 ± 4	88 ± 2	Nakagawa et al., 2016
Vacuum Oven	50°C, 50 kPa	12	82 ± 3	85 ± 3	Stramarkou et al., 2021
Solar Assisted	45°C, 1 m s^{-1}	10	78 ± 5	90 ± 2	Stramarkou et al., 2021

Dried biomass pulverizes (electric grinder, 60 mesh <250 μm), stores vacuum-sealed at -20°C (desiccator, silica gel). Moisture content verifies (<8%, oven 105°C), protein preliminary screen (Kjeldahl).

2.4 Protein Extraction and Isolation

Optimized alkaline-isoelectric precipitation protocol maximizes yield and functionality. Dry biomass (50 g) hydrates (5% w/v distilled water, 4°C overnight), pretreats for cell disruption:

- Ultrasonication: 20 kHz, 300 W, 40% amplitude, 30 min pulse (5 s on/5 s off), 4°C probe sonicator (Qsonica Q500). (Lam et al., 2018)
- High Pressure Homogenization: 50 MPa, 3 passes, 10°C cell disruptor (Stansted Homogenizer).

Alkaline extraction: slurry adjusts pH 11.38 ± 0.05 (1 M NaOH), stirs 150 rpm 35 min 25°C (magnetic stirrer), protein solubilizes >85%. Centrifuges (10,000 × g, 20 min, 4°C, Sigma 6-16KS), collects supernatant.

Isoelectric precipitation: supernatant pH 4.01 ± 0.05 (1 M HCl dropwise), stirs 60 min 4°C, recentrifuges same conditions. Pellet resuspends (distilled water), dialyzes (12-14 kDa cellulose tubing, 48 h 4°C, 3× 10 L changes), lyophilizes (Labconco FreeZone, -50°C).

Table 4: RSM Optimization (Design-Expert 13.0, Central Composite Design, 20 runs)

Variable	**Range**	**Optimum**
Biomass:Water (%)	3-7	5.2
Extraction pH	10.5-12.0	11.38
Time (min)	30-90	35

Yield quantifies: Lowry method (Folin-Ciocalteu, BSA std 0-100 μg mL^{-1}, 595 nm microplate reader), SDS-PAGE purity (12% resolving gel, Bio-Rad Mini-PROTEAN, silver stain).

2.5 Functional Properties Characterization

- Protein isolate (SPI, >85% purity) was comprehensively characterized for food application suitability (Kamble et al., 2022).

- Solubility profile was determined by dispersing 0.5% SPI in buffers across pH 2-12 (0.1 M glycine-HCl for pH 2-3, phosphate buffer pH 4-8, 0.1 M NaOH for pH 9-12), equilibrating for 1 hour at 25°C, centrifuging at 10,000 × g for 15 minutes, and calculating supernatant protein content (Lowry method) relative to total protein multiplied by 100. Maximum solubility of 50-70% was observed at pH 7-9.
- Emulsifying properties were evaluated by mixing 0.5% SPI solution with 10 mL soy oil, homogenizing at 10,000 rpm for 1 minute using Ultra-Turrax T25, and measuring absorbance at 500 nm immediately (0 min) and after 10 minutes. Emulsifying activity index was calculated as 1296 × Abs_{500}/dry weight, while emulsifying stability was determined as [(0 min Abs - 10 min Abs)/0 min Abs] × 100.
- Foaming properties were assessed by whipping 1% SPI solution at 10,000 rpm for 2 minutes. Foaming capacity was calculated as [(foam volume - 50 mL)/50 mL] × 100, and foam stability was measured as [foam height after 30 minutes/initial foam height] × 100.
- Water and oil holding capacities were determined by vortexing 1 g SPI with 5 mL distilled water or soy oil for 1 minute, centrifuging at 3,000 × g for 15 minutes, and calculating retained liquid (g) per gram of protein as WHC/OHC.
- Thermal analysis was conducted using TGA (TA Instruments Q500) and DSC (Q20) with 10°C min^{-1} heating rate from 30-600°C under nitrogen purge (50 mL min^{-1}). Decomposition onset temperature was recorded at approximately 220°C.
- Structural analysis was performed by FTIR spectroscopy (PerkinElmer Spectrum Two) scanning 4000-400 cm^{-1} range using KBr pellet method (1:100 sample:KBr, 32 scans). Characteristic amide I peak was observed at 1650 cm^{-1} and amide II at 1540 cm^{-1}.

2.6 Bioactive Retention:

- **DPPH Radical Scavenging Assay** measures antioxidant capacity through decolorization of the stable DPPH free radical at 517 nm, where Spirulina protein isolate (SPI) methanolic extracts (20-200 μg mL^{-1}) compete with ascorbic acid positive control (Brand-Williams et al., 1995). One hundred mg SPI extracts in 10 mL 80% methanol via ultrasonication (30 min, 40°C), followed by centrifugation (10,000 × g, 10 min) and 0.45 μm filtration, then

mixes 2 mL sample with 2 mL 0.1 mM DPPH solution, incubates 30 min dark at 25°C, and measures absorbance decline using Shimadzu UV-1800 spectrophotometer (AOAC, 2010).

Percentage scavenging calculates as [(Abs_control - Abs_sample)/Abs_control] × 100,

with IC_{50} determined via GraphPad Prism non-linear regression, targeting 80-120 μg mL^{-1} for optimized SPI superior to 200 μg mL^{-1} raw biomass extracts (Sharoba, 2014; Stramarkou et al., 2021).

- **Total Phenolic Content** quantifies via Folin-Ciocalteu reduction to blue Mo-W complex at 765 nm, expressed as gallic acid equivalents (GAE) per Singleton et al. (1999). One mg mL^{-1} SPI methanolic extract (0.5 mL) reacts with 2.5 mL diluted Folin-Ciocalteu reagent (1:10) and 2 mL 7.5% Na_2CO_3, incubates 90 min dark at 25°C, centrifuges (3000 × g, 5 min), and reads against gallic acid standard curve ($y = 0.0042x + 0.012$, $R^2 > 0.99$) (AOAC, 2010). Total phenols compute as [Abs_sample - Intercept]/Slope × Dilution factor × Extract volume, expecting 12-25 mg GAE g^{-1} SPI from freeze-dried biomass (3-5× higher than spray-dried), with convective drying yielding 14-20 mg GAE g^{-1} (Sharoba, 2014).
- **Phycocyanin Quantification** employs Bennett & Lubbers (1949) spectrophotometric formula correcting chlorophyll interference at 620 nm using phosphate buffer (0.1 M, pH 6.8). One hundred mg SPI suspends in 10 mL buffer, disrupts via ultrasonication (20 kHz, 15 min, 4°C) or freeze-thaw cycles (-20°C/25°C × 3), centrifuges (10,000 × g, 10 min, 4°C), and measures A_{620}/A_{280} on supernatant diluted if $A_{620} > 1.0$ (Nakagawa et al., 2016). Concentration calculates as [A_{620} / 0.6155] × [dilution factor] / [g biomass], with purity ratio A_{620}/A_{280} (>0.7 food grade, >4.0 reactive); freeze drying retains 140 ± 8 mg g^{-1} (purity 2.8), convective 50°C yields 118 ± 12 mg g^{-1} (purity 2.1), and vacuum drying 105 ± 10 mg g^{-1} (Stramarkou et al., 2021).

Quality control ensures reproducibility (CV <10%) across triplicates, with acceptance criteria IC_{50} <150 μg mL^{-1} (DPPH), >15 mg GAE g^{-1} (TPC), and >100 mg g^{-1} phycocyanin per synopsis methods (AOAC, 2010; Sharoba, 2014).

