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Development of "Field Level" Chromogenic Assay for Aflatoxin M1 Detection in Milk

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Abstract

Out of eighteen *Bacillus* spp. screened for aflatoxin M1 (AFM1) sensitivity *Bacillus brevis* MTCC 3136 was found most sensitive followed by *B. megaterium* ATCC 9885 at 0.5 ppb (Codex limit). *Bacillus brevis* 3136 was found weakly positive for lipase activity while *B. megaterium* 9885 showed significant acetyl esterase activity. Maximum sporulation was achieved in Arret and Krishbaum sporulation agar (90.63%) followed by tryptone yeast extract broth, tryptone glucose yeast extract broth, minimal media and sucrose salt medium. A field level chromogenic assay for AFM_1 in milk has been developed using spores of *B. megaterium* 9885 grown in Arret and Krishbaum sporulation agar. Spores were added with milk containing germinants (dextrose, sucrose, tryptone) and chromogenic substrate followed by incubation at 37°C for 75 ± 5 min. The colour change from colourless to sky blue colour within 75 ± 5 min is indication of spore germination followed by release of enzyme and its action on chromogenic substrate. However, no colour change indicates that milk is positive for AFM_1 at 0.5 ppb. The developed assay was validated with raw, pasteurized and dried milks with a 96% correlation by comparing with radioimmunoassay i.e., Charm 6602 assay. The reliability of assay has also been checked by spiking AFM_1 in milk. The developed assay was also validated by certified reference materials of heavy metals and pesticides for checking the cross reactivity of these with our developed assay. The developed chromogenic assay is cost effective, portable, having no interference of antibiotics, detergents, sanitizers, heavy metals and pesticides. The developed assay can be used in dairy farm and laboratories with fewer resources for AFM_1 detection in milk.

Keywords: Aflatoxin M_1; Sensitivity; *Bacillus megaterium*; Chromogenic assay

Introduction

Aflatoxin M_1 is the 4-hydroxy derivative of aflatoxin B_1 and is secreted in the milk of mammals that consume aflatoxin B_1 containing feed [1]. Toxicity of aflatoxin M_1 (AFM_1) has now been classified as Group 1 carcinogen [2]. AFM_1 is responsible for hepatotoxicity, cancer, nutritional interference, immunosuppressive in humans and if aflatoxin levels are high enough, liver damage can occur [3,4]. AFM_1 is resistant to thermal inactivation, pasteurization, autoclaving [5,6]. According to Codex Alimentarius Commission (CAC) permissible level of AFM_1 is 0.5 ppb [7] whereas United States Food Drug Administration (USFDA) and European Union (EU) limit is 0.5 ppb and 0.05 ppb, respectively for milk [8].

Different commercial methods are available for detection of AFM_1 in milk include Enzyme linked immunosorbent assay [9], Radioimmunoassay i.e., AOAC approved Charm 6602 [10], HPLC with fluorescence detection [11]. Different biosensors are also available i.e., Flow injection immunoassay with amperometric detection [12], Electrochemical immunosensor using screen-printed electrodes [13], Chemiluminescent Enzyme Immunoassay [14], Biosensor based on surface Plasmon – enhanced fluorescence spectroscopy [15], Impedimetric biosensor based on a DNA probe and gold nanoparticles [16], Miniaturised hybrid immunoassay with luminal substrate [17], Electrochemi-luminescent Immunoassay [18] with their advance technology, specificity and reliability. But all these methods are costly, need huge infrastructure, require experienced personnel.

Therefore, efforts were undertaken to develop an assay for detection AFM_1 in milk based on spore germination inhibition. After germination de novo enzyme was released from the core of the spore which act upon substrate and the signal can be captured using optical device to quantify the presence of target analyte [19]. This process of sporulation and germination can be used as biosensing mechanism for monitoring of broad spectrum antibiotic, specific β-lactam residues and *Enterococci* in milk at dairy farm level, reception dock and manufacturing unit of dairy industry [20-22]. *Bacillus* spp. has been reported highly sensitive to Aflatoxins [23]. A new aflatoxin, aflatoxin B_{2b}, exhibited antimicrobial activity against *Escherichia coli*, *Bacillus subtilis* and *Enterobacter aerogenes* [24].

