**Vechur cow milk yoghurt- Response surface methodology-based process optimization and storage studies**

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SUPPLEMENTARY FILE

**Materials and Methods**

*Preparation of yoghurt samples*

Standardized (3% fat and 8.5% SNF) and two stage homogenized (2500 psi and 500 psi) Vechur cow milk was heat treated at (90°C/5min), cooled to the incubation temperature, inoculated with the culture NCDC-260 (a mixed culture containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus*, procured from National Collection of Dairy Cultures (NCDC, Karnal) and incubated at the specified temperatures for specified periods. The products obtained were subjected to sensory analysis (color and appearance, flavor, body and texture and overall acceptability) using a nine point hedonic scale where 9 points indicate ‘extremely like’ and 1 point indicates ‘extremely dislike’ (Larmond,1977). Control samples were prepared from cross-bred cow milk sample standardized to 3.0 per cent fat and 8.5 per cent solids not fat (SNF). The minimum and maximum values for the three production parameters were decided by comparing the sensory scores of the treatment samples with the corresponding control sample.

*Generation of optimized solution*

Data obtained from sensory analysis were fed to RSM software (Design-Expert® Software Version 8.0.1.0) to generate the regression coefficients and ANOVA of fitted polynomial models for sensory parameters and responses to get the optimized combinations of production parameters. For this, the desired goals for each production parameter and responses were chosen and different weights were assigned to each goal. The responses were kept maximum and the production parameters in range, during the course of optimization. The optimized combinations of the products were validated by comparing observed values of sensory data with the predicted values generated by RSM software. To ensure that there is no significant lot to lot variation in sensory attributes of optimized products the triangle test was carried out using two coded samples (A and B) of yoghurt prepared separately from the same lot of Vechur cow milk adopting the optimized solution.

*Physico-chemical analysis*

The physico-chemical properties analysed were Fat, SNF, total solids, Lactose, Protein , titratable acidity , syneresis percentages and colour characteristics. Fat content of yoghurtwas measured by standard Gerber method (IS: SP: 18,1981). Protein content of yoghurt samples was determined by semi-micro Kjeldahl method described by Menefee and Overman (1940) and the total solids by the standard method IS: 12333 (1997) . Lactose contents of yoghurt samples were determined by Lane Eynon method (IS: SP: 18, Part XII (1981). IS: 1166 (1986) with some modifications was followed for determining the titratable acidity. Syneresis of yoghurtsamples were determined as per the method of Goncalvez *et al.* (2005). Colour of yoghurt samples were measured using a Color flex colorimeter (Hunter Associated Laboratory, Inc., VA, USA, software version 4.10.).

*Microbiological analysis*

The optimized samples were assessed for total viable, coliform, yeast and mold, lactic acid bacteria counts. For the enumeration of the microbial population, appropriate dilutions of the samples were pour plated using their respective growth medium and incubated at the particular temperature time combinations. The total viable bacteria of yoghurt samples were enumerated by pour plat­ing appropriate dilutions on nutrient agar (HiMedia, Mumbai) and incubation at 37±0.5°C for 48 h (APHA, 1978). Violet red bile agar (VRBA, HiMedia, Mumbai) was used for the enumeration of coliform bacteria, and the plates were incubated at 37±0.5°C for 24 h (IS: 5401, 2002). Yeast and mold counts were determined using potato dextrose agar (PDA, HiMedia, Mumbai) and subsequent incubation at 25±1°C for 5 days. Starter culture count in terms of lactococci and lactobacilli counts was determined using M17 agar, deMan Rogosa Sharpe Agar (MRS agar) (HiMedia, Mumbai) respectively and subsequent incubation at 37±0.5°C for 48 h (Downes and Ito, 2001). The counts obtained were added and expressed as Lactic acid bacteria count. All the counts were expressed as log 10 CFU/g.

*Analysis of the optimized product during room and refrigerated storage*

Tyrosine values of yoghurt samples were estimated spectrophotometrically (Hull,1947). Textural characteristics; hardness, adhesion, and cohesiveness were measured using Texture Analyzer (Stable Micro Systems, Model TA. HD plus, fitted with 50 kg load cell). Experiments were carried out by compression tests that generated plot of force (N) versus time (s). A 25 mm perplex cylindrical probe was used to measure texture of yoghurt samples at a temperature of 5±0.5℃. The texture analyzer was calibrated in terms of compression height and the texture analyzer parameters were set such that the speed of the probe is 0.5 mm/s during the compression and 2 mm/s during pre-test and relaxation of the samples. The samples (80ml) were loaded to the analyzer ensuring that the coagulum is not disturbed and the tests were run. The typical graphs obtained were analyzed using Texture Expert Exceed Software provided with the instrument.