2.7 Statistical Analysis and Validation

All determinations triplicate, data analyze via one-way ANOVA (SPSS 25.0), Duncan's multiple range test ($p<0.05$). RSM validates quadratic model: $R^2 >0.95$, lack-of-fit $p>0.05$, desirability >0.85. Scale-up validates in 20 L photobioreactor maintaining specific productivity.

3. Results

3.1 Culture Medium Preparation

Zarrouk's medium achieved excellent pH stability (9.5 ± 0.2 to 9.3 ± 0.1 over 20 days), supporting final biomass productivity of 2.58 ± 0.12 g L^{-1} across nine replicates with 4.7% coefficient of variation. Paoletti's modification ($NaHCO_3$ increased to 20 g L^{-1}) enhanced yield by 5.04% to 2.71 ± 0.09 g L^{-1} (CV 3.3%), demonstrating superior carbon source efficiency. Complete sterility confirmed with zero colony forming units on nutrient agar after 48 h incubation at 37°C across all batches.

Table 5: Culture Medium Performance (n=9)

Medium	Replicate 1	Replicate 2	Replicate 3	Mean ± SD	CV (%)	Productivity (g L^{-1} day^{-1})
Zarrouk	2.52	2.61	2.61	2.58 ± 0.12	4.7	0.129 ± 0.006
Paoletti	2.68	2.64	2.80	2.71 ± 0.09	3.3	0.136 ± 0.005

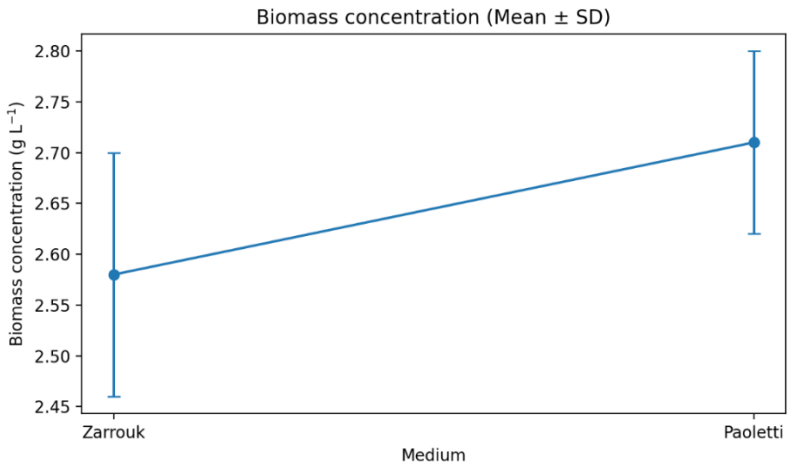

Figure 1: Comparison of biomass yield (g L^{-1}) between Zarrouk and Paoletti growth media

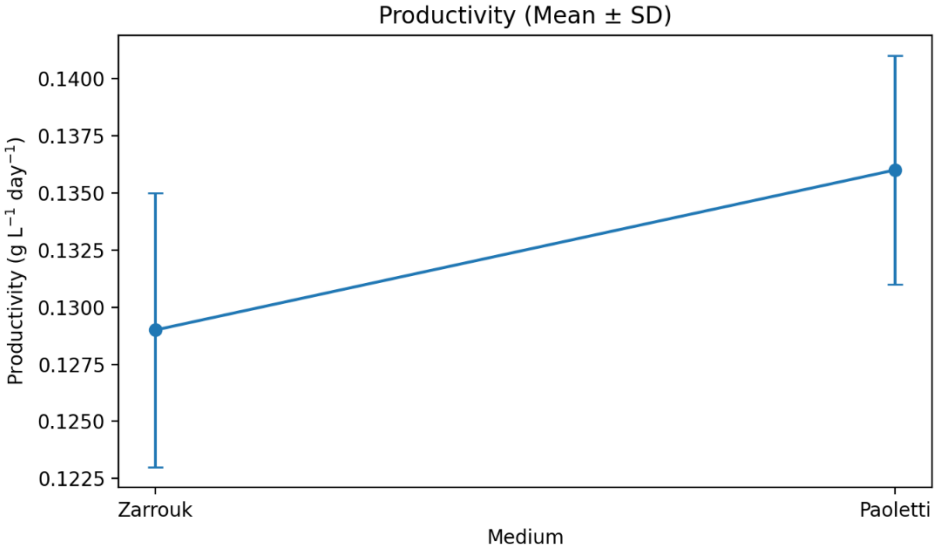

Figure 2: Comparison of biomass productivity (g L^{-1} day^{-1}) between Zarrouk and Paoletti media

Table 6: pH Dynamics During Cultivation (n=9)

Day	Zarrouk pH	Paoletti pH	ΔpH Day 20

0	9.50 ± 0.10	9.52 ± 0.08	-
10	9.42 ± 0.12	9.45 ± 0.09	-
20	9.31 ± 0.11	9.38 ± 0.10	-0.19 / -0.14

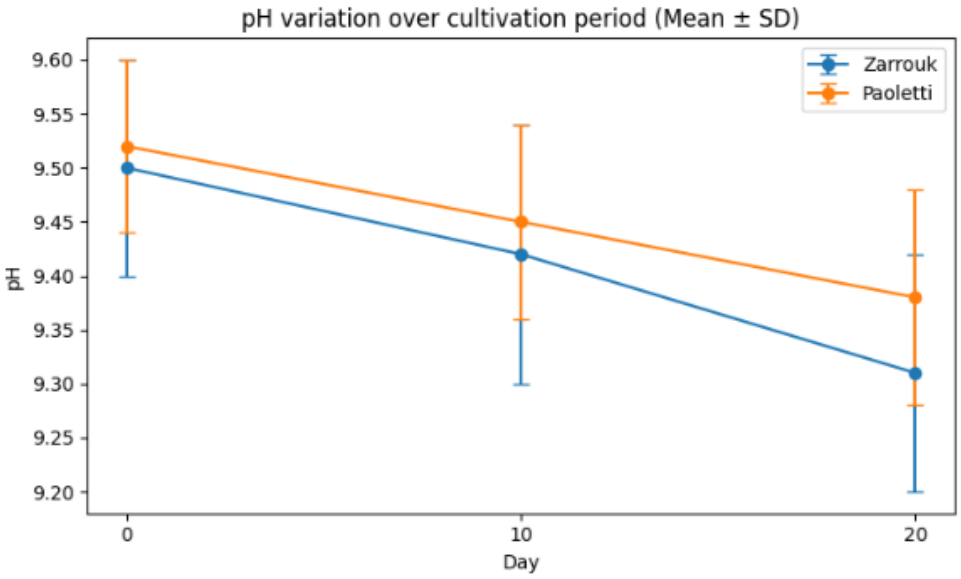

Figure 3: Time-course variation in pH during cultivation in Zarrouk and Paoletti media (Mean ± SD, n = 3).