Material and Methods

Chemicals and instrumentation

AFM_1 procured from Sigma Aldrich (India). Acetone was obtained from Rankem (India). Indoxyl acetate obtained from Hi-media, Mumbai (India). Medias and germinants (tryptone, sucrose) obtained by Hi-media, Mumbai (India). Dextrose procured from Sigma, Mumbai, India. Disks and strips from enzyme screening were obtained from Fluka, Mumbai (India). Antibiotics were procured from Hi-media, Mumbai (India) while detergents and sanitizers from Qualigens and CDH, New Delhi (India). Pesticides (methyl Parathion, chlorpyriphos) and heavy metals (cadmium, copper, lead) were procured from Sigma Aldrich (India). Certified reference materials of heavy metals and pesticides were obtained by Fluka, Mumbai (India). Milk samples were

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procured from Ambala (Haryana) and Punjab (India). Incubation was done in eppendorf Innova 42R (Germany). Distilled water used during experiments was produced in Bioage-Labpure ultra plus (BIO-AGE™, Punjab, India). pH meter from Century (India) was used to prepare buffers and growth medium. For spore production centrifuge from eppendorf North America, Inc. USA, 2801R was used. For absorbance measurement, VictorX3 2030 microbiological plate reader from Perkin Elmer (USA) was used. Validation work was done by using Charm system 6602 (Charm Science Inc., USA) and the data processing was done by using Microsoft excel (Window 7) data analysis.

Selection of indicator strain against AFM_1

Fourteen *Bacillus* spp. (MTCC 2444, 428, 453, 2412, 1684, 3165, 4911, 6129, 868, 869, 3136, 430 ATCC 9885, 14581) procured from Microbial Type Culture Collection (MTCC) IMTECH, Chandigarh, India and American Type Culture Collection (ATCC) and four isolated strains from milk [25] were screened for AFM_1 sensitivity at 0.5 ppb by modified protocol [23].

Screening of enzyme in indicator strain

Indicator strain i.e., most sensitive strain against AFM_1 was screened for different enzymes namely acetyl esterase, tryptophanase, esculinase, ß-galactosidase, ß-lactamase, and lipase using disk and strips of fluka by their given protocol. Colonies of *Bacillus* were wiped off from a petri dish by the function zone of diagnostic strip or bacterial suspension (0.5-1 mL) was prepared in saline followed by incubation at 37˚C for 2 seconds to 20 hrs (according to enzyme) and color change was observed.

Sensitivity of indicator strain against non-microbial contaminants

Non-microbial contaminants include antibiotics, detergents, sanitizers, preservatives, pesticides and heavy metals. Sensitivity of selected indicator strain has been evaluated against different antibiotics namely penicillin ampicillin, cloxacillin, amoxicillin, tetracycline, oxytetracycline, sulphamethazine, gentamycin, streptomycin, neomycin, erythromycin, bacitracin, lincomycin at their maximum residue limit (MRL) set by codex [23,26].

Recommended level of sanitizers i.e., Quaternary ammonium compound (QAC) and iodophorused for sanitization is 150- 200 ppm and 12.5-25 ppm [27], respectively. On that basis 1.0, 2.5, 5, 10, 20, 30 ppm concentrations (conc.) of iodophor and 0.5, 2.5, 5, 25, 50, 100 ppm of QAC has been studied. As recommended level of detergents i.e., sodium hydroxide and sodium hypochlorite is 1-1.5% and 50-100 ppm [28], respectively. So 0.1, 0.3, 0.5, 0.7, 1.0, 2.0% conc. of sodium hydroxide and 0.5, 2.5, 5, 25, 50, 100 ppm of sodium hypochlorite has been studied. However, 0.01, 0.02, 0.03, 0.04% conc. of formalin and 0.025, 0.05, 0.1, 0.2, 0.3, 3 ppm of hydrogen peroxide has been studied based on their recommended level of preservatives i.e., formalin and hydrogen peroxide i.e., 0.03% and 8 ppm, respectively.

Indicator strain has been evaluated against pesticides namely methyl parathion and chlorpyriphos based on their wide use for food crops [29,30]. Food Safety and Standards Authority of India [31] established maximum residue limit of 1000 ppb and 1 ppb for methyl parathion and chlorpyriphos, respectively in milk, carbonated water and vegetables. On that basis 1000, 100, 10 ppb conc. of methyl parathion and 2.0, 1.5, 1.0, 0.5 ppb of chlorpyriphos has been studied.

