

## Efficacy of volatile compounds and food preservatives in the control of citrinin production by three strains of *Aspergillus Terreus*

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### Abstract

In the present study, volatile compounds and food additives were tried for their effectiveness in the control of the development of biomass and citrinin production by three strains of *Aspergillus terreus*. Fumes of phenols and formic acid altogether repressed citrinin production in three strains of *Aspergillus terreus*. Among food additives, sodium acetic acid and sodium metabisulphite inhibited citrinin production at 150 µg/ml concentration. The amount of citrinin was measured using high performance liquid chromatography. The results of the above work are presented in this communication.

**Keywords:** *aspergillus terreus*, citrinin, high performance liquid chromatography

### Introduction

A typical poultry feed is formulated from many grains and may contain a unique mycotoxin. If more than one mycotoxins are present in a diet it may result in either an additive or synergistic or antagonistic (Casarett, 1986) [4]. Varieties of studies are conducted to gauge the effect of multiple mycotoxins. However, these studies are primarily centered on the acute effects of these mycotoxins. The numbers of mycotoxins consumed in these acute studies were comparatively high and are rarely seen in industrial poultry rations. In routine animal feed screening, mycotoxins are typically found at comparatively low levels (Watts *et al.*, 2003) [37]. However, limited data are available to low levels of multiple mycotoxins in stock (Berry, 1998; Peraica *et al.*, 1999; Mafri, 2006) [2, 28, 22]. It's been observed low concentrations of mycotoxins may have a synergistic effect on the health of animals variety of approaches have been made to evaluate fungal toxicity of animal feeds. The proliferation of moulds in animal feeds is reported to scale down nutrients like vitamins and amino acids. The control of mycotoxin contamination of food commodities has been the subject of great concern. Different strategies such as prevention, protection and detoxification have been adopted for this purpose (Park, 1993) [27]. Development of commercially acceptable crop varieties that restrict toxin production is one of the preventive approaches (Hundley, 2001; Juglal, 2002; Paranagama *et al.*, 2003; Muntvold, 2003; Reddy *et al.*, 2004; Guihua Bai and Gregory Shaner, 2004; Reddy *et al.*, 2008; Vinod Kumar *et al.*, 2008 and Ahmed Tayel, 2010) [15, 16, 26, 24, 31, 14, 32, 36, 1]. are also being advocated to arrest mould growth and inactivation of mycotoxins elaboration. Food preservatives in the management of mycotoxins have received considerable attention of microbiologists. However, no information is available with the management of citrinin production. Hence, it was considered worthwhile to investigate the efficacy of some food preservatives in the management of citrinin production by three strains of *Aspergillus terreus*. Simultaneously efficacy of some

antioxidants, vitamins and growth regulators against the growth and citrinin production by three strains of *Aspergillus terreus* was assessed. The contamination of foods and feeds stuff by citrