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Development of Selective Enrichment Broth for Coliforms using Response Surface Methodology

Kunal M. Gawai, Jashbhai B. Prajapati[1], Govind P. Tagalpallewar[2]

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ABSTRACT

Background: Food Safety and Standard Authority of India has adopted conventional IS-5887 (Part-I) 1976 and IS-5401 Part-1 (2012) protocol for monitoring of *E. coli* and coliforms in dairy products respectively. These methods are time consuming, relying on bacteriological media and sometimes require further isolation and confirmation to finalize the true contaminant. The current investigation was carried out to develop such a broth media which will support growth of coliforms and inhibit other organisms associated within the micro-environment of coliforms and get entry in dairy products due to wrong processing practices and post production contaminant.

Methods: The present investigation involved formulation of selective broth for coliforms by optimizing the rate of addition of Sodium lauryl sulphate salt, Gentamicin sulphate+Amoxycillin (in 1:1 ratio) and Cefsulodin by Response Surface Methodology (RSM).

Result: Formulation of selective broth for coliforms and *E.coli* consisting of Sodium lauryl sulphate salt, Gentamicin sulphate+ Amoxycillin and Cefsulodin which was added at the rate of 0.25 g, 10 µl and 312.5 µl respectively per 100 ml of broth. This combination of ingredients along with base composition of broth were able to increase the growth of coliforms as well as able to inhibit population of *Salmonella typhi* ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923.

Key words: Coliforms, Dairy products analysis, *E.coli,* Media formulation, Selective broth.

INTRODUCTION

Food safety is a global health concern and has become interesting subject, eliciting a great deal of public concern all over the world (Velusamy *et al.* 2010). This is a result of emerging food borne pathogens that continue to cause outbreak of food borne diseases (Lefoka, 2009). Contamination of food and water causes thousands of deaths and each year millions sickens worldwide (Naratama and Santoso, 2020). Many microorganisms get access to milk and milk products, but among them recovery of *E. coli* is used as reliable indicator of faecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms. Most *E. coli* are harmless, but some are also known to be pathogenic, causing severe intestinal diseases (Karpac *et al*. 2008). *E. coli* is a part of coliform group and the entire group is indicator of general hygienic condition while manufacturing dairy/food products. *E. coli* infection is the most common in developing countries. In past decades, many outbreaks in Europe and Northern America have been attributed to a strain of *E. coli* which has been identified among the most common causes of diseases related to food safety (Lawaniya, 2014). Regulatory agencies at global level are in process of developing universal food safety standards through Codex Alimentarius Commission (CAC) and their suggestions are adopted by National Regulatory Agency in India.

Indian dairy industry is mostly following IS 5401: part-1 (2012) and ISO 4832 (2006) standard procedure for enumeration of coliforms. Main concern is with the interpretation of results as it is not mentioned which types of colonies have to be counted and which need not be. Gazette notification of Government of India in 2016, has specified limits of coliforms in dairy products like pasteurized butter, milk powders, ice cream cheese, fermented milk and traditional Indian dairy products. Along with this, FSSAI has mentioned to analyze coliform count by 5401 Part 1/ISO: 4832 and *E. coli* count by IS 5887: Part 1 or ISO: 16649-2. Conventional enrichment and isolation methods for detecting coliforms in foods are generally very reliable, but they are expensive, laborious and time consuming, requiring at least 3-4 days protocol for presumptive identification (Teramura *et al*. 2019). Alternative methods based on nucleic acid, fluorescent antibody or immunology based techniques needed additional equipments and expensive devices as well as enrichment steps for identification. Violet Red Bile

Department of Dairy Microbiology, SMC College of Dairy Science, Kamdhenu University, Anand-388 110, Gujarat, India.
[1]SMC College of Dairy Science, Anand Agricultural University, Anand-388 110, Gujarat, India.
[2]Department of Food Processing Technology, College of Food Processing Technology and Bio Energy, Anand Agricultural University, Anand-388 110, Gujarat, India.