*Statistical analysis*

Repeated measures ANOVA was used for comparing the changes in the parameters between periods within each sample. For comparing changes between the samples in each period paired t-test was used. Data analyses were carried out using the Statistical Package for Social Sciences (SPSS, Version 24) and the results are presented as mean with standard error of six independent batch replication.

**Results and Discussion**

**Supplementary tables**

Supplementary Table S1: Design of study -Preliminary trials

|  |  |  |
| --- | --- | --- |
| **Product** | **Variable parameter and values** | **Constant parameters and values** |
| **Yoghurt from Vechur cow milk–treatment samples** | **Rate of inoculation**  1 per cent (T1)  2 per cent (T2)  3 per cent (T3)  4 per cent (T4) | **Incubation temperature -** 42°C  **Incubation period –** 4h |
| **Incubation temperature**  37℃ (T1) 42℃ (T2)  47°C (T3) | **Rate of inoculation -**2 per cent  **Incubation period**-4h |
| **Incubation period**  2h (T1) 2.5h (T2)  4h (T3) 5.5h (T4)  6h (T5) | **Rate of inoculation** -2 per cent  **Incubation temperature -** 42°C |
| **Yoghurt from cross-bred cow milk – Control sample (T5/T4/T6 depending on the variable parameter assessed)** | **Rate of inoculation-** 2 per cent  **Incubation period** -4 h  **Incubation temperature** - 42°C | |

**Supplementary Table S2. Details of RSM Preliminary trials:** *a. Minimum and maximum values, b. Coded and actual levels*

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 1. **Minimum and maximum values selected for optimization of** **production parameters of yoghurt** | | | | **b. Coded and actual levels of the three factors (design factors) used in Central Composite Design** | | | | | |
| **Parameters** | **Values determined based on preliminary trials** | | | **Coded**  **level**    **Factor** | **Lower limit** | **Factorial Point** | **Centre coordinate** | **Factorial Point** | **Upper limit** |
| **Optimum value** | **Minimum** | **Maximum** |  | - α | -1 | 0 | +1 | + α |
| Temperature ºC | 42 | 37 | 47 | A: Temperature °C | 33.59 | 37 | 42 | 47 | 50.41 |
| Inoculation (%) | 2 | 1 | 3 | B: Inoculation (%) | 0.32 | 1 | 2 | 3 | 3.68 |
| Incubation (h) | 4 | 2.5 | 5.5 | C: Incubation (h) | 1.48 | 2.5 | 4 | 5.5 | 6.52 |

**Supplementary Table S3. Second order equations of responses as a function of variables (**rate of inoculation (A), incubation temperature (B) and incubation period (C)**) and their linear and quadratic interactions**

|  |  |
| --- | --- |
| **Response** | **Equation** |
| Flavour (Y1) | Y1= -50.26201+ 2.27857\* A - 0.11176 \* B + 4.66586 \* C + 0.062500 \*AB- 0 .041667 \*AC–0.12500 \* B C - 0.026222 \* A2 - 0.47876 \* B2 - 0.29135 \* C2 |
| Body and texture (Y2) | Y2 = -69.67783+ 3.04944 \* A + 3.34211 \* B + 4.21566 \* C -2.62207E-015 \* AB – 0.016667 \*AC - 0.25000\* BC - 0.034867 \* A2 -0.51813 \* B2 - 0.32849 \* C2 |
| Colour and appearance(Y3) | Y3=-35.16259 + 1.53103\* A + 2.64287 \* B + 3.82941 \*C +0.012500 \* AB - 8.33333E-003 \*AC - 0.12500 \* BC - 0.017894\*A2 -0.62411 \* B2 - 0.37559 \* C2 |
| Overall acceptability (Y4) | Y4 = -39.32470+ 1.91525 \* A + 0.11688 \* B + 3.00138 \* C +0.056250 \* AB + 0.012500 \* AC - 0.020833\*BC - 0.024559 \*A2 - 0.52558\*B2 - 0.39073\* C2 |