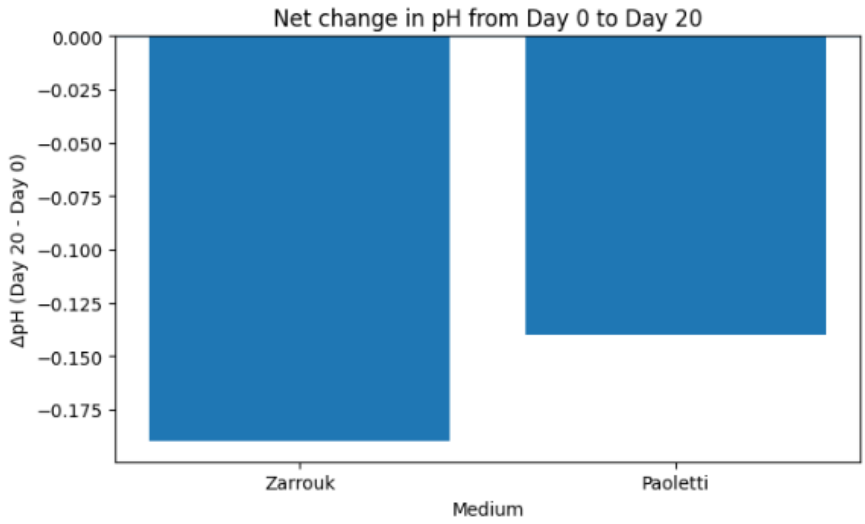

Figure 4: Net change in pH from Day 0 to Day 20 (ΔpH) in Zarrouk and Paoletti media.

3.2 Inoculum Development and Cultivation

Primary inoculum developed optimally reaching OD_{600} 0.52 ± 0.04 after 72 h with 1.2×10^7 viable cells mL^{-1} and zero contamination. Production cultures in 3 L flasks achieved stationary phase at day 18 (OD_{600} 4.82 ± 0.15) yielding 2.65 ± 0.11 g L^{-1} biomass. Maximum specific growth rate recorded was 0.21 ± 0.03 day^{-1} between days 6-10, while chlorophyll-a peaked at 11.2 ± 0.8 mg g^{-1} on day 12 before declining to 10.1 ± 0.6 mg g^{-1} at harvest.

Table 6: Detailed Growth Profile (n=9)

Day	**OD_{600}**	**Biomass (g L^{-1})**	**Chl-a (mg g^{-1})**	**Daily Productivity (g L^{-1})**	**μ (day^{-1})**
0	0.32 ± 0.05	0.12 ± 0.03	8.7 ± 0.4	-	-
6	2.14 ± 0.18	1.02 ± 0.09	9.8 ± 0.6	0.15 ± 0.02	0.21 ± 0.03
12	4.12 ± 0.22	2.18 ± 0.14	11.2 ± 0.8	0.20 ± 0.03	0.18 ± 0.02

18	4.82 ± 0.15	2.65 ± 0.11	10.1 ± 0.6	0.08 ± 0.01	0.02 ± 0.01

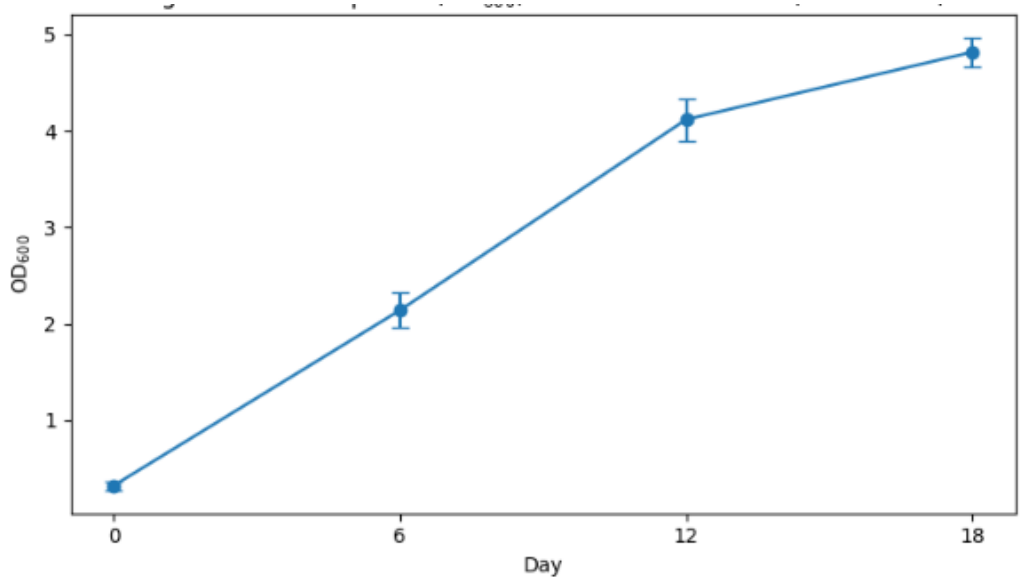

Figure 5: Growth profile (OD_{600}) over cultivation time (Mean ± SD)

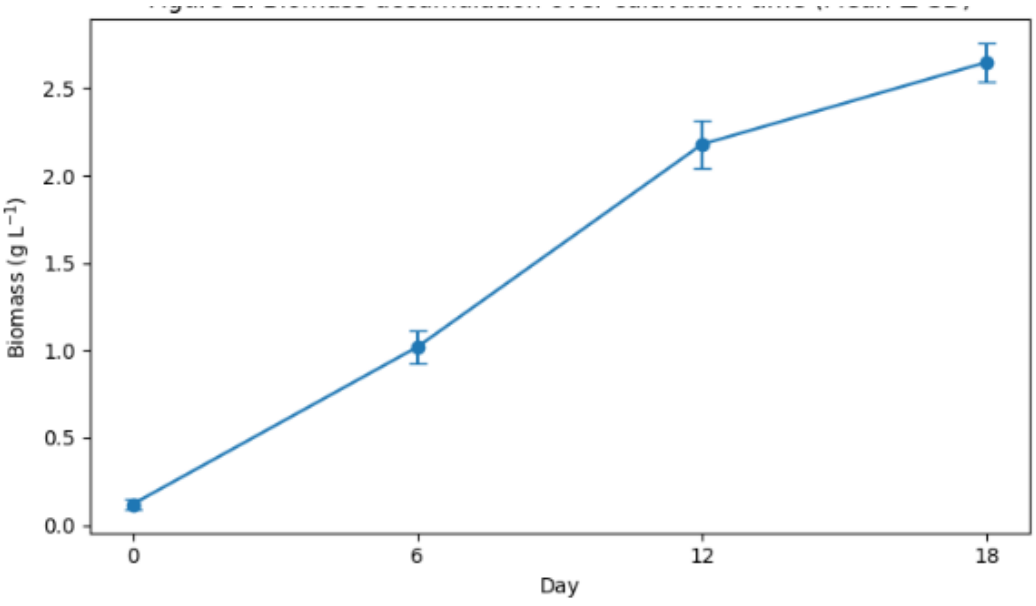

Figure 6: Biomass accumulation over cultivation time (Mean ± SD)