Corresponding Author: Kunal M. Gawai, Department of Dairy Microbiology, SMC College of Dairy Science, Kamdhenu University, Anand-388 110, Gujarat, India.
Email: kunalgawai@kamdhenuuni.edu.in

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agar or MacConkey's agar are not truly selective and for confirmation needs further evaluation and identification. A selective enrichment broth with better promoting coliforms would be useful the other detection methods which can reduce the time and improve precision of the test.

Development of rapid methods which must be accurate, efficient and can give results in less time would be highly appreciated by the industry and researchers. Such developed enrichment media would favour the growth of coliforms largely and make subsequent detection easier. It is possible to formulate coliform enrichment media that will support the growth of almost all genera of coliforms with inhibition of salmonella and shigella. Such media would be helpful in precise identification and differentiation of coliforms without a confirmation step.

MATERIALS AND METHODS

The study under investigation was planned to develop a selective enrichment broth for coliforms group. It was conducted in Department of Dairy Microbiology, SMC College of Dairy Science, Kamdhenu University, Anand in the year 2020.

The cocktail of coliform culture was prepared by mixing contents from three positive tubes of MacConkey's broth from Most Probable Number (MPN) experiment conducted on milk sample. This culture was propagated in nutrient broth medium and incubated at 37°C for 24 h and then stored at 5±2°C. Sub culturing was done at an interval of 7 days during the course of the study.

Salmonella typhimurium ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were procured from Hi-media Laboratories, Mumbai and stored at 5±2°C. These cultures were maintained by routine sub-culturing in 20 ml test tubes containing 5 ml of sterilized recommended broth and on agar slant tubes (Table 1).

Selective enrichment broth was formulated by keeping in view that the coliforms are lactose fermenters and some essential nutrients are required for their growth. Selection of ingredients was based on promoting growth of coliform and inhibition of gram positive and non-lactose fermenters. Ingredients which are essential for growth of coliforms like bile salts (0.25 g), NaCl (0.25 g), Di-sodium phosphate (0.24 g), mono sodium phosphate (0.15 g), Yeast extract powder (0.3 g), Tergitol @ 0.01 g (AFNOR, 1990) and lactose (1 g) were kept constant at standard rate per 100 ml of the broth as a base ingredients for broth formulation (Manafi, 2003; Difco and BBL Manual, 2009).

Ingredients which were decided to optimize in the formulation were: Sodium lauryl sulphate salt (range 0.1 - 0.4 g/100 ml), Gentamicin sulphate + Amoxycillin (solution of each, 1 mg/ml in 1:1 proportion) range 5 µl -15 µl/100 ml and Cefsulodin (10 mg/10ml) range 125-500 µl/100 ml).

To optimize the best rate of addition of these ingredient Response Surface Methodology was used.

Response surface design (RSD) is one of the most advanced design used in providing statistical process control of various formulations. It gives freedom to the users to develop, improve and optimize the process constraints by controlling the required responses (Mahapatra *et al*. 2020). Screening of factors and sequential experimentation was an important task hence we used the central composite design. Optimization of ingredients was done by measuring optimized base ingredients and dissolving them in 100 ml of sterilized distilled water and heated to boiling for 5 min. Then allowed them to cool at 37°C and then pH was adjusted to 7.4±1 by addition of 0.1 N Hydrochloric acid solution.

Gentamycin sulphate: Amoxycillin (1 mg/ml of each in 1:1 proportion per 100 ml) and Cefsulodin was added just before distribution of broth in Eppendorf tubes. Target organism was spiked @100 cells/10 ml and response measured after incubation of 12 h at 37±1°C. Spiking protocol suggested by Gawai *et al*. (2017) was used. Optimization was done by measuring optical density at 590 nm wavelength for the growth of coliforms. Inhibition of spiked targeted organisms *viz*. *Salmonella typhimurium* ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 (spiked amount chosen was 100 cells/10 ml) was detected by pour plate method on respective agar after 12 h of incubation.