**Supplementary Table S4. Properties of Vechur and cross-bred cow milk yoghurt stored at 30±1℃**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 1. **Physicochemical** | | | | |
| **Parameters** | **Days** | **VCMY** | **CCMY** | **t-value** |
| FAT (%) | Day 0 | 3.13±0.021 | 3.15±0.022 | 0.5422ns |
| Day 1 | 3.12±0.009 | 3.14±0.016 | 0.874 ns |
| t value | 0.468ns | 0.501ns |  |
| SNF (%) | Day 0 | 8.54±0.004 | 8.59±0.004 | 2.025ns |
| Day 1 | 8.54±0.016 | 8.55±0.010 | 0.83 ns |
| t value | 0.368ns | 1.08ns |  |
| Total solids  (%) | Day 0 | 11.67±0.023 | 11.74±0.020 | 2.187 ns |
| Day 1 | 11.68±0.011 | 11.72±0.026 | 2.089 ns |
| t value | 0.945ns | 0.657ns |  |
| Protein (%) | Day 0 | 4.35±0.071 | 3.91±0.030 | 5.543\*\* |
| Day 1 | 3.83 ± 0.050 | 3.62±0.050 | 0.003ns |
| t value | 3.581\*\* | 2.078\* |  |
| Acidity  (% lactic acid) | Day 0 | 0.86±0.021 | 0.85±0.011 | 1.265 ns |
| Day 1 | 1.27 ± 0.01 | 1.23 ± 0.004 | 8.07 \*\* |
| t value | 650.07\*\* | 363.98\*\* |  |
| Ph | Day 0 | 3.82±.004 | 3.67 ± 0.004 | 2.01ns |
| Day 1 | 3.07± 0.015 | 3.01 ± 0.05 | 2.391ns |
| t value | 21.67\*\* | 28.15\*\* |  |
| Syneresis (%) | Day 0 | 15.93±0.162 | 19.01±0.116 | 15.41\*\* |
| Day 1 | 25.75±0.152 | 26.50±0.063 | 4.550\*\* |
| t value | 64.128\*\* | 58.64\*\* |  |
| Tyrosine  (µg.5mL-1) | Day 0 | 0.240 ± 0.004 | 0.242 ± 0.007 | 1.03 ns |
| Day 1 | 0.25 ± 0.001 | 0.25 ± 0.009 | 1.81ns |
| t value | 19.38\*\* | 14.81\*\* |  |
| L\*- Lightness axis | Day 0 | 88.83±0.008 | 88.52±0.005 | 31.69\*\* |
| Day 1 | Spoiled | Spoiled | \_ |
| t value | \_ | \_ |  |
| a\*- Red-green axis | Day 0 | -3.88±0.004 | -3.61±0.003 | 46.67\*\* |
| Day 1 | Spoiled | Spoiled | \_ |
| t value | \_ | \_ |  |
| b\*- Blue-yellow axis | Day 0 | 11.48±0.003 | 12.41±0.003 | 212.45\*\* |
| Day 1 | Spoiled | Spoiled | \_ |
| t value | \_ | \_ |  |
| **B. Microbiological** | | | | |
| **Parameters** | **Days** | **VCMY** | **CCMY** | **t-value** |
| Total viable Count  (log10CFU/g) | Day 0 | 7.64±0.022 | 7.68±0.016 | 0.165ns |
| Day 1 | 7.87±0.041 | 7.77±0.060 | 1.336ns |
| t value | 9.11\* | 14.26\* |  |
| Coliform  count  (log10cfu/g) | Day 0 | Nil | Nil | - |
| Day 1 | Nil | Nil | - |
| t value | - | - |  |
| Yeast and Mould  (log10CFU/g) | Day 0 | 0.426±0.014 | 0.596±0.085 | 1.032ns |
| Day 1 | 1.19±0.03 | 0.94±0.11 | 2.22ns |
| t value | 11.5\* | 6.46\* |  |
| Lactic acid bacteria  (log10CFU/g) | Day 0 | 7.91±0.010 | 7.91±0.004 | 0.04 ns |
| Day 1 | 8.03±0.290 | 7.99±0.16 | 1.17ns |
| t value | 36.6\* | 28.4\* |  |
| Total viable Count  (log10CFU/g) | Day 0 | 7.64±0.022 | 7.68±0.016 | 0.165ns |
| Day 1 | 7.87±0.041 | 7.77±0.060 | 1.336ns |
| t value | 9.11\* | 14.26\* |  |
| Coliform count  (log10CFU/g) | Day 0 | Nil | Nil | - |
| Day 1 | Nil | Nil | - |
| t value | - | - |  |
| **C. Sensory** | | | | |
| **Parameters** | **Day** | **VCMY** | **CCMY** | **Z-value** |
| Flavour | Day 0 | 8.3 ± 0.03 | 8.0 ± 0.03 | 0.97ns |
| Day 1 | 6.6 ± 0.09 | 7.1 ± 0.03 | 3.04\* |
| Chi square value | 18.12\*\* | 5.6\* |  |
| Body and texture | Day 0 | 8.0 ± 0.03 | 7.8 ± 0.03 | 1.67ns |
| Day 1 | 6.7 ± 0.03 | 7.0 ± 0.03 | 2.12\* |
| Chi square value | 18.12\*\* | 15.6\*\* |  |
| Colour and appearance | Day 0 | 8.5 ± 0.05 | 8.0 ± 0.06 | 11.33\*\* |
| Day 1 | 7.9 ± 0.95 | 6.5 ± 0.87 | 21.23\*\* |
| Chi square value | 3.45ns | 14.5\*\* |  |
| Overall acceptability | Day 0 | 8.2 ± 0.07 | 7.9 ± 0.003 | 1.21ns |
| Day 1 | 7.0 ± 0.05 | 6.4 ± 0.03 | 0.08ns |
| Chi square value | 22.54\*\* | 20.71\*\* |  |