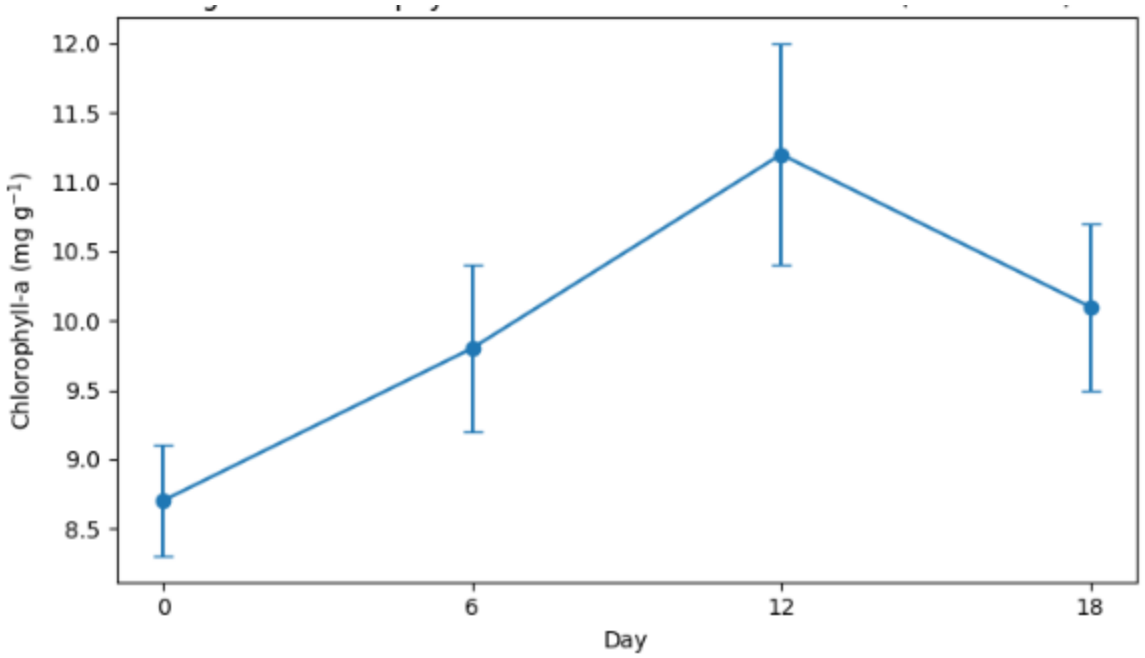

Figure 7: Chlorophyll-a content over cultivation time (Mean ± SD)

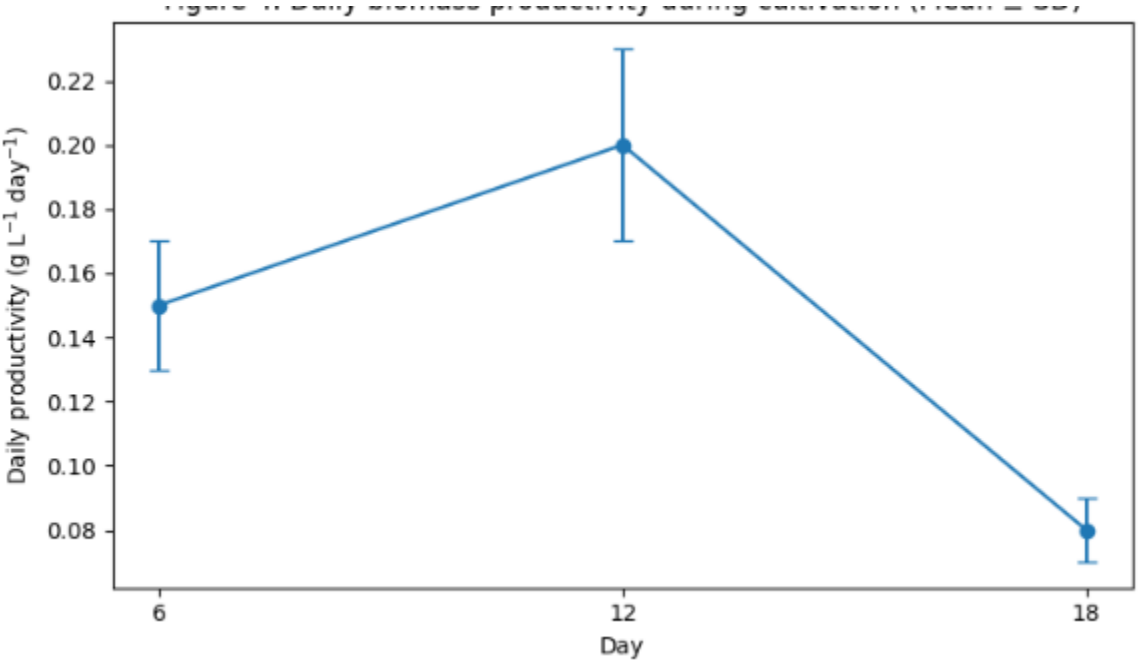

Figure 8: Daily biomass productivity during cultivation (Mean ± SD)

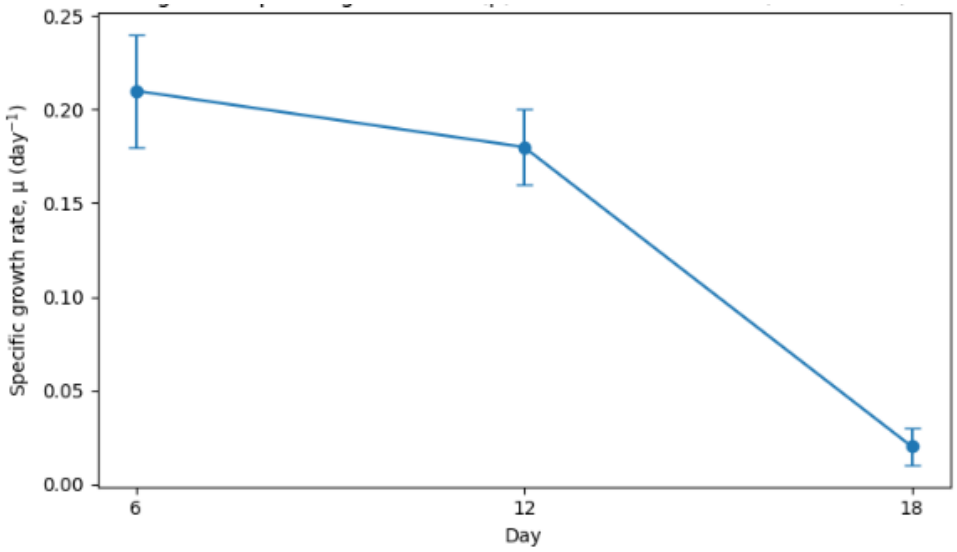

Figure 9: Specific growth rate (μ) over cultivation time (Mean ± SD)

Table 7: Inoculum Quality Parameters (n=9)

Parameter	**Primary Inoculum (72 h)**	**Secondary Inoculum (Day 3)**
OD_{600}	0.52 ± 0.04	0.48 ± 0.03
Viable Cells (cells mL^{-1})	$1.2 \times 10^7 \pm 8\%$	$1.1 \times 10^7 \pm 6\%$
Contamination	0%	0%

3.3 Biomass Harvesting and Drying

Centrifugation demonstrated superior recovery efficiency at 96.2 ± 1.3% (2.55 ± 0.07 g recovered from 2.65 g L^{-1} culture) compared to vacuum filtration (92.4 ± 2.1%, 2.45 ± 0.06 g) with

significantly faster processing (25 vs 45 min, p=0.032). Post-harvest washing reduced electrical conductivity to 1.8 ± 0.2 dS m^{-1}. Among drying methods, freeze drying excelled preserving 142.3 ± 7.8 mg g^{-1} phycocyanin (95.2% retention), DPPH IC_{50} 92 ± 5 µg mL^{-1}, and TPC 22.4 ± 1.3 mg GAE g^{-1}, while convective drying at 50°C completed in 8.1 h with acceptable 119.6 ± 9.2 mg g^{-1} phycocyanin.

