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RESEARCH ARTICLE

Development of lateral flow enzyme substrate assay strip for qualitative estimation of coliforms

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Abstract: FSSA 2011 has adopted a 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 and sometimes requires further isolation and confirmation to finalize the true contaminant. The current investigation was carried out to minimize these limitations in detection of coliforms and *Escherichia coli* and to give a real time test to the industry that meets legal limits on day to day basis. The present investigation comprised of development of protocol for qualitative detection of coliforms in spiked samples using sonication technique and enzyme substrate assay. A protocol developed for sample preparation by extracting β-galactosidase (β-gal) using ultra sonicator (amplitude 80% and time 15 minutes) to give a positive ONPG test results within 20 minutes. After finalizing the sample preparation protocol, specific amount of the extract was used to check the presence of coliforms by using X-gal impregnated absorbent strip. Sensitivity of X-gal test was very good as it could detected minimum 10 cells/10 ml of spiked coliforms sample. Spiked cells (10 cells/10 ml of broth) need maximum 10 hours of incubation time and 30 mins for sample preparation and processing (sonication and centrifugation) before testing with ONPG and X-gal assay. It turned white strip in to blue colour indicating qualitative presence of coliforms within 20 minutes at 37 ºC. This developed protocol could be able to reduce the actual detection time from 24 hrs by conventional Indian Standards method to nearly 11 hours with accuracy of 1 cells/ml.

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Introduction

There are few limitations in selection of commercially available bacteriological media in detection and enumeration of coliform mainly for testing water and food products. Hence, there is a scope for the easier technique which would help to recover the maximum genera of coliforms group and inhibit rest all *i.e.* lactose non-fermenters including Salmonella and Shigella (Gawai, 2022a, b). The biochemical tests used for bacterial identification and enumeration in classical cultural methods are generally based on metabolic reactions (Tavakoli et al. 2008). For this reason, these are not completely specific and requires many additional tests to obtain precise confirmation. The use of microbial enzyme profiles to detect indicator bacteria is an attractive alternative to classical methods. Enzymatic reactions can be group-, genus- or species-specific, depending on the enzyme targeted. Moreover, reactions are rapid and sensitive. Thus, the possibility of detecting and enumerating coliforms through specific enzymatic activities has been under investigations since long time.

Enzyme based methods (*e.g.* chromogenic and fluorogenic media) have been developed and certified. These methods have rendered rapid and much easier measurement of *E.coli* and coliforms than the methods approved in the past, hence these are attracting greater interest from researchers and industries (Kanangire, 2013). These methods concurrently detect the total coliforms and *E.coli* which increasingly make possible the quantification of *E.coli*, rather than simply 'thermotolerant coliforms' (Siegrist, 2020). In the past decade, diverse methods using chromogenic and/or fluorogenic substrates to reveal β-d-glucuronidase and β-d-galactosidase activity on culture media have been reported to determine whether a strain belongs to the coliform group and/or *E. coli* (Siegrist, 2020;Gawai, 2022c). Various commercial media containing chromogenic substrates for the identification and enumeration of *E. coli* and Total Coliforms (TC) are available. Among them, Chromo-agars (Alonso et al. 1999) and Chromocults (Geissler et al. 2000) are able to give results in less than 24 hrs.

Some reviews on the use of fluorogenic and chromogenic substrates for bacterial qualitative detection are available (Chro¨st, 1991). These indicates that the use of these substrates has led to improve accuracy and faster detection. Methods for detection or enumeration may be performed in a single medium, thus bypassing the need for a time consuming isolation procedure prior to identification. Regarding enzyme substrate assays, ISO compiled and recommended a set of such test methods, including β-D-glucuronidase activity for *E. coli* detection (Anon, 2016).

Way back in 1988, Edberg and Edberg proposed a combined substrate technology where in ONPG used as a substrate for the constitutive enzyme β-galactosidase present in all coliforms and the substrate MUGlu was used for the specific detection of *E. coli*. The defined substrate method was basically constituted as a presence or absence test. The tubes, which are colourless after samples addition, are incubated at 35 °C. Development of yellow colour in the test tube (indicating the hydrolysis of ONPG) was taken as a positive for TC. Yellow tube is then exposed to long wave UV light, and blue white fluorescence demonstrates the presence of *E. coli*. No additional confirmatory test were needed (Anon, 2020a).