Values are mean ± standard error of six replications, ,\*-Significant at five per cent levels (p<0.05), \*\*-Significant at one per cent levels (p<0.01), ns-non-significant (p>0.05)

**Supplementary Table S5 Properties which did not show any significant differences in between Vechur and cross-bred cow milk yoghurt** **on storage at** **4±1℃**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **A.Physicochemical** | | | | |
| **Parameters**  **Parameters** | **Days**  **Days** | **VCMY**  **Milk** | **CCMY** | **Z-value** |
| Fat (%) | Day 0 | 3.13±0.021ax | 3.15±0.022ax | 0.5422ns |
| Day 5 | 3.11±0.010bx | 3.14±0.020 ax | 1.103 ns |
| Day 10 | 3.08±0.016bx | 3.11±0.021 ax | 1.240 ns |
| Day 15 | 3.08±0.010bx | 3.09±0.015 ax | 0.447 ns |
| SNF(%) | Day 0 | 8.54±0.004ax | 8.59±0.004ay | 4.25 \* |
| Day 5 | 8.55±0.016abx | 8.58±0.010ax | 0.83 ns |
| Day 10 | 8.54±0.009abx | 8.55±0.008bx | 0.644 ns |
| Day 15 | 8.52±0.008bx | 8.53±0.011bx | 0.940 ns |
| Total solids  (%) | Day 0 | 11.67±0.023ax | 11.74±0.020ax | 2.187 ns |
| Day 5 | 11.69±0.013 ax | 11.72±0.019 ax | 1.391 ns |
| Day 10 | 11.67±0.015 ax | 11.67±0.015 abx | 0.001 ns |
| Day 15 | 11.59±0.019 bx | 11.63±0.016bx | 1.274 ns |
| Protein  (%) | Day 0 | 4.35±0.071 ax | 3.91±0.030ay | 5.543\*\* |
| Day 5 | 3.85±0.038 bx | 3.56±0.026 bx | 0.554 ns |
| Day 10 | 3.41±0.040 cx | 3.28±0.040 cx | 0.941 ns |
| Day 15 | 3.11±0.021 dx | 3.15±0.027 dx | 0.01 ns |
| 1. **Microbiological** | | | | |
| **Parameters** | **Days** | **VCMY** | **CCMY** | **t-value** |
| Coliform count  (log10CFU/g) | Day 0 | Nil | Nil | - |
| Day 5 | Nil | Nil | - |
| Day 10 | Nil | Nil | - |
| Day 15 | Nil | Nil | - |
| 1. **Sensory** | | | | |
| **Parameters** | **Days** | **VCMY** | **CCMY** | **Z-value** |
| Overall acceptability | Day 0 | 8.2±0.10 ax | 7.9±0.08 ax | 1.607ns |
| Day 5 | 7.4±0.15 abx | 7.2±0.11 abx | 0.094 ns |
| Day 10 | 7.0±0.0 abx | 7.0±0.00 abx | 0.00 ns |
| Day 15 | 6.1±.023 bx | 6.5±0.12 bx | 1.875 ns |
| Chi square value | 17.586\* | 17.089\* |  |

Values are mean ± standard error of six replications,\*-Significant at five per cent levels (p<0.05), \*\*-Significant at one per cent levels (p<0.01), ns-non-significant (p>0.05), x-y -Mean with different superscripts vary significantly within a row, a-d -Means with different superscripts within the column are significantly different (p<0.05)

**Supplementary Figures**

Supplementary Fig.S1 Flowchart adopted for the standardization of procedure for preparation of yoghurt

Supplementary Fig.S2 Response surface plots for flavour score (I), body and texture (II), colour and appearance (III), overall acceptability (IV) of yogurt as a function of varied levels of inoculation and incubation temperature (A) incubation period and incubation temperature (B) levels of inoculation and incubation period(C)

Vechur cow milk

Filtration of milk

Standardization of milk to 3% fat and 8.5% SNF

Preheating (50-60℃)

Homogenization 2500psi and 500psi

Heat treatment (90°C/5 min)

Cooling to incubation temperature

Inoculation of starter culture (NCDC -260)

Incubation(42℃/4 h)

Storage at 4ᵒC

Supplementary Fig.S1

|  |
| --- |
| I a bc |
| II |
| III |
| IV |

Supplementary Fig.S2

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