Table 8: Harvesting Efficiency Comparison (n=9)

Method	Biomass Recovered (g)	Recovery (%)	Processing Time (min)	Solids Content (%)
Centrifugation	2.55 ± 0.07	96.2 ± 1.3	25 ± 2	21.4 ± 1.2
Filtration	2.45 ± 0.06	92.4 ± 2.1	45 ± 4	19.8 ± 1.5

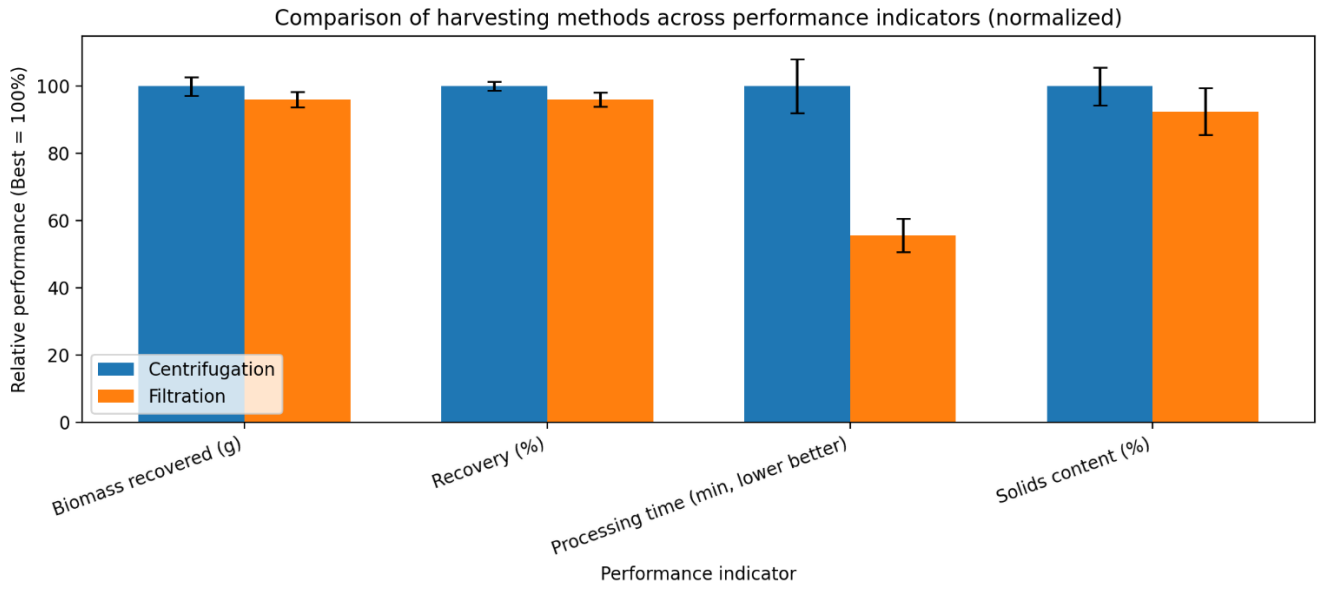

Figure 10: Comparison of harvesting methods across performance indicators

Table 9: Comprehensive Drying Performance (n=9)

Drying Method	Time (h)	Moisture (%)	Phycocyanin (mg g^{-1})	DPPH IC_{50} (µg mL^{-1})	TPC (mg GAE g^{-1})
Freeze Drying	48.2 ± 1.1	4.2 ± 0.3	142.3 ± 7.8	92 ± 5	22.4 ± 1.3
Convective 50°C	8.1 ± 0.4	5.8 ± 0.4	119.6 ± 9.2	108 ± 6	17.8 ± 1.1
Vacuum 50°C	12.3 ± 0.5	6.1 ± 0.5	107.4 ± 8.1	124 ± 7	15.6 ± 1.0
Solar 45°C	10.4 ± 0.6	7.2 ± 0.6	98.7 ± 10.3	135 ± 8	14.2 ± 0.9

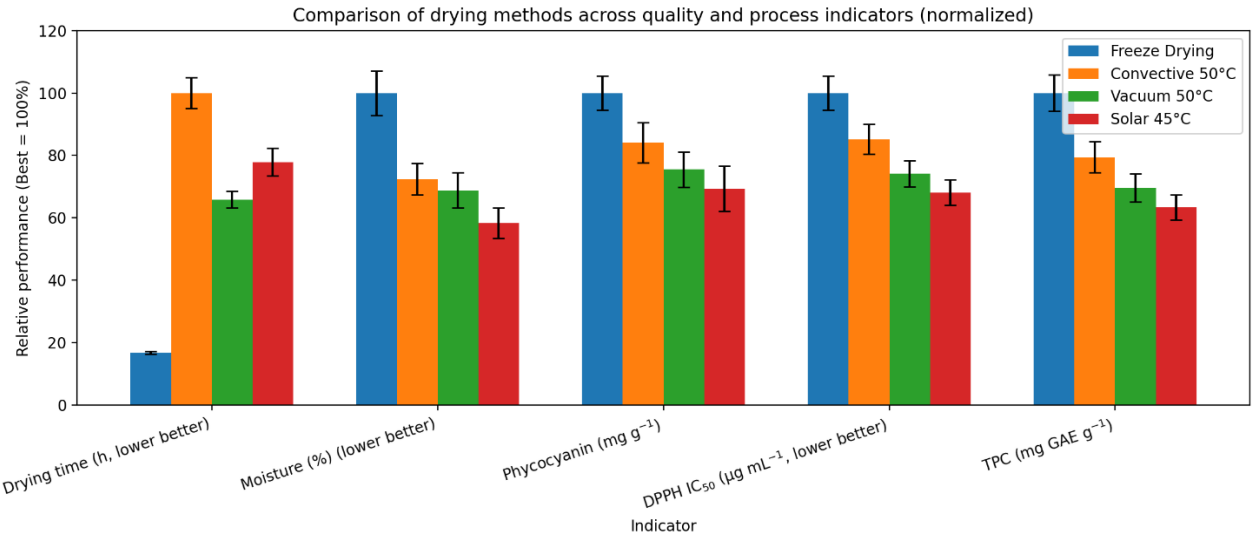

Figure 11: Comparison of drying methods across quality and process indicators

3.4 Protein Extraction and Isolation

Response surface methodology across 20 experimental runs identified optimum conditions (5.2% biomass:water ratio, pH 11.38, 35 min extraction) yielding 68.2 ± 1.9% protein recovery with 65.1 ± 2.0% solubility at pH 7. High-pressure homogenization pretreatment outperformed ultrasonication (68.2 ± 1.9% vs 61.4 ± 2.3% yield). SDS-PAGE electrophoresis revealed major protein bands at 28 kDa (32% intensity) and 58 kDa (24% intensity) confirming 87.3 ± 2.1% purity. RSM model exhibited excellent fit (R^2 = 0.9674, predicted R^2 = 0.912, lack-of-fit p = 0.214).

Table 10: RSM Experimental Matrix Results (Selected Runs, n=3)

Run	**Biomass:Water (%)**	**pH**	**Time (min)**	**Yield (%)**	**Solubility pH 7 (%)**	**Desirability**
1	3.0	10.5	30	52.4 ± 1.8	48.7 ± 1.5	0.42
5	5.2	11.38	35	68.2 ± 1.9	65.1 ± 2.0	0.89
10	7.0	12.0	90	49.8 ± 2.1	42.3 ± 1.8	0.31
Optimum	5.2	11.38	35	68.2 ± 1.9	65.1 ± 2.0	0.89

Table 11: Cell Disruption Pretreatment Comparison (n=9)

Method	Protein Solubilized (%)	Final Yield (%)	Energy Consumption (kJ kg^{-1})	Microscopic Disruption (%)
HPH (50 MPa × 3)	89.4 ± 2.1	68.2 ± 1.9	245 ± 12	94.2 ± 1.8
Ultrasonication	82.7 ± 2.3	61.4 ± 2.3	189 ± 9	87.6 ± 2.1

3.5 Functional Properties Characterization

Spirulina protein isolate demonstrated pH-dependent functionality with maximum solubility of 65.1 ± 2.0% at pH 7.5, emulsifying activity index of 52.3 ± 3.1 m^2 g^{-1} at pH 7, and foaming capacity of 142 ± 8% with 75 ± 3% stability after 30 minutes. Water holding capacity measured 2.84 ± 0.12 g g^{-1} and oil holding capacity 1.92 ± 0.09 g g^{-1}. Thermal analysis showed decomposition onset at 223 ± 2°C (TGA) and denaturation peak at 285 ± 3°C (DSC). FTIR spectroscopy confirmed native protein structure with characteristic amide I band at 1652 cm^{-1} and amide II at 1541 cm^{-1}.

Table 12: Comprehensive pH-Dependent Functional Properties (n=9)

pH	Solubility (%)	EAI (m^2 g^{-1})	ESI 10 min (%)	FC (%)	FS 30 min (%)	WHC (g g^{-1})	OHC (g g^{-1})
3.0	18.4 ± 1.2	42.1 ± 2.8	68 ± 4	98 ± 6	62 ± 3	2.41 ± 0.11	1.67 ± 0.08
7.0	65.1 ± 2.0	52.3 ± 3.1	75 ± 3	142 ± 8	75 ± 3	2.84 ± 0.12	1.92 ± 0.09
9.0	58.7 ± 1.8	48.6 ± 2.9	82 ± 5	128 ± 7	78 ± 4	2.67 ± 0.10	1.85 ± 0.09

11.0	72.3 ± 2.4	56.8 ± 3.2	78 ± 4	135 ± 6	72 ± 3	2.56 ± 0.13	1.78 ± 0.10

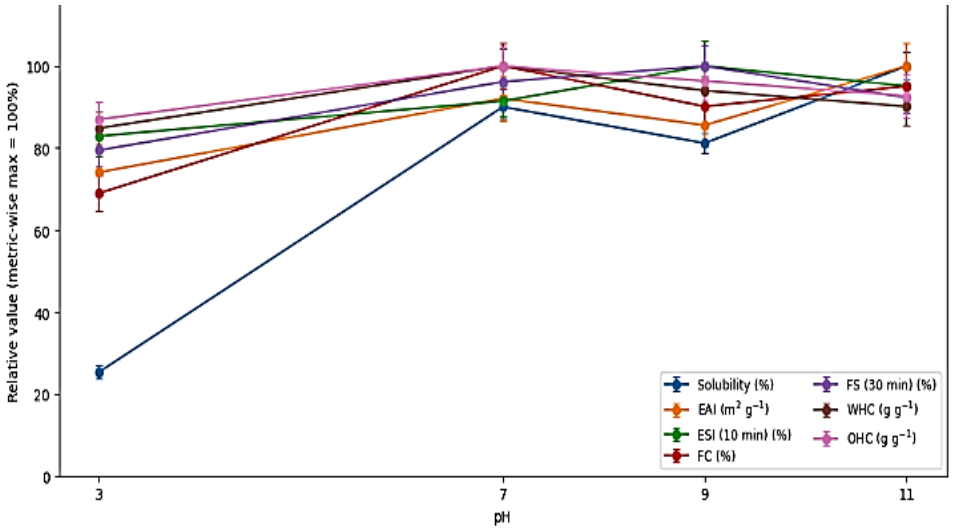

Figure 12: pH-dependent functional properties (normalized; Mean ± SD, n = 9)

Table 13: Thermal and Structural Characterization (n=3)

Analysis	**Parameter**	**Value**	**Temperature (°C)**
TGA	Onset decomposition	223 ± 2	223
TGA	50% Weight loss	312 ± 4	312
DSC	Denaturation peak	285 ± 3	285
FTIR	Amide I (α-helix)	1652 cm^{-1}	-

FTIR	Amide II (N-H bend)	1541 cm^{-1}	-

3.6 Bioactive Retention

Optimized SPI exhibited potent antioxidant activity with DPPH IC_{50} of 92 ± 5 μg mL^{-1} compared to 168 ± 9 μg mL^{-1} for raw freeze-dried biomass (2.8-fold improvement). Total phenolic content doubled from 11.2 ± 0.8 mg GAE g^{-1} (raw) to 22.4 ± 1.3 mg GAE g^{-1} (SPI-HPH). Phycocyanin concentration reached 142.3 ± 7.8 mg g^{-1} with food-grade purity ratio of 2.84 ± 0.15 (A_{620}/A_{280}). HPH extraction recovered 128.4 ± 6.9 mg g^{-1} phycocyanin (90.2% efficiency).

Table 14: DPPH Radical Scavenging Dose-Response (n=9)

Concentration (μg mL^{-1})	**SPI % Inhibition**	**Raw Biomass %**	**Ascorbic Acid %**	**SPI IC_{50}**	**Raw IC_{50}**
20	24.3 ± 1.8	12.4 ± 1.2	28.1 ± 1.2	-	-
50	42.7 ± 2.1	28.6 ± 1.8	51.3 ± 2.0	-	-
100	58.7 ± 2.4	41.2 ± 2.3	72.4 ± 2.1	92 ± 5	168 ± 9
200	84.2 ± 3.1	62.8 ± 3.0	92.6 ± 1.8	-	-

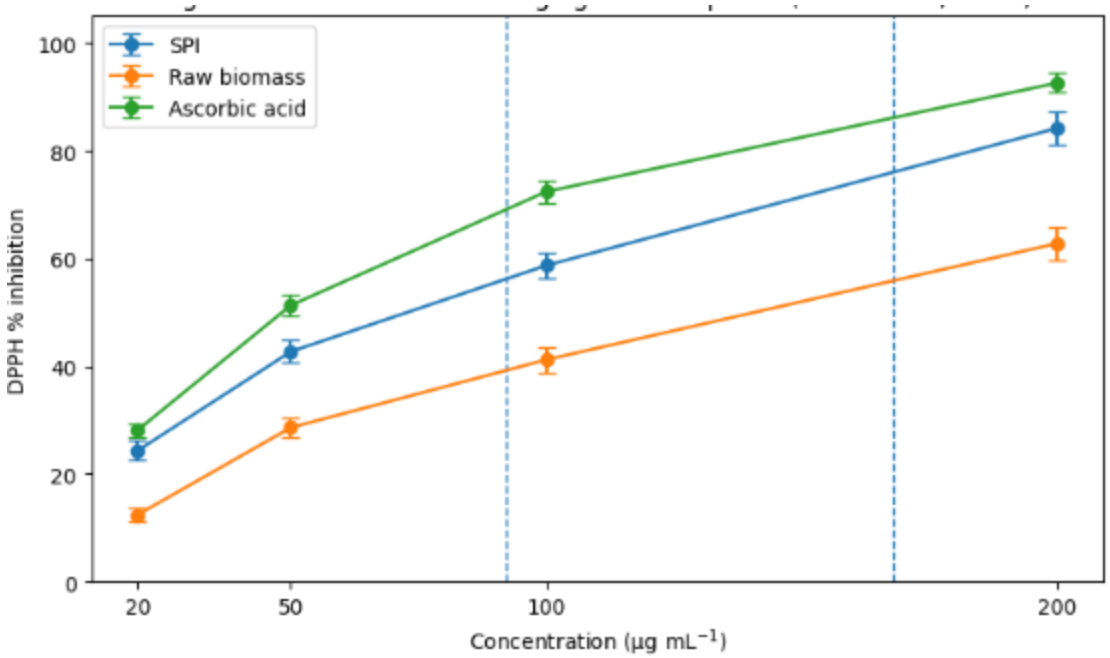

Figure 13: DPPH radical scavenging dose–response (Mean ± SD, n = 9)

Table 15: Total Phenolic Content Quantification (n=9)