RESULTS AND DISCUSSION

MacConkey's broth, Nutrient broth and Violet Red bile broths are most commonly used for the growth of coliforms in the analysis of dairy and food products. With an aim to develop competitive broth, lactose was kept as main essential ingredient. On the basis of preliminary trials Bile salts, Sodium chloride, Di-sodium phosphate, Mono sodium

Table 1: Growth media and conditions for growth and maintenance of cultures.

Name of cultures	Gram test	Growth medium	Nature	Selective media used for purity and cell count	Optimum Temperature	Incubation time (h)
Salmonella typhimurium ATCC 14028	-ve	Brain heart Infusion broth	Aerobe	Xylose lysine deoxycholate agar	37°C	24
Enterococcus faecalis ATCC 29212	+ve	Nutrient broth	Aerobe	M-enterococcus agar	37°C	24
Staphylococcus aureus ATCC 25923	+ve	Brain heart Infusion broth	Aerobe	Baird parker Agar	37°C	24
Coliforms cocktails	-ve	Nutrient broth	Aerobe	Violet red bile agar	37°C	24

phosphate, Yeast extract powder lactose monohydrate and Tergitol were considered for base broth formulation.

Design Expert 10.0.1 used responses of the preliminary trials for formulation of broth with some range of parameters. These factors were Sodium lauryl sulphate salt (in the range 0.1-0.4 g/100 ml), Gentamicin sulphate + Amoxycillin (1 mg/ml of each in 1:1 proportion per 100 ml) ranged from 5 µl - 15 µl/100 ml and Cefsulodin (10 mg/10ml) ranged from 125-500 µl/100 ml). The software suggested 20 runs which are given in Table 2.

Influence of varying levels of ingredients on the growth of coliforms and targeted organisms

Sodium lauryl sulphate salt usually added as an inhibitors of gram positive organisms. Gentamycin has a wide spectrum activity due aminoglycoside group against *Pseudomonas aeruginosa*, *E. coli*, *Proteus spp*., *Klebsiella spp*., *Enterobacter spp*., *Serratia spp*., *Providencia spp*., *Acinetobacter spp*., *Citrobacter spp*., *Morganella spp*., *S. aureus, Staphylococcus spp*., *Viridansstertococci, Enterococcus spp*. and Mycobacterium *spp*. (www.antimicrobe.org). Gentamicin sulphate is effective against gram positive bacteria and gram negative bacteria hence added with Amoxycillin and allowed the design expert to decide at what rate it would be effective against targeted organism Ilomuanya *et al*. (2018). Amoxycillin is effective against many different bacteria including *H. influenzae*, *N. gonorrhoea*, *E. coli*, *Pneumococci*, *Streptococci* and certain strains of *Staphylococci* (www.medicinenet.com). Cefsulodin is useful selective agent against *Aeromonas spp*. and suggested to promote coliforms (Alonso *et al*. 1996). Slack *et al*. (1979) put forward combinations of Cefsulodin and Gentamicin to have additive or synergistic activity against a strains of *Pseudomonas aeruginosa*. Supporting to this finding, Price and Wildeboer (2016) suggested that false positive coliform results were due to the presence of *Aeromonas spp.* which could be eliminated by using Cefsulodin in the media formulation.

LMX broth first described by Manafi and Kneifel (1989) was modified later by Ossmer (1993) to improve substrate utilization, sensitivity and reduction of incubation time up to 24 h. In this broth added Sodium lauryl sulphate @ 0.1 g/liter was good in inhibition of gram positive microflora. MI agar method recommended by U.S. Environmental Protection Agency for determination of total coliforms and *Escherichia coli* uses Cefsulodin, an antibiotic, to inhibit non-targeted growth which likely to cause false positive reactions (oh.water.usgs.gov).

Brenner *et al.* (1993) on the same line of work developed a new membrane filter agar medium consisting cefsulodin in levels of 2, 3, 4, 5, 7, 10, 15 and 25 µg/ml. In that study, they found that addition of cefsulodin @ 5 ml of a freshly prepared 1 mg/ml filter sterilized solution greatly reduced the background counts of *Flavobactenium* and *Aeromonas* species. Geissler *et al.* (2000) compared the performance of Lauryl sulfate MUG X-gal (LMX) broth, Chromocult Coliform agar (CC) and Chromocult Coliform agar plus cefsulodin (10 mg/ml) (CC-CFS), with standard multiple tube

Table 2: Experimental design matrix and responses recorded for coliforms and other contaminants at different rates of Sodium lauryl sulphate salt, Gentamicin sulphate+Amoxycillin and Cefsulodin.

Run	A: Sodium lauryl sulphate salt (g/100 ml)	B: Gentamicin sulphate+Amoxycillin µl/100 ml)	C: Cefsulodin (µl/100 ml)	Coliforms growth (Optical Density)	*Salmonella typhi* ATCC 14028(cfu/ml)	*Enterococcus faecalis* ATCC 29212 (cfu/ml)	*Staphylococcus aureus* ATCC 25923 (cfu/ml)
1	0.40	15.00	500.00	0.90	30	280	70
2	0.25	10.00	312.50	1.80	4	35	22
3	0.10	05.00	500.00	1.30	10	160	51
4	0.10	10.00	312.50	1.70	7	56	38
5	0.10	15.00	125.00	0.80	15	120	32
6	0.25	05.00	312.50	1.60	8	50	25
7	0.25	10.00	125.00	1.40	12	90	29
8	0.40	15.00	125.00	0.80	17	130	47
9	0.10	05.00	125.00	0.90	26	210	44
10	0.25	10.00	312.50	2.15	5	22	14
11	0.10	15.00	500.00	1.00	9	180	49
12	0.40	05.00	500.00	0.70	20	300	40
13	0.25	10.00	312.50	1.90	3	28	17
14	0.25	15.00	312.50	1.60	10	45	25
15	0.25	10.00	312.50	2.10	2	30	23
16	0.40	05.00	125.00	0.80	17	150	38
17	0.40	10.00	312.50	1.50	15	36	40
18	0.25	10.00	500.00	1.50	15	250	30
19	0.25	10.00	312.50	2.15	3	38	16
20	0.25	10.00	312.50	2.20	2	35	17

fermentation (MTF), for the enumeration of total coliforms and *Escherichia coli* from marine recreational waters. Overall CC-CFS showed that total coliforms recovered as 2.63, 1.95 and 1.90 times in LMX, CC and MTF respectively indicting potential use of cefsulodin.

The quadratic model for evaluating a formulated broth; parameters under investigations like changes in the population of coliforms, inhibition of *Salmonella typhi* ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were obtained through successive regression analysis. The Model F, the coefficient of determination (R^2) and the adequate precision value (APV) for changes in coliforms, inhibition of *Salmonella typhi* ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 suggested a better fit of the quadratic model highlighting the suitability of it to navigate the design. Anova for regression analysis of these changes in given in Table 3.

Effect of varying levels of ingredients on the growth of coliforms

The values presented in Table 3 revealed that addition of Sodium lauryl sulphate salt had significant (P<0.05) positive effect on the growth of coliforms at linear level while interaction of sodium lauryl sulphate salt and Gentamicin sulphate+Amoxycillin (AB), sodium lauryl sulphate salt and Cefsulodin (AC) and Gentamicin sulphate+Amoxycillin and Cefsulodin (BC) had a non-significant effect on the inhibition of coliforms. At quadratic level all the ingredients showed highly significant (P<0.01) negative effects on the growth of coliforms.

The response surface plot generated multiple regression equation for Coliforms, *Salmonella typhi* ATCC 14028, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and corresponding graphs for these equations are shown in Fig 1 to 4.