Indicator strain has been evaluated against heavy metals namely lead, cadmium and copper due to their highly toxic effects [32]. However, lead and cadmium are listed in top 10 hazardous substances by Agency for Toxic Substances and Disease Registry [33]. Based on European communities [34], standard of lead in milk 100, 50, 20, 10 ppb conc. of lead has been studied. United State Environmental Protection Agency [35] established limits of cadmium and copper in drinking water i.e., 5 ppb and 1.3 ppm, respectively. So, 10, 5, 2.5, 1 ppb conc. of cadmium and 2.0, 1.5, 1.3, 1.0 ppm of copper has been studied.

Spore production

B. megaterium 9885 was streaked on nutrient agar and incubated at 37˚C for 12 hrs. Single colony of *B. megaterium* 9885 was inoculated in 5 mL nutrient broth and incubated at 37˚C for 24 ± 2 h. Then from nutrient broth @ 1% inoculated into 100 mL of different sporulation media namely tryptone yeast extract broth, tryptone glucose yeast extract broth, minimal media, and sucrose salt medium [36] followed by incubation at 37 ± 2˚C for 48 h. After sporulation, spores were centrifuged at 10,000 rpm for 10 mins at 10˚C and pellet was washed, re-centrifuged by using normal saline (0.85 % NaCl, pH 7.0). The optical density of the final spore suspension was adjusted to 0.3 at 595 nm by using microbiological plate reader (Perkin Elmer) and total viable count (TVC) and spore count (SC) were enumerated [37].

In case of Arret and Krishbaum (A.K.) agar *B. megaterium* was streaked on A.K. agar (300 mL) in reagent bottles and incubated horizontally at room temperature for 7 days. Growth was harvested from bottles by 20-30 sterile glass beads and sterilized distilled water (25 mL). Bacterial suspension was transferred into centrifuge tube and heated in boiling water at 100˚C for 15 mins. Heated suspension was centrifuged at 5˚C/20 mins/16000 rpm and washed 3 times by 20 mL sterilized distilled water. Optical density was adjusted 0.3 at 595 nm using microbiological plate reader (Perkin Elmer, model 2030) [36] and suspension was analyzed for TVC and SC [37]. Further AFM_1 sensitivity has been analyzed against *B. megaterium* 9885 by above protocol. Growth and sporulation experiments on *B. megaterium* 9885 were designed using CRD (Critical random design) with six replicates (n=6) [38].

Optimization of germinant

Tryptone, dextrose and sucrose were optimized as germinant for *B. megaterium* ATCC 9885 [19,39]. Optimization of germinant has been done by incorporating different concentration of tryptone (10-100 mg), sucrose (5-35 mM) and dextrose (5-200 mM) individually in buffer (1 mL) containing spores (100 µL) and chromogenic substrate (25 µL), followed by incubation at 37 ± 2˚C for 60 minutes. Color change from colourless to sky blue was taken as criteria for spore germination and release of enzyme.

Chromogenic assay

The optimal conditions for the chromogenic assay for the detection of AFM_1 in milk was optimized i.e., quantity of spore suspension, milk and chromogenic substrate, incubation time. Milk (10 mL) containing optimized concentration of germinant (tryptone, sucrose and dextrose) was pre heat treated by boiling in oven from 10-30 seconds to minimize the background interference contributed by microorganisms present in milk. However, heating process results in denaturation of whey protein which provides more germinants for spores to germinate. Chromogenic assay was optimized based on color change inpre-heat treated milk, spore suspension and chromogenic substrate.

Validation of chromogenic assay by Radioimmunoassay

Validation of developed assay was done with radioimmunoassay

(RIA) i.e., charm 6602 system [10] analysing raw, pasteurized and dried milk samples (n-25) procured from different organised or private dairies in the area of Haryana and Punjab in India. Reliability of assay has been done by spiking different concentration of AFM_1 (0.1-1 ppb) in aflatoxin free milk.

Validation of chromogenic assay by certified reference materials

Chromogenic assay was also validated by certified reference material of heavy metal and pesticides. It include heavy metals (Cu-0.602 µg/g, Pb-18.5 ng/g, Zn-49.0 µg/g, Fe-2.32 µg/g) and pesticides (DDT-69 µg/kg, Aldrin-6.2 µg/kg, Dieldrin-36.1 µg/kg, β-HCH-12 µg/kg, Y-HCH-45.4 µg/kg, β-HEPO-32 µg/kg, HCB-37.4 µg/kg, DDE- 51 µg/kg at their European Union limit. Certified reference materials were reconstituted in 1:10 ratio and heat treated after adding germinant. Chromogenic assay was performed with this heat treated milk by addition of spores and chromogenic substrate and color change was observed.