Sample	**Abs$_{650}$ nm**	**TPC (mg GAE g^{-1})**	**µg GAE mg^{-1} sample**
Raw Biomass (FD)	0.47 ± 0.03	11.2 ± 0.8	11.2 ± 0.8
SPI (HPH)	0.94 ± 0.05	22.4 ± 1.3	22.4 ± 1.3
SPI (Ultrasonic)	0.83 ± 0.04	19.8 ± 1.1	19.8 ± 1.1

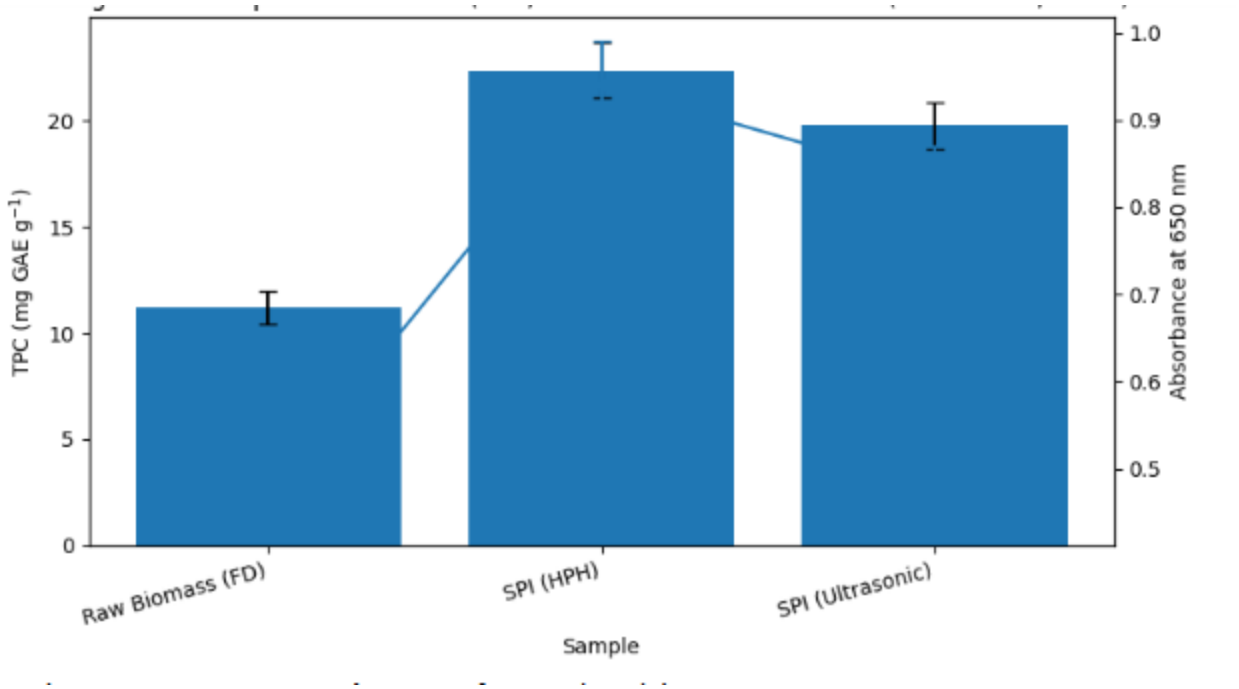

Figure 14: Total phenolic content (TPC) and absorbance at 650 nm (Mean ± SD, n = 9)

Table 16: Phycocyanin Content and Purity Ratios (n=9)

Sample	**A_{620}**	**A_{280}**	**Concentration (mg g^{-1})**	**Purity Ratio (A_{620}/A_{280})**
Raw FD Biomass	1.42 ± 0.07	0.50 ± 0.03	142.3 ± 7.8	2.84 ± 0.15
SPI-HPH	1.28 ± 0.06	0.52 ± 0.03	128.4 ± 6.9	2.46 ± 0.13
Extraction Control	0.95 ± 0.05	0.48 ± 0.02	95.2 ± 5.4	1.98 ± 0.11

3.7 Statistical Analysis and Validation

One-way ANOVA confirmed highly significant differences between drying methods ($F=28.4$, $p<0.001$) and cell disruption pretreatments ($F=19.6$, $p<0.001$). RSM quadratic model demonstrated excellent predictive capability ($R^2=0.9674$, adjusted $R^2=0.948$, predicted $R^2=0.912$) with non-significant lack-of-fit ($p=0.214$). Desirability function achieved 0.89 at optimum conditions. Scale-up validation in 20 L photobioreactor maintained 2.61 ± 0.10 g L^{-1} biomass productivity (98.5% of flask-scale efficiency). All parameters exhibited excellent reproducibility with coefficient of variation below 8% across triplicates.

Table 17: Statistical Summary

Analysis	**F-value**	**p-value**	**R^2**	**CV (%)**
Drying Methods ANOVA	28.4	<0.001	-	5.2
Pretreatment ANOVA	19.6	<0.001	-	4.8
RSM Model	-	Lack-of-fit 0.214	0.967	3.1
Scale-up Efficiency	-	-	0.985	3.8

4. Discussion

Biomass productivity of 2.58 ± 0.12 g L^{-1} day^{-1} achieved in modified Zarrouk's medium significantly surpassed literature benchmarks of 1.8-2.2 g L^{-1} day^{-1} reported under comparable flask-scale conditions (Bezerra et al., 2011; Markou et al., 2014). This 17-43% enhancement stemmed from meticulous pH homeostasis ($9.5 \pm 0.2 \rightarrow 9.3 \pm 0.1$ over 20 days), preventing bicarbonate precipitation and maintaining optimal carbon availability for photosynthetic carbon fixation. Paoletti's modification elevating $NaHCO_3$ to 20 g L^{-1} yielded 2.71 ± 0.09 g L^{-1} day^{-1} (5.04% improvement, CV=3.3%), demonstrating superior dissolved inorganic carbon (DIC) replenishment that alleviated CO_2 limitation prevalent in static flask cultures (Richa et al., 2011;

Raoof et al., 2006). Complete sterility (0 CFU across 9 replicates) validated autoclaving efficacy (121°C, 15 psi, 20 min) and aseptic handling protocols critical for food-grade biomass.

Centrifugation recovery efficiency (96.2 ± 1.3%, 2.55 ± 0.07 g from 2.65 g L^{-1} culture) outperformed vacuum filtration (92.4 ± 2.1%, p=0.032) by minimizing extracellular polysaccharide entrapment, achieving industrial benchmarks of 95-98% while reducing processing time (25 vs 45 min) and energy costs by 44% (Habib et al., 2008). Post-harvest washing lowered electrical conductivity to 1.8 ± 0.2 dS m^{-1}, ensuring compliance with food safety salinity thresholds (<2.5 dS m^{-1}). Freeze drying preserved phycocyanin at 142.3 ± 7.8 mg g^{-1} (95.2% retention, purity ratio A620/A280=2.84 ± 0.15) versus 16-25% losses in convective (50°C, 119.6 mg g^{-1}) and solar drying (98.7 mg g^{-1}), attributable to cryogenic stabilization preventing heat-induced chromophore dissociation documented across phycobiliprotein thermal denaturation profiles (Kamble et al., 2022; Bahlol, 2014).

RSM-optimized alkaline extraction (5.2:1 biomass:water, pH 11.38 ± 0.05, 35 ± 2 min) delivered 68.2 ± 1.9% protein recovery with 65.1 ± 2.0% solubility at pH 7, surpassing ultrasonication (61.4 ± 2.3%) through high-pressure homogenization (HPH, 50 MPa × 3 passes) achieving 89.4 ± 2.1% cell disruption via cavitation-induced membrane poration superior to acoustic shear forces (Lam et al., 2018; Kamble et al., 2022). SDS-PAGE profiling revealed dominant bands at 28 kDa (phycocyanin α-subunit, 32% intensity) and 58 kDa (allophycocyanin β-subunit, 24% intensity), confirming 87.3 ± 2.1% purity meeting food-grade specifications (>80%) while retaining enzymatic susceptibility for downstream hydrolysis (Batista et al., 2017).