Effect of varying levels of ingredients on the growth of *Salmonella typhi* ATCC 14028

It was observed that addition of Sodium lauryl sulphate salt (A) had highly significant (P<0.01) effect on the inhibition of *Salmonella typhi* ATCC 14028 at linear level and significant effect (P<0.05) at quadratic level (Table 3). The interactive effect of ingredients Sodium lauryl sulphate salt and Gentamicin sulphate+Amoxycillin (AB) and Sodium lauryl sulphate salt and Cefsulodin (AC) had a highly significant (P<0.01) effect while interaction of Gentamicin sulphate+ Amoxycillin and Cefsulodin (BC) had a significant (P<0.05) effect on the inhibition of *Salmonella typhi* ATCC 14028. The square of factors indicated that all the ingredients decreased growth of *Salmonella typhi* ATCC 14028. Sodium lauryl sulphate salt (A) had a significant effect (P<0.05) and Cefsulodin (C) had a highly significant effect (P<0.01) on the inhibition of *Salmonella typhi* ATCC 14028.

Effect of varying levels of ingredients on the growth of *Enterococcus faecalis* ATCC 29212

The values presented in Table 3 revealed that addition of ingredient Cefsulodin (C) had significant effect (P<0.01) on the inhibition of *Enterococcus faecalis* at linear level while other ingredients Sodium lauryl sulphate salt (A) and Gentamicin sulphate+Amoxycillin (B) had a non- significant effect. Interaction of Sodium lauryl sulphate salt and Cefsulodin (AC) had a highly significant (P<0.01) effect on the inhibition of *Enterococcus faecalis* ATCC 29212. The square of factor (quadratic) indicated the Cefsulodin (C) had highly significant effect (P<0.01).

Table 3: Partial coefficients of regression equations of suggested models for changes in coliforms and other contaminants at different rates of Sodium lauryl sulphate salt, Gentamicin sulphate + Amoxycillin and Cefsulodin.

Factor		Change in coliforms in terms of optical density	Change in *salmonella typhi* ATCC 14028 (cfu/ml)	Change in *Enterococcus faecalis* ATCC 29212 (cfu/ml)	Change in *Staphylococcus aureus* ATCC 25923 (cfu/ml)
Linear	A	-0.10#	3.20*	17.00	2.10
	B	-0.020	0.000	-11.50	2.50
	C	0.070	-0.30	47.00*	5.00*
Interactive	AB	0.075	2.75*	3.75	6.63*
	AC	-0.075	4.75*	36.25*	0.13
	BC	0.000	2.50#	13.75	3.87#
Quadratic	A^2	-0.31*	4.50#	11.18	16.91*
	B^2	-0.31*	2.50	12.68	2.91
	C^2	-0.46*	7.00*	135.18*	7.41#
R^2		0.9666	0.9532	0.9590	0.9498
Model F value		31.13	22.61	26.00	21.04
Intercept		1.98	4.50	32.73	19.74
APV		13.970	16.119	15.072	15.079
Model		Quadratic	Quadratic	Quadratic	Quadratic

*P<0.01; #0.05; APV= Adequate precision value; R^2= Coefficient of determination A: Sodium lauryl sulphate salt, B: Gentamicin sulphate + Amoxycillin and C: Cefsulodin.

Effect of varying levels of ingredients on the growth of *Staphylococcus aureus* ATCC 25923

The addition of Cefsulodin (C) had a highly significant (P<0.01) effect on the inhibition of *Staphylococcus aureus* ATCC 25923 at linear level; however Sodium lauryl sulphate salt (A) and Gentamicin sulphate+Amoxycillin (B) had a non-significant effect. Again as shown in Table 3, the interactive effect of Sodium lauryl sulphate salt and Gentamicin sulphate+Amoxycillin (AB) had a highly significant positive effect at P<0.01 while interactive effect of Gentamicin sulphate+Amoxycillin and Cefsulodin (BC) had a significant positive effect (P<0.05) on the inhibition of *Staphylococcus aureus* ATCC 25923. The square of factor (quadratic) model indicated the Sodium lauryl sulphate salt (A) had highly significant effect (P<0.01) while Cefsulodin (C) had a significant effect (P<0.05) on the inhibition of *Staphylococcus aureus* ATCC 25923.