Chromogens and fluorogens substrates produce color and fluorescence respectively and compounds such as o-nitrophenyl-3-D-galactopyranoside (ONPG), p-nitrophenyl-3-D-galactopyranoside (PNPG), and 4-methylumbelliferyl-p-D-galactopyranoside (MUGal) have been included in a variety of media to demonstrate the presence of β-galactosidase, an enzyme produced by coliforms and 4-methylumbelliferyl-I3-D-glucuronide (MUG) as a substrate to detect the presence of *E. coli* in milk and dairy products (Brenner et al. 1993).

Several commercially available liquid presence-absence or most-probable-number media Colilert [Environetics, Inc., Branford, Conn.], Colisure [Millipore Corp., Bedford, Mass.] and ColiQuick [Hach, Loveland, CO, USA] have been developed to detect TC and *E. coli* in water samples within 24 to 28 h. Among them Colilert, Modified Colitag and Readycult test are few who have been approved for drinking water analysis (Rompre et al. 2002, Anon, 2020).

The objectives of the present study were to develop such a method which will provide results of coliform test in just 12 hrs by using enzyme substrate interactions. Along with this, it was attempted to develop an interpretation chart for quantitative detection of coliforms which would be helpful in decision making when counts laid in a specific range.

Materials and methods

The study was planned to develop a lateral flow enzyme substrate assay strip for qualitative estimation of coliforms. The work was conducted in the Department of Dairy Microbiology, SMC College of Dairy Science, Kamdhenu University, Anand. It was planned to use formulated selective broth to test the performance of coliforms detection strip from spiked samples.

Formulated selective coliforms broth was developed with addition of Sodium lauryl sulphate salt @ 0.2g, Gentamicin sulphate + Amoxycillin (1:1 ratio) @ 10 µl and Cefsulodin @ 312.5 µl per 100 ml which exhibited strong inhibition of targeted organism like *Salmonella typhi* ATCC 14028 while promoted the growth of coliforms cocktail and *Escherichia coli* ATCC 25922 (Gawai, 2022b). The composition of the broth is given in Table 1. This formulated selective broth was used to inoculate spike coliforms and later its presence was detected using developed enzyme substrate assay. The cocktail of coliform culture was prepared by mixing equal contents from three positive tubes of MacConkey's broth. 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.

Protocol standardization for a sample processing

Sample processing protocol was standardized after slight modification in the method described by Prasad et al. (2013) and Makwana et al. (2019). Sample prepared with addition of fixed cells of coliforms as per the spiking protocol in the formulated coliforms broth (Gawai et al. 2017). B-galactosidase and other enzymes present in coliforms are intracellular type, hence to extract these ultra-sonicator was used. Amplitude and time for

Table 1: 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)

ultra sonication were standardized using a statistical program software Response Surface methodology.

Protocol for crude enzyme extraction

Coliforms cells were added by spiking in 9 ml formulated selective broth and incubated at 37 °C up to 10 h. After incubation, test tube was removed and mixed carefully. The cells were harvested at 5000 rpm for 10 min at 4°C in refrigerated centrifuge. The supernatant discarded as it contains extracellular enzymes and the remained cell pellet was mixed with 5 ml of 0.05 M sodium phosphate buffer (pH 6.8). The cell pallets suspension was mixed thoroughly with vortex for 2-3 min. Further to extract intracellular enzyme, cell disintegration method *i.e.* sonication treatment was used. The cell suspensions were sonicated for 15 minutes (pulse 10 seconds off/15 seconds on and 75 % amplitudes) in ice bath using ultra sonicator (LABMAN, India). After sonication, the extract was centrifuged at 5000 rpm at 4°C for 10 min and obtained the supernatant containing the crude enzymes. This was further used to check the presence of coliforms using ONPG test. The flow chart for crude enzyme extraction for coliform testing using X-gal strip or ONPG assay is given in fig 1.