Quantitative detection of aflatoxin M_1

Quantitative detection of aflatoxin M_1 by chromogenic assay has been tried. AFM_1 (5 ppb) was spiked in skim milk powder and diluted from 1:2 to 1:6 ratio and by applying dilution factor recovered concentration of aflatoxin M_1 has been estimated. Percentage recovery was calculated based on results of concentration spiked and recovered.

Results and Discussion

Selection of indicator strain against AFM_1

Out of eighteen *Bacillus* spp. four strains showed sensitivity against AFM_1. *Bacillus brevis* MTCC 3136 was showed better sensitivity at 0.5 ppb recommended by codex maximum residue limit (MRL) limit with an inhibition zone 10 mm (excluding disc diameter i.e., 6 mm) compared to 8 mm zone of *B. megaterium* ATCC 9885. The response of ATCC 14581 and MTCC 430 in terms of sensitivity was poor with the inhibition zone of 6 and 5mm, respectively at 0.5 ppb. Other fourteen strains i.e., MTCC 2444, 428, 453, 2412, 1684, 3165, 4911, 6129, 868, 869 and isolated strains 1,2,3,4 were found resistant against AFM_1 showing no inhibition zone. Therefore, it was concluded that *Bacillus brevis* MTCC 3136 was most sensitive followed by *B. megaterium* 9885, so these two strains were selected for further investigation. *Bacillus brevis* and *B. megaterium* were most sensitive to aflatoxin, being inhibited at 10 and 15 g/mL, respectively [40]. Most sensitive organism inhibited by as low as 7.5 µg aflatoxins/mL was *B. megaterium* giving an inhibition zone of 10.5 mm [41]. *Bacillus brevis* was found sensitive to eight mycotoxins such as AFB_1, ochratoxin A, citrinin, patulin, penicillic acid, cyclopiazonic acid, penitrem A and zearalenone [23].

Screening of enzyme in indicator strain

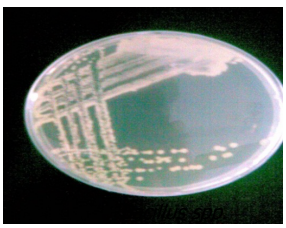
Bacillus brevis MTCC 3136 and *Bacillus megaterium* ATCC 9885 was screened for enzymes namely acetyl esterase, tryptophanase, esculinase, ß-galactosidase, ß-lactamase and lipase using chromogenic strips. *Bacillus brevis* MTCC 3136 was found weakly positive for lipase enzyme activity while *Bacillus megaterium* ATCC 9885 showed significant acetyl esterase activity (Figure 1). Acetyl esterase enzyme produced by cells and spores of *B. megaterium* [42,43]. Existence of extracellular lipase and a cell-bound esterase was reported in *B. megaterium* [44]. On the basis of sensitivity and enzyme activity ATCC 9885 was selected for further investigation.

Sensitivity of indicator strain against other non-microbial contaminants

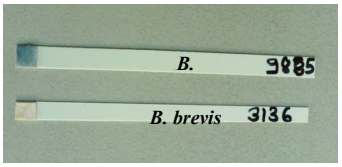
Sensitivity of indicator strain against non-microbial contaminants i.e., antibiotics, detergents, sanitizers, preservatives, pesticides and heavy metals has been studied to avoid interference of these in our chromogenic assay. Most commonly used antibiotics such as penicillin, ampicillin, cloxacillin, amoxicillin, tetracycline, oxytetracycline, sulphamethazine, gentamycin, streptomycin, neomycin, erythromycin, bacitracin and lincomycin for treatment of diseases in dairy animals at their MRL concentration in our study were found no inhibitory effects on indicator strain.

The detergents and sanitizers used in dairy industry for cleaning and sanitization of dairy equipments were found to be ineffective against indicator strains at recommended level except hydrogen peroxide i.e., sensitive at 3 ppm. A proper sanitizer used in milk plant at recommended dosage of hydrogen peroxide is usually 8 ppm. However, under practical situation a maximal residual level of 0.5 ppm could be traced in milk when treated at 10 ppm level for sanitation purpose [45]. So hydrogen peroxide would not affect our assay. Sanitizers namely iodophor and quaternary ammonium compound highly effective on bacterial cell protein [27].

Indicator strain ATCC 9885 has been evaluated against pesticides residues namely methyl parathion and chlorpyriphos, heavy metals such as lead, copper and cadmiumat varying concentrations according



Indoxyl Strip



Positive reaction : **A blue-green spot develops at the position of wiped colony eg. *B. megaterium 9885***

Negative reaction: **No color change develops at the position of wiped colony eg. *B. brevis 3136***

Figure 1: *B. megaterium* ATCC 9885 showing acetyl esterase activity.

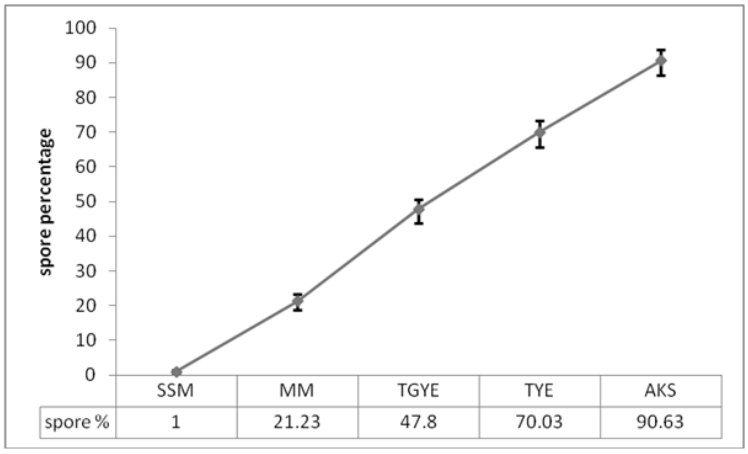
to their MRL limit as mentioned earlier have no inhibitory effect on *Bacillus megaterium*.

Spore production

Out of five different sporulation media maximum sporulation was achieved in A.K. sporulation agar followed by tryptone yeast extract broth, tryptone glucose yeast extract broth, minimal media, and sucrose salt medium i.e. 90.63, 70.03, 47.8, 21.23, 1%, respectively (Figure 2) [36]. Spores grown in A.K. medium showed similar pattern of sensitivity like vegetative cells against aflatoxin M_1 because spores by utilizing nutrients of sensitivity medium germinate and converted into vegetative cells.

Optimization of germinant

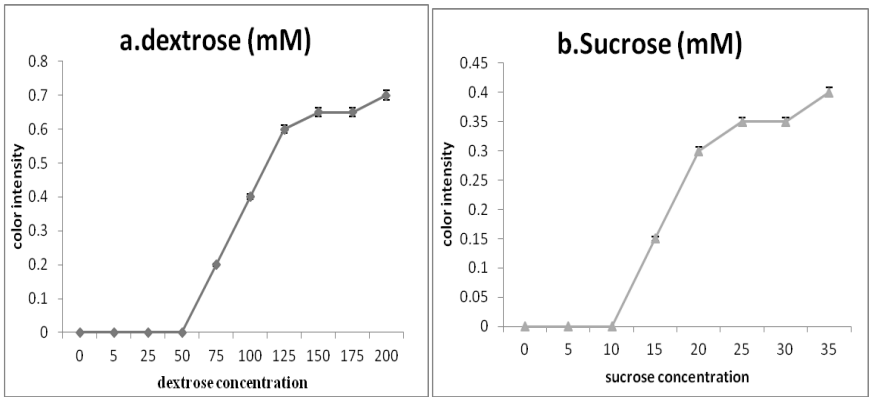
Dextrose, sucrose and tryptone were optimized as germinant for *B. megaterium* ATCC 9885. During study of dextrose it was observed that, there was no color development up to 5-50 mM concentration. Color intensity increased gradually from 75-150 mM, after that it get



	SSM	MM	TGYE	TYE	AKS
spore %	1	21.23	47.8	70.03	90.63

Note: SSM-sucrose salt medium, MM-Minimal media, TYE- tryptone yeast extract broth TGYE-tryptone glucose yeast extract broth, A.K.S.- Arret & Krishbaum sporulating agar.

Figure 2: Effect of different sporulation media on sporulation of *B. megaterium* 9885.