Optimization of selected ingredients in the developed selective broth

RSM suggested that Sodium lauryl sulphate salt @ 0.2226 µl/100 ml, Gentamicin sulphate+Amoxycillin (1:1 ratio) @ 10.1344 µl/ 100 ml and Cefsulodin @ 301.951 µl/100 ml as the most suitable solution with desirability of 0.92%. However, during actual trials, it was observed that Sodium lauryl sulphate salt @ 0.2, Gentamicin sulphate + Amoxycillin (1:1 ratio) @ 10 µl and Cefsulodin @ 312.5 µl per 100 ml were found best for the maximum coliforms growth response and best in the inhibition of *Salmonella typhi* ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923. The process was replicated seven

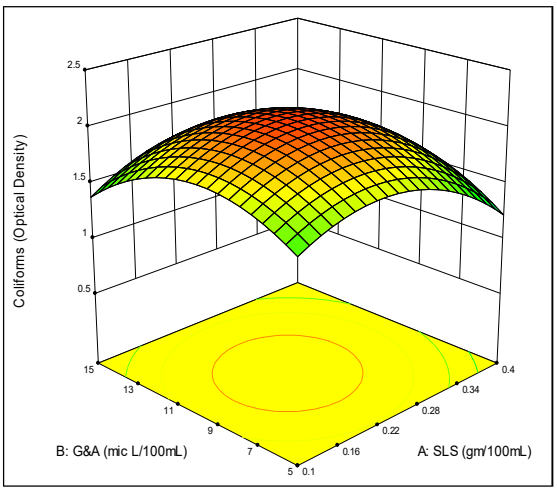

Fig 1: Response surface of changes in coliforms in terms of optical density at different rates of Sodium lauryl sulphate salt, Gentamicin sulphate+Amoxycillin and Cefsulodin.

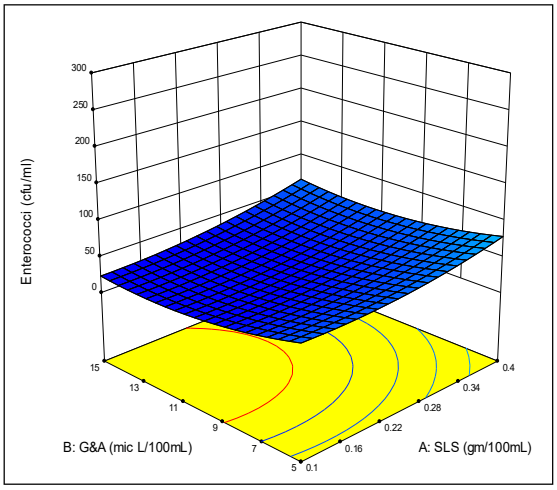

Fig 3: Response surface of changes in *Enterococcus faecalis* ATCC 29212 (cfu/ml) at different rates of Sodium lauryl sulphate salt, Gentamicin sulphate+Amoxycillin and Cefsulodin

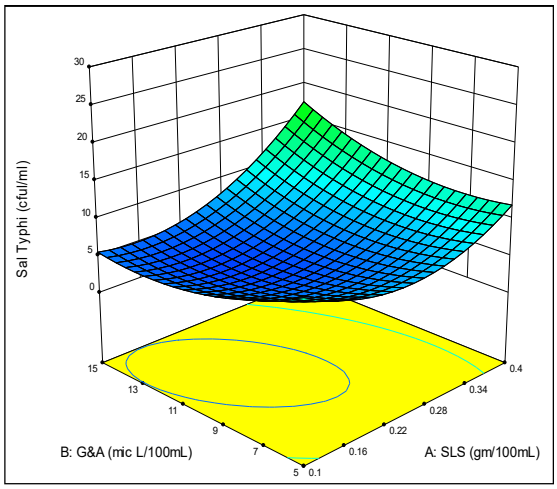

Fig 2: Response surface of changes in *Salmonella typhi* ATCC 14028 (cfu/ml) at different rates of Sodium lauryl sulphate salt, Gentamicin sulphate+Amoxycillin and Cefsulodin.