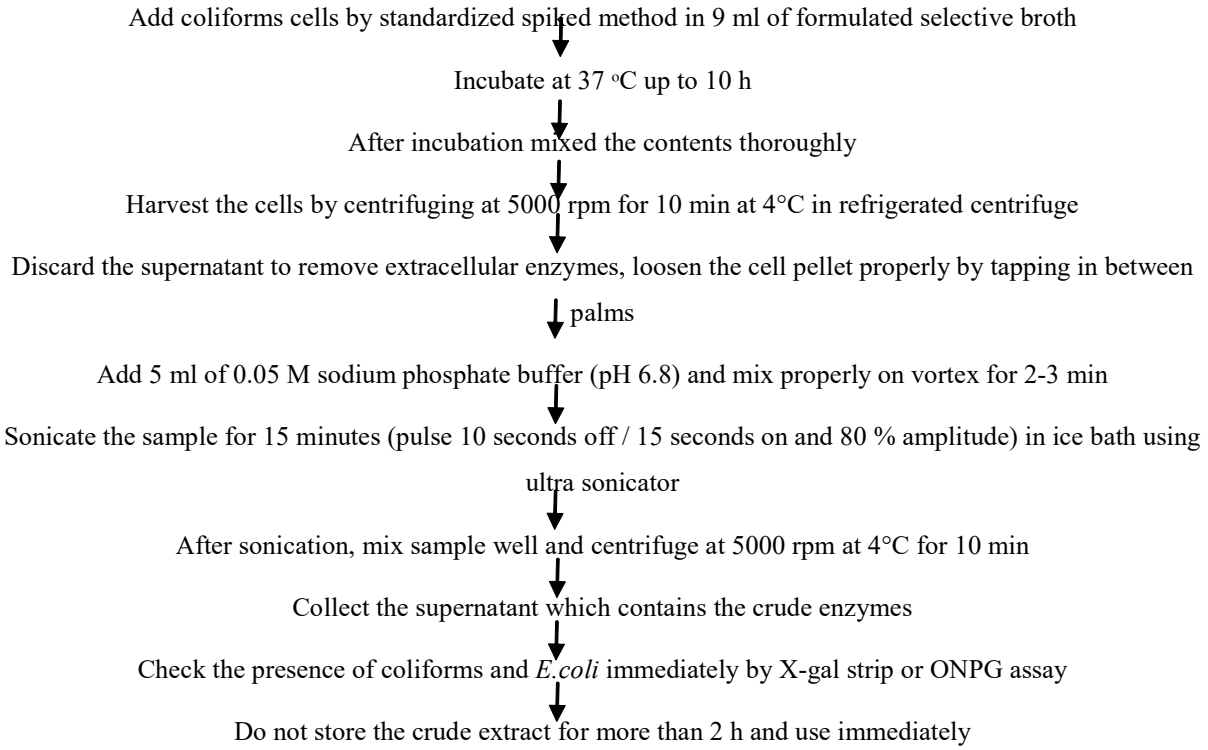

Fig. 1 Flow chart for crude enzyme extraction for coliform testing using X-gal strip or ONPG assay

Table 2: Experimental design matrix (CCRD) for levels of factors: Amplitude and time of sonicator and the results of ONPG test responses

Run	Standard	A: Amplitude of Sonicator	B: Time of Sonication	Response of ONPG test in min
1	7	80.00	07.99	140
2	8	80.00	22.07	55
3	9	80.00	15.00	16
4	4	95.00	20.00	75
5	6	101.21	15.00	110
6	13	80.00	15.00	28
7	10	80.00	15.00	20
8	3	65.00	20.00	60
9	1	65.00	10.00	80
10	2	95.00	10.00	60
11	5	58.78	15.00	40
12	11	80.00	15.00	22
13	12	80.00	15.00	30

Preparation of enzyme substrate assay strip test

A strip was used to make interaction of enzymes extracted from the sample and impregnated dried substrate. For preparation of a strip, an absorbent pad (Axiva Chemicals Limited, New Delhi) was used. It was cut in size of H"8 cm x .8 cm. Precautions were taken to avoid any contamination of strip from sweat and other chemicals which may interfere with the results. On the strip, X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (100 mg/ 4 ml Dimethyl sulfoxide) solution was added @ 20 ul using 2.5 ml medical grade syringe and allowed it to dry for 4 h. These strips were stored in cool and dry place till onset of experiment. A properly dried dip strip aseptically added in sterilized empty test tube. To this, 1000 µl of prepared crude extract was added and incubated the test tube at 37°C for 15 min in an incubator and observed for change in the colour of a strip from white to blue.

Results and Discussion

Optimization protocol for the use of ultra sonicator

Endogenous enzyme extraction was carried out in formulated coliform broth medium using Sonicator (LABMAN, India). Prasad et al. (2013) developed method for extraction and characterization of β-galactosidase produced by *Bifidobacterium animalis spp. lactis* Bb12 and *Lactobacillus delbrueckii spp. bulgaricus* ATCC 11842 grown in whey using 15 minutes (pulse 10 seconds off / 15 seconds on and 75 % amplitudes) in ice bath using ultra Sonicator (LABMAN, India). For optimization of best suitable condition for extracting β-galactosidase enzyme from coliforms an advanced statistical software programme named Design Expert 10.0.1 was employed. Here, amplitudes of sonicator were selected from the range of 65 to 95, time from 10 to 20 min. Among the various combination of amplitude and time, it was considered the best wherein ONPG test took the minimum time to indicate a positive result.

Influence of varying levels of amplitude and time of sonicator on early positive ONPG test

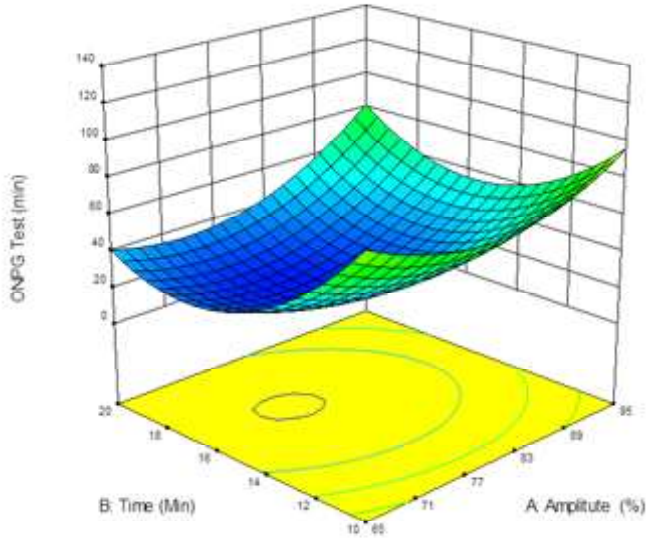

Fig. 2 Response surface for changes in amplitude of sonicator with different time intervals

In all the runs of the experiments suggested by RSM software, spiking of cells of coliforms were kept constant *i.e.* 10 cells per 10 ml of formulated selective broth. The data of the time require to get positive ONPG test is given in Table 2.

Time require to get positive result of ONPG test was observed in the range of 16 to 140 min for all the runs. The coefficient of determination (R^2) is the proportion of variability in the data explained or accounted for the model and high value (0.7751) of R^2 (Table 3) indicated a better fit for the model to the data. Also the adequate precision value (APV) of 5.572 recommended the use of this response to navigate the design.

The values presented revealed that time of sonication (A) and amplitude of sonicator (B) had non-significant effect on the test result of ONPG at linear level. Interaction effect of time of sonication and amplitude of sonicator (AB) also had a non-significant effect on the test result of ONPG.

Table 3: Partial coefficients of regression equations of suggested models for early positive result of ONPG at different amplitudes and time intervals

Factor		ONPG test Results (Times in min)
Linear	A	11.75
	B	-15.65
Interactive	AB	+8.75
Quadratic	A^2	21.53#
	B^2	32.77*
R^2		0.7751
Model F value		4.83
Intercept		23.20
APV		5.572
Model		Quadratic

*P < 0.01; #P<0.05; APV= Adequate Precision Value; R^2= Coefficient of determination
A:amplitude of sonication and B: to time chose for sonication process

The square of factor (quadratic) of sonication amplitude (A) had a significant effect ($P<0.05$) while time of sonication (B) had a highly significant effect ($P<0.01$) effect on the response of ONPG test. Multiple regression equation generated to predict the result of ONPG test as affected by amplitude and time of sonication is given in fig 2.