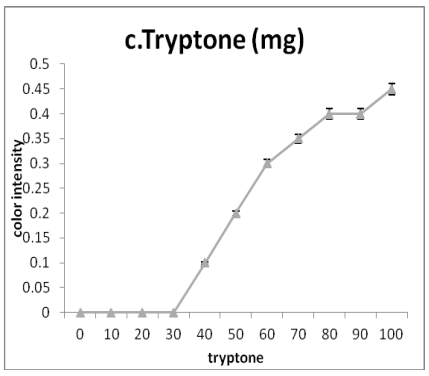


Figure 3: Optimization of germinant.

stabilized or slightly increased up to 200 mM (Figure 3a). During study of sucrose it was observed that, there was no color development up to 10 mM concentration. Color intensity increased gradually from 15-25 mM, after that it get stabilized or slightly increased up to 35 mM (Figure 3b). In case of addition of tryptone in saline 0.85% from 10-100 mg, there was no color development up to 10-30 mg. Color intensity increased gradually from 40-80 mg after that it get stabilized or slight increase in intensity up to 100 mg (Figure 3c).

Based on the above observation, tryptone (80 mg), dextrose (150 mM) and sucrose (25 mM) concentration was selected as germinant for germination of spores in chromogenic assay. Glucose and L-proline were previously reported as a germinant for *Bacillus megaterium* [19,39].

Chromogenic assay

Milk was pre heat treated by boiling in oven for 10-30 seconds to avoid background interference. The background interference of milk at 20 and 30 seconds boiling was minimum as there was no color change in milk containing germinant with 50 µL of 57 mM chromogenic substrate i.e., indoxyl acetate compared to 10 seconds (sky blue color change due to bacteria present in milk). Therefore, 20 seconds was selected for pre heat treatment of milk. Based on the color intensity different parameters i.e., spore suspension, milk, substrate and incubation time with positive (containing AFM_1) and negative milk chromogenic assay was optimized. In spore suspension up to 300 µL of 0.3 optical density (O.D.) in positive milk samples there was no color development and in negative samples color intensity increased significantly up to 250 µL i.e., 0.8 after that there was slight increase. The color development in spore's suspension at a level of more than 300 µL was ≥ 0.3 in positive samples and for negative sample was higher (Figure 4a). The colour intensity developed at a level of 50 and 75 µL of 57 mM chromogenic substrate i.e., indoxyl acetatewas 0.8-0.85 for negative milk and for positive sample there was no color development. The chromogenic substrate above 75 µL color intensity was increased gradually for both positive and negative samples (Figure 4b). In case of volume of milk, the intensity of color development was higher at 1.5 mL and color intensity was decreases gradually where the milk sample increases while there was no color development in positive samples (Figure 4c). In case of

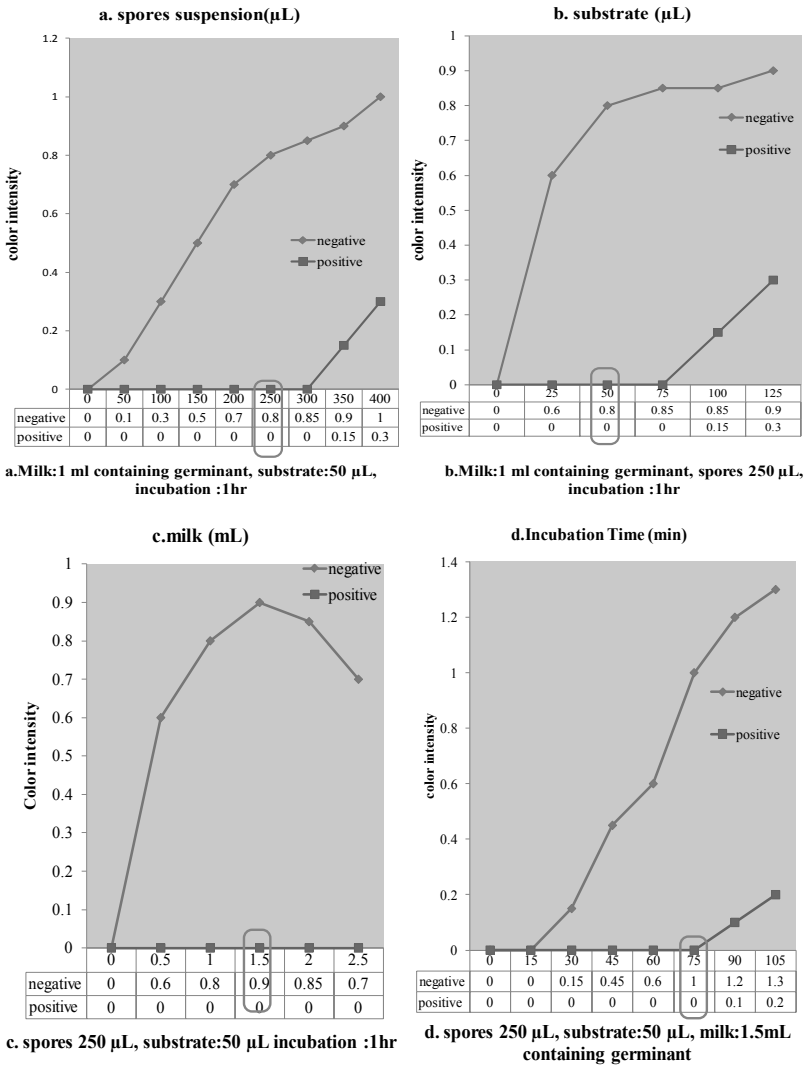
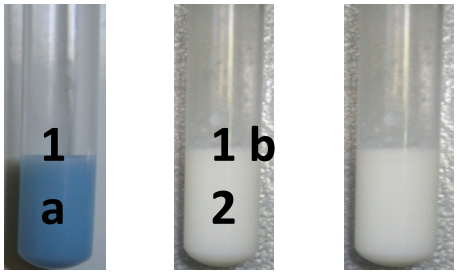