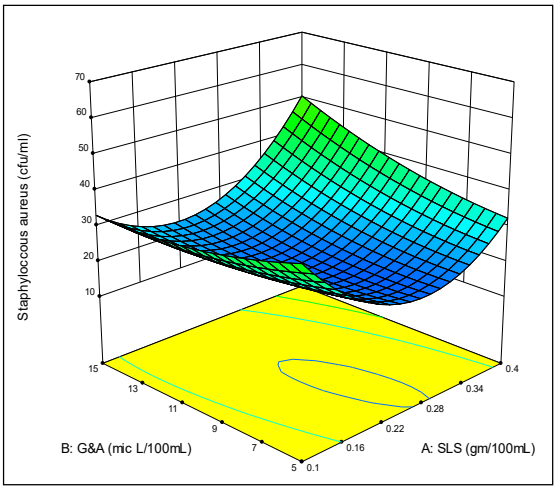

Fig 4: Response surface of changes in *Staphylococcus aureus* ATCC 25923 (cfu/ml) count at different rates of Sodium lauryl sulphate salt, Gentamicin sulphate+Amoxycillin and Cefsulodin.

Table 4: Comparison of predicted v/s actual values of responses used for ingredient optimization.

Responses	P-value	Predicted Value	Actual value	Calculated t -value	Level of significance
Coliforms growth measured by Optical density	0.46	1.98	2.01	0.80	NS
Salmonella typhi ATCC 14028 cfu/ml	0.01	4.14	3.16	2.02	NS
Enterococcus faecalis ATCC 29212 (cfu/ml)	0.20	27.89	31.33	1.47	NS
Staphylococcus aureus ATCC 25923 (cfu/ml)	0.34	19.69	18.17	1.05	NS

Predicted values of Design Expert 10.0.1 package @Actual values (average of seven trials) of the optimized product t-values at 5% level of significance NS = Non Significant Tabulated t-value = 2.57.

Table 5: Optimized final formulation for preparation of selective broth for Coliforms.

Ingredients	Quantity per 100 ml
Bile salt	0.25 g
Sodium chloride	0.25 g
Di-sodium phosphate	0.24 g
Mono sodium phosphate	0.15 g
Tergitol	0.01 g
Yeast extract	0.30 g
Lactose	1.00 g
Sodium lauryl sulphate salt	0.25 g
Cefsulodin 10 mg/1 vial	312.5 µl
Gentamicin sulphate+Amoxycillin (1:1 ratio)	10 µl (5+5 µl)
pH adjustment	7.4 (adjusted with 0.1 N HCl)

Fig 5: Photographs of formulated selective Coliforms broth.

times. The selected factors and the average values of the results were derived. The values of the selected constraints shown in Table 4 were compared statistically using paired t-test with that of the predicted values. Level of importance 3 for all three constraints indicated that addition of each ingredient is moderately important in broth formulation. The calculated values of all these selected constraints suggest that the calculated values of 't' for all the constraints were less than the table values, thus it was inferred that there was no significant (P>0.05) difference between the predicted and actual values of responses.

It was therefore confirmed that the selected combination of the factors was the best in terms of the responses delineated at the study. Final optimized formulation of the coliforms broth is provided in Table 5 and photograph of the formulated broth is shown in Fig 5.

CONCLUSION

A selective broth for coliforms growth was developed by optimizing three factors with some base ingredients commonly used in commercial formulation were kept at fixed level. It was observed that addition of Sodium lauryl sulphate salt @ 0.2 g, Gentamicin sulphate+Amoxycillin (1:1 ratio) @ 10 µl and Cefsulodin @ 312.5 µl per 100 ml, with desirability of 0.92 showed strong inhibition of targeted organisms like *Salmonella typhi* ATCC 14028, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 while promoted the growth of coliforms. It is further needed to evaluate this formulated broth with commercially available broths to know better the exact inhibition of targeted organism while growth support to coliforms and that will truly justify the findings of this research.

Conflict of interest: None.

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