Sonication is one of the most widely used methods for disruption of the bacterial cell walls (Engler, 1985). Among the three methods tested; sonication, bead milling and high-pressure homogenizer, sonication was found to be more effective for releasing β-gal (Sakakibara et al. 1994). Berger et al. (1995) compared two physical disruption methods for the extraction of intracellular β-gal enzyme from Thermus species and found that the sonication was superior to the glass-bead milling.

Many workers have suggested different protocol for sonication of coliforms depending on different work like extraction of β-galactosidase, certain type of protein *etc*. Jing (2016) demonstrated that 60 min of sonication duration with 80% amplitude released the highest concentration of NS1 protein from the *E. coli* cells.

Cruz-Cansino et al. 2016 investigated the effectiveness of ultrasound for the inactivation of *Escherichia coli* inoculated into cactus pear juices (green and purple). The kinetics of *E. coli* in cactus pear juices treated by ultrasound (60%, 70%, 80% and 90% amplitude levels for 1, 3 and 5 min) were evaluated over 5 days. They reported ultrasound treatment at 90% amplitude for 5min resulted in non-detectable levels of *E. coli* in cactus pear juice for 2 days.

Optimization of varying level of amplitude of sonicator and time to get positive ONPG Test

In the process of optimization of levels of amplitude and time, RSM suggested the best solution with 75.03% for amplitude and 16.41 min for time with desirability of 0.975. However, in case of actual trials, it was observed that amplitude of sonication @ 80, time for sonication @ 15 min was found the best for the positive ONPG test response. The process was replicated seven times. The selected factors and the average values of the results were derived. The values of the selected constraints/responses were compared statistically using paired t-test with that of the predicted values as shown in Table 4. 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 non-significant ($P>0.05$) difference between the predicted and actual values of responses. Thus it was confirmed that the selected combination of the factors (amplitude of sonication @ 80 %, time for sonication @ 15 min) was the best in terms of the responses delineated at the study.

Testing of samples using enzyme substrate assay based strip for coliforms detection

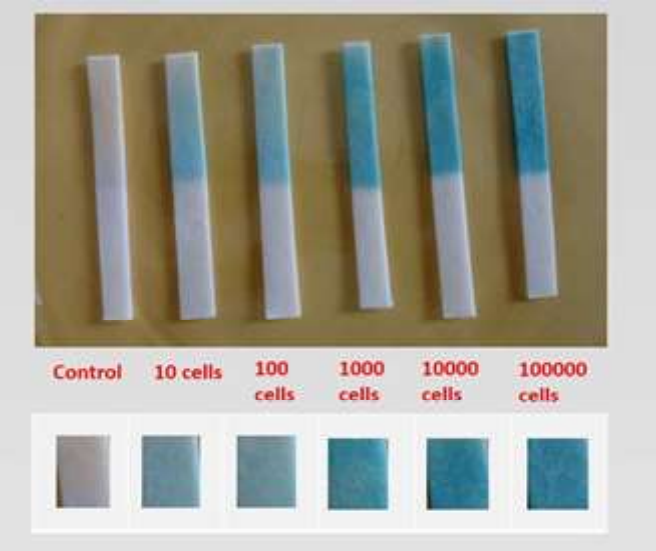

Fig.3 Interpretation chart of the results accessed by X-gal strip method

Spiked samples of coliforms @ 10, 100, 1000, 10000 and 100000 cells were tested with developed strips impregnated and dried with X-gal substrate as per the protocol described earlier. The strip without addition of the cells of coliforms acted as a control and did not develop any change in its colour and remained white. Rest of the strips showed incremental development of blue colour. As numbers of coliforms cells spiked were increased, the intensity of colour also increased. Interpretation chart for use of this developed strip to quantify the population of coliforms in the sample is given in fig 3.

In food hygiene, coliforms acts as index indicator organisms and it is important basis for the assessment of good manufacturing practice. X-gal (5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside), a soluble colorless compound consisting of galactose linked to a substituted indole reacts with β-galactosidase extracted from coliforms and is the best standard for this interaction (Hahn and Wittrock, 1991). There is high specificity for the galactose part of β-galactose substrates but low specificity for its organic moiety. Thus, an insoluble intensely blue product is produced as hydrolyzed X-gal product, releasing the substituted indole that spontaneously dimerizes. In chromogenic medium containing X-gal, colonies of *E. coli* that have an active β-galactosidase become blue because of this reaction (Sedzro et al. 2018, Pala et al. 2020).

Kilian and Bulow (1976) surveyed the *Enterobacteriaceae* and reported that glucuronidase activity was mostly limited to *E. coli*. The prevalence of this enzyme and its utility in the detection of *E. coli* in water were later reviewed by Hartman (1989). β-D-glucuronidase-positive reactions were observed in 94-96% of the *E. coli* isolates tested (Kilian and Bulow, 1976), while Chang et al. (1989) found a higher proportion of β-D-glucuronidase-negative *E. coli* (a median of 15% from *E. coli* isolated from

Table 4: Comparison of predicted v/s actual values of responses used for process optimization of varying level of amplitude and time of sonicator suggested to get positive ONPG Test

Responses	P-value	Predicted Value	Actual value	Calculated t value	Level of significance
ONPG test	0.18	19.04	23.2	1.60	NS

Predicted values of Design Expert 8.0.3 package
@Actual values (average of seven trials) of the optimized process protocol
t-values at 5 % level of significance
NS = Non Significant
Tabulated t-value = 2.776

human fecal samples). In contrast, β-D-glucuronidase activity is less common in other *Enterobacteriaceae* genus, such as Shigella (44 to 58%), Salmonella (20 to 29%) and Yersinia strains and in Flavobacteria (Kilian and Bulow, 1976). β-D-galactosidase, catalyzes the breakdown of lactose into galactose and glucose and has been used mostly for enumerating the coliform group within the *Enterobacteriaceae* family.

In the line of the present study Gunda et al. (2017) developed strip test device to detect presence of coliforms from water sample. In their study, the reaction zone was formed below the hydrophobic barrier by depositing the 100 µl of custom formulated chemical composition (Red-Gal, B-PER and LTB) using pipette and the resulting paper strips were completely dried for one hour under a fume hood before dipping into test sample. They reported that a test with dip time of 2 min, it was able to detect as low as 200 cfu/ml in 180±20 min and higher concentrations such as 2×10^5cfu/ml within 75±12 min. However, for a dip time test of 90 min, the developed DipTest device was able to detect as low as 200 cfu/ml in 54±8 min and higher concentrations such as 2×10^5cfu/ml within 28±5 min.

Gunda et al. (2016) used a novel hydrogel based porous matrix to encapsulate the optimized chemical compounds and incorporated it within a readily available plunger-tube assembly. This overall system allows efficient, field deployable, rapid testing of water samples by simultaneously pre-concentrating and detecting *E. coli* within one integrated unit. They were able to detect *E. coli* concentrations of 4×10^6 cfu/ml to 4×10^5 cfu/ml within 5 min and 4×10^4 cfu/ml to 400 cfu/ml within 60 min using the integrated plunger-tube assembly containing the hydrogel matrix.

Rapid response time and simplicity of use are important for point of use systems. To achieve rapid response times and simple 'dip and use' utility, the enzyme and a colorimetric substrate were not allow contact on strip to prevent mixing before immersion. The close proximity of enzyme and substrate provides a rapid sensing platform. To create the colorimetric response needed for visual detection of bacteria, can adopt a sensing construct that uses β-galactosidase and some specific substrate complimentary to it.

Dasgupta et al. (2016) developed a paper strip to detect the presence of *E.coli* made of a Grade GB003, Whatman absorbing gel blotting paper with one edge of the strip coated with wax hydrophobic barrier and the opposite edge (attraction zone) coated with D-glucose (dextrose) solution. Under the hydrophobic barrier it contains Red-Gal substrate (6-Chloro-3-indolyl-β-D-galactoside), bacterial enzyme (protein) extracting reagent (B-PER) and nutrient medium Lauryl Tryptose Broth (LTB).

When compared the present work with the work in the same line, this developed method is rapid and could gave results in less time. This could be better alternative to industry people to release a lot of products in approximately 50 % less time in comparison of the results obtained by conventional methods. This method needs lesser capital investment and operating cost wise also affordable.

Conclusion

In the present investigation, the developed formulated broth was used to cultivate coliforms culture and a protocol was standardized for testing presence of coliforms in the spiked broth. It was observed that ultra-sonication technique was the best to extract the intra-cellular enzymes. This method takes approximately 12 hrs to get the results and there is not any need to get further confirmation of results. Use of this technique can provide help to researchers and Indian Dairy Industry in saving time, space and affordable alternative to conventional method. However, for better precision and authenticity, it needs testing of large number of samples. This developed protocol needs evaluations and confirmation with raw and pasteurized milk samples.

References

Alonso JL, Soriano A, Carbajo O, Amoros I, Garelick H (1999) Comparison and recovery of *E. coli* and thermotolerant coliforms in water with a chromogenic medium incubated at 41 and 44.5 °C. Appl Environ Microbiol 65:3746-3749

Anon (2016) New standard to detect coliforms. Microbiol Focus 8.3:1-12

Anon (2020) Guild lines for Canadian Drinking water quality. Guild line Technical Document: Total coliforms. Published by Water and Air Quality Bureau, Healthy Environments and Health Canada (2020). On line Access: https://www.canada.ca/en/health-canada/services/publications/healthy-living/guidelines-canadian-drinking-water-quality-guideline-technical-document-total-coliforms.html

Anon (2020a). BAM Chapter 4: Enumeration of *Escherichia coli* and the Coliform Bacteria. Authors: Feng P, Weagant SD, Grant MA, Burkhardt W. Bacteriological Analytical Manual, 8th Edition, Revision A, (1998). Content updated: Sept 2020. Online access: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-4-enumeration-escherichia-coli-and-coliform-bacteria

Berger JL, Lee BH, Lacroix C (1995). Identification of new enzyme activities of several strains of Thermus species. Appl Microbiol Biotechnol 44(1-2):81-87

Brenner KP, Rankin CC, Roybal YR, Stelma Jr. GN, Scarpino PV, Dufour AP (1993). New medium for the simultaneous detection of total coliforms and *Escherichia coli* in water. Appl Environ Microbiol 59(11):3534-354

Chang GW, Brill J, Lum R (1989). Proportion of β-D-glucuronidase-negative *Escherichia coli* in human faecal samples. Appl Environ Microbiol 55:335-339

Chro¨st RJ (1991) Microbial enzymes in aquatic environments. Springer-Verlag, New York, pp.317

Cruz-Cansino NDS, Reyes-Hernández I, Delgado-Olivares L, Jaramillo-Bustos DP, Ariza-Ortega JA, Ramírez-Moreno E (2016) Effect of ultrasound on survival and growth of *Escherichia coli* in cactus pear juice during storage. Brazilian J Microbiol 47:431-437

Dasgupta S, Gunda NSK, Mitra SK (2016) Fishing, trapping and killing of *Escherichia coli* in potable water. Environ Sci Water Res 2(6):931-941

Edberg SC, Edberg MM (1988) A defined substrate technology for the enumeration of microbial indicators of environmental pollution. Yale J Biol Med 61:389-399

Engler CR (1985) *Disruption of microbial cells in comprehensive biotechnology*. In: Moo-Young M Cooney CL (Eds.), 2:305-324, United Kingdom. Pergamon

Gawai KM, Khan S, Prajapati JB (2017) Comparison of 3M Petrifilm *E. coli* / Coliform Count (EC) Plates vs. IS methods for enumeration of Coliforms (IS-5401 Part-1) and *E. coli* (IS 5887: Part-1) to evaluate quality of Indian milk and milk products. Indian J Dairy Sci 70(2):193-199