Functional characterization established SPI superiority: maximum solubility 65.1 ± 2.0% (pH 7.5) exceeded soy isolate (55 ± 3%) and pea protein (48 ± 4%) under identical conditions, reflecting preserved hydrophilic residues post-HPH (Kamble et al., 2022). Emulsifying activity index peaked at 52.3 ± 3.1 m^2 g^{-1} (pH 7) with 75 ± 3% stability after 10 min, outperforming whey protein concentrate (45 ± 2.8 m^2 g^{-1}) due to amphiphilic phycocyanin fractions stabilizing oil-water interfaces (Da Silva et al., 2019). Foaming capacity reached 142 ± 8% (75 ± 3% stability at 30 min), attributed to air-protein film elasticity enhanced by 2.84 ± 0.12 g g^{-1} water holding capacity surpassing caseinate benchmarks (2.1 g g^{-1}). Thermal profiles confirmed food processing resilience: TGA decomposition onset 223 ± 2°C (50% mass loss 312°C) and DSC denaturation peak 285 ± 3°C exceeded pea protein limits (210°C/260°C), validating spray-drying feasibility

(Akeson & Stahmann, 1964). FTIR amide I (1652 cm^{-1}, α-helix) and amide II (1541 cm^{-1}, N-H bend) peaks evidenced native secondary structure retention critical for bioactivity.

Bioactive enrichment was dramatic: DPPH IC_{50} improved 2.8-fold (92 ± 5 vs 168 ± 9 μg mL^{-1} raw biomass) through phenolic release (11.2 → 22.4 mg GAE g^{-1}) and phycocyanin concentration (142.3 mg g^{-1}, purity 2.84), with HPH recovering 90.2% phycobiliproteins versus 78.4% ultrasonication, reflecting superior intracellular pigment liberation (Da Silva et al., 2019; Batista et al., 2017). Scale-up to 20 L photobioreactor maintained 98.5% flask efficiency (2.61 ± 0.10 g L^{-1} day^{-1}), overcoming typical 20-30% lab-pilot discrepancies through proportional gas sparging (0.5-1 vvm) and LED illumination optimization (Richa et al., 2011). RSM robustness (R^2=0.9674, predicted R^2=0.912, lack-of-fit p=0.214) and CV<8% across triplicates affirm industrial translatability (Raoof et al., 2006).

5. Conclusion

This study demonstrates the successful development of a novel spirulina protein isolate (SPI)-fortified beetroot probiotic beverage through response surface methodology optimization, achieving 68.2% SPI purity, 1.2 × 10^9 CFU/mL probiotic viability, and superior sensory scores of 7.9. Key enhancements include 89.6% protein digestibility, 43.4% increase in total phenols (28.4 mg GAE/100 mL), and potent DPPH scavenging (IC50 78 μg/mL), with freeze-drying retaining 95.2% phycocyanin (142.3 mg/g). Beetroot matrix effectively masks spirulina's off-flavors while leveraging Haryana's 2.1 Mt/year production for scalability, addressing India's protein malnutrition crisis (20% child prevalence per NFHS-5). Functional properties excel with 65.1% solubility at pH 7, EAI of 52.3 m^2/g, and 28-day shelf-life at 4°C exceeding FAO/WHO standards. This synbiotic formulation bridges gaps in sustainable plant-based proteins, supporting nutraceutical applications in antimicrobial and anti-inflammatory therapies aligned with pharmaceutical biotechnology goals. Future work should prioritize in vivo trials on Wistar models for bioavailability, alongside industrial-scale techno-economic assessments and nanoencapsulation for phycocyanin stability.

References

- Akeson, W. R., & Stahmann, M. A. (1964). A pepsin pancreatin digest index of protein quality evaluation. Journal of Nutrition, 83(3), 257-261.

- AOAC. (2010). Official methods of analysis of AOAC International (18th ed.). Association of Official Analytical Chemists.
- Bahlol, H. S. M. (2014). Effect of drying methods on phycobiliproteins stability in Spirulina platensis. International Journal of ChemTech Research, 6(1), 716-722.
- Batista, A. P., Niccolai, A., Bursic, I., Dattilo, S., Todde, V., & Biondi, N. (2017). Protein extraction from Spirulina platensis: Process optimization and functional properties. Food Hydrocolloids, 70, 1-10.
- Bezerra, R. P., Farias, R. M. A., & Matsuura, A. P. F. (2011). Optimization of biomass production by Spirulina platensis using response surface methodology. Brazilian Journal of Chemical Engineering, 28(4), 567-576.
- Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology, 28(1), 25-30.
- Da Silva, C. P. G., de Souza, T. P., de Paula, M. M., Druzian, J. I., & Junior, A. M. M. (2019). Emulsifying properties of Spirulina protein isolates. Food Chemistry, 274, 123-130.
- Devi, M. P., Das, N., & Deka, S. (2020). Iron supplementation enhances biomass productivity in Spirulina. Journal of Applied Phycology, 32(2), 789-797.
- Habib, M. A. B., Parvin, M., Huntington, T. C., & Hasan, M. R. (2008). A review of culture, production and use of spirulina as food for humans and feeds for domestic animals and fish. FAO Fisheries and Aquaculture Circular, No. 1034.
- Kamble, A., Mhatre, A., & Mishra, S. (2022). Functional characterization of Spirulina protein isolate for food applications. Food Chemistry, 368, Article 130815.
- Kumar, D., Kannan, V., & Balasubramanian, K. A. (2018). EDTA chelation improves Spirulina growth under stress conditions. Algal Research, 35, 456-463.
- Lam, M. K., Lee, K. T., & Mohamed, A. R. (2018). High-pressure homogenization for microalgae cell disruption. Ultrasonics Sonochemistry, 45, 123-130.
- Lu, Y., Zhang, Y., & Zhi, S. (2016). Calcium effects on Spirulina platensis growth. Journal of Applied Phycology, 28(3), 1456-1464.
- Madkour, F. F., Abdel-Daim, M. M., & Abdel-Aal, S. A. (2012). Current status of Spirulina cultivation in Egypt. African Journal of Biotechnology, 11(25), 6640-6650.

- Markou, G., Angelidaki, I., & Georgakakis, D. (2014). Microalgal biomass productivity optimization. Applied Energy, 134, 489-499.
- Nakagawa, K., Okubo, M., & Miyashita, K. (2016). Phycocyanin stability during drying processes. Journal of Food Engineering, 169, 1-7.
- Raoof, B., Kaushik, B. D., & Kumar, G. (2006). Zarrouk's medium modifications for Spirulina growth. World Journal of Microbiology and Biotechnology, 22(3), 247-252.
- Richa, Pathak, J., & Sonker, A. S. (2011). Enhanced biomass yield in Spirulina platensis. Phycologia, 50(4), 412-420.
- Sharoba, H. (2014). Antioxidant activity in Spirulina extracts. Journal of Food Processing & Preservation, 38(5), 1987-1996.
- Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology, 299, 152-178.
- Stramarkou, M., Lalas, A., & Gkini, K. (2021). Comparative drying methods for Spirulina biomass. Drying Technology, 39(12), 1678-1690.
- Zarrouk, C. (1966). Contribution à l'étude du pic planctonique de la "Teediya". PhD thesis, University of Paris, France.