Figure 4: Optimization of chromogenic assay.



[1a]Absence of Aflatoxin M1 in milk; [1b]Presence of Aflatoxin M1 at 0.5 ppb codex limit
[2]Control without spore with no color change (To check the proper heat treatment of milk)

Figure 5: Chromogenic assay.

incubation time there was no color development up to 75 minutes in positive milk while color intensity was 1.0 in negative milk (Figure 4d). Based on the above observation, the spore suspension of 250 ± 25 µL of 0.3 O.D., 1.5 ± 0.25 mL milk, 50 ± 5 µL substrate of 50 mM stock and 75 minutes incubation time was selected for chromogenic assay (Figure 5). A novel colorimetric microbial bioassay has been developed with particular sensitivity to trichothecene mycotoxins, utilizing inhibition of expression of beta-galactosidase activity within the yeast *Kluyveromyces marxianus* as a sensitive toxicity indicator [46]. A warfarin treated patient unexpectedly presented with an elevated international normalized ratio (INR). The chromogenic assay of factor X was used to determine the correct INR result [47].

Validation of chromogenic assay by Radioimmunoassay

Chromogenic assay was validated for the presence of AFM_1 residues withraw (n-10), pasteurized (n-10) and dried (n-5) milk samples procured from different organized or private dairies from Haryana (Ambala) and Punjab, India as depicted in Figure 6. Out of twenty five samples, six samples was found positive at codex limit (0.5 ppb), showed 24% incidence of AFM_1 residues by chromogenic assay while five samples was found positive i.e., 20% incidence by RIA i.e., Charm 6602 system. There was only one false positive sample by chromogenic assay. The correlation of chromogenic assay with RIA i.e., was 96%. Ninty raw milk samples investigatedfor the presence of AFM_1 by ELISA andtwenty eight samples (31.11%) were found higher than the maximum tolerance limit (0.05 ppb) accepted by the European countries [48].

Reliability of chromogenic assay has been checked by spiking aflatoxin M_1 in aflatoxin free milk from 0.1-1 ppb (Figure 7). Intensity of color change observed was inversely proportional to aflatoxin M_1 concentration means color intensity decreased with increase in AFM_1 from 0.1-0.5 ppb. However, there was no color change observed at higher concentration of AFM_1 i.e., 0.5 ppb-1 ppb.

Validation of chromogenic assay by certified reference materials

Chromogenic assay was performed with certified reference material containing various pesticides and heavy metalsat their European Union limit by reconstitution of reference powder in 1:10 ratio in distilled water. Sky blue color change with reference material showed that there was no interference of pesticides (DDT-69 µg/kg, Aldrin-6.2 µg/kg, Dieldrin-36.1 µg/kg, β-HCH-12 µg/kg, Y-HCH-45.4 µg/kg, β-HEPO-32 µg/kg, HCB-37.4 µg/kg, DDE- 51 µg/kg) and heavy metals (Cu-0.602 µg/g, Pb-18.5 ng/g, Zn-49.0 µg/g, Fe-2.32 µg/g)

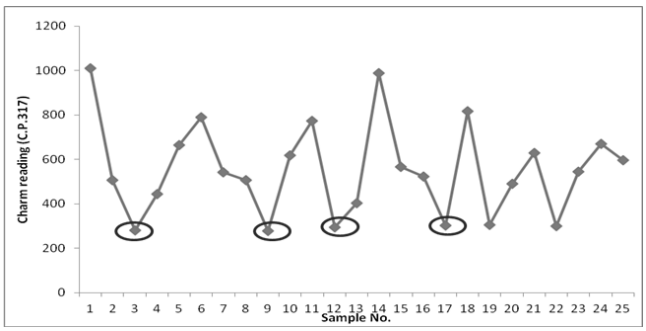


Figure 6: Validation of chromogenic assay with Radioimmunoassay.

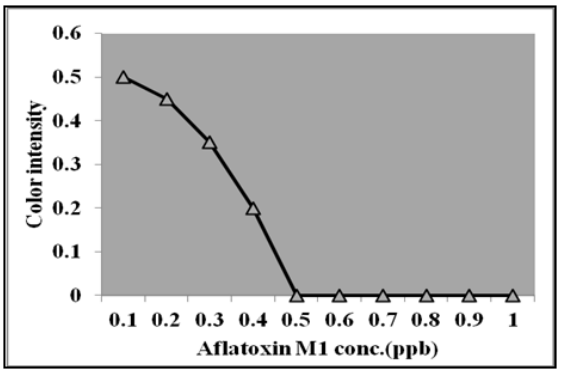


Figure 7: Comparison of Aflatoxin M_1 conc. with chromogenic assay color intensity.

in our developed chromogenic assay. Generally pesticides inhibit the acetylcholine esterase enzyme but in our case pesticides are not interfering because there was spore inhibition by AFM_1 not enzyme inhibition and enzyme used in our study is acetyl esterase (EC 3.1.1.6) not acetylcholine esterase (EC 3.1.1.7).

Quantitative detection of aflatoxin M_1 by chromogenic assay

High concentration of AFM_1 i.e., 5 ppb was spiked in skim milk powder and dilutions were prepared in aflatoxin free milk from 1:2 to 1:6 ratios. By applying dilution factor recovered concentration was estimated by charm rosa system. Percentage recovery was also calculated based on results of concentration spiked and recovered (Table 1). If sample comes positive means it contain ≥ 0.5 ppb then by diluting milk sample and applying dilution factor we can detect quantity of AFM_1 in sample. One sample was found positive by chromogenic assay means no color change so we have diluted it 1:2 ratio further it there it was found by chromogenic assay means it contains more than 1 ppb. Then we have diluted this sample in 1:3 ratio then the sample was found negative with sky blue color change means sample contains 1.5 ppb aflatoxin. So based on recovery the concentration of aflatoxin M_1 should be 1.51-1.41 ppb in milk sample (Table 2).

Conclusion

B. megaterium ATCC 9885 has been selected for development of chromogenic assay based on their higher sensitivity against aflatoxin M_1 and significant acetyl esterase enzyme activity. Acetyl esterase activity has been used for measuring early responses of spores during germination. A chromogenic assay has been developed based on the inhibition mechanism of spores by AFM_1. The developed assay

Dilution	Dilution factor	Concentration Spiked in ppb (5X Dilution Factor)	Recovered Conc.	% Recovery
1:2	0.5	2.5	2.5	100
1:3	0.33	1.65	1.60	96
1:4	0.25	1.25	1.21	96.8
1:5	0.2	1.0	0.95	95
1:6	0.166	0.83	0.78	93.9

Table 1: Spiked aflatoxin M_1 concentration recovered after dilution.

Sample	Chromogenic assay	Dilution factor	Concentration in milk sample	% Recovery
(0.5ppb/dilution factor)	0.5	2.5	2.5	100
Original	No color change	0	≥ 0.5 ppb	96
1:2	No color change	0.5	>1 ppb	96.8
1:3	Color change	0.33	1.51-1.41 ppb (range based on above data of recovery 100-94%)	95
1:6	0.166	0.83	0.78	93.9

Table 2: Quantitative detection of aflatoxin M1 by chromogenic assay.

was validated with natural as well as spiked milk samples and good correlation (96%) of results was obtained with radioimmunoassay i.e., charm 6602 system. In comparison with existing commercially available methods for aflatoxin M_1 i.e., HPLC, Charm, ELISA the present assay has the advantage of portability, simplicity, cost effectiveness, realtime analysis with no interference of antibiotics, detergents, sanitizers, heavy metals and pesticides on its performance. So, it may be concluded that chromogenic assay has the commercial potential to detect AFM_1 in milk which can be applied at farm level and also in dairy with fewer resources.

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