Gawai KM, Prajapati JB, Tagalpallewar, GP (2022a) Development of selective enrichment broth for coliforms using Response Surface Methodology (RSM). Asian J Dairy Food Res. 10.18805/ajdfr.DR-1911

Gawai KM, Prajapati JB, Tagalpallewar GP (2022b) Comparison study and evaluation of selective enrichment broth for coliforms with commercial broth media. Asian J Dairy Food Res. 10.18805/ajdfr.DR-1912

Gawai KM, Hati S, Prajapati JB (2022c) Development of fluorescence-based method for qualitative and quantitative detection of *E. coli*. Int J Fermented Foods 11(01): 01-08.

Geissler K, Manafi M, Amorós I, Alonso JL (2000) Quantitative determination of total Coliforms and *Escherichia coli* in marine waters with Chromogenic and Fluorogenicmedia. Comparative Study. J Appl Microbiol 88(2):280-285

Gunda NSK, Mitra SK. (2016) Rapid water quality monitoring for microbial contamination. Electrochem Soc Interface Winter 25(4):73-78

Gunda NSK, Dasgupta S, Mitra SK (2017) DipTest: A litmus test for *E. coli* detection in water. PLoS ONE 12(9):e0183234

Hahn G, Wittrock E. (1991) Comparison of chromogenic and fluorogenic substances for differentiation of coliforms and *Escherichia coli* in soft cheese. Comparative Study. Acta Microbiol Immunol Hung 38(3-4):265-271

Hartman PA (1989) The MUG (glucuronidase) Test for *E. coli in* Food and Water, In: Balows, A., Tilton, RC, Turano, A. (Eds.), Proceedings of the 5th International Symposium on Rapid Methods and Automation In: Microbiology and Immunology, Florence, Italy. November 4-6, 1987

Jing KL (2016) Optimization of ultra-sonication and lysozyme disruption methods for the release of NS1 protein of influenza H5N1 virus from *E. coli* Nova blue. A project report submitted to the Department of Biomedical Science Faculty of Science Universiti Tunku Abdul Rahman

Kanangire O (2013) *Laboratory validation of low cost methods for the measurement of E. coli and total coliforms.* M.Sc. Thesis. UNESCO-IHE, Institute for water education. https://www.dora.lib4ri.ch/eawag/islandora/object/eawag:13611

Kilian M, Bulow P (1976) Rapid diagnosis of *Enterobacteriaceae*: I. Detection of bacterial glycosidases. Acta Microbiol Immunol Scandinavica Sect B. 84:245-251

Makwana S, Hati S, Modha H (2019) Preparation of lactose hydrolysed milk using β-galactosidase enzyme extracted from potential Lactobacillus cultures. Indian J Dairy Science 72(1): 76-84.

Pala L, Sirec T, Spitz U (2020) Modified Enzyme Substrates for the Detection of Bacteria: A Review. Molecules 25(16): 3690

Prasad LN, Ghosh BC, Sherkat F, Shah NP (2013) Extraction and characterisation of β-galactosidase produced by *Bifidobacterium animalis spp. lactis* Bb12 and *Lactobacillus delbrueckii spp. bulgaricus* ATCC 11842 grown in whey. Internal Food Res J 20(1):487-494

Rompre A, Servais P, Baudart J, De-Raubin MR, Laurent P (2002) Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods 49, 31-54

Sakakibara M, Wang DZ, Ikeda K, Suzuki K (1994) Effect of ultrasonic irradiation on production of fermented milk with *Lactobacillus delbrueckii*. Ultrason Sonochem 1:107-110

Sedzro DM, Bellah SMF, Akbar H, Billah SMS (2018) Structure, function, application and modification strategy of β-galactosidase. J Multidiscip Res Rev 1(1):10-16

Siegrist J (2020) Selective growth media for differentiation and detection of *Escherichia coli* and other coliforms. AnalytiX 8(4):1-5

Tavakoli H, Bayat M, Kousha A, Panahi P (2008) The application of chromogenic culture media for rapid detection of food and water borne pathogen. Am-Eurasian J Agric Environ Sci 4(6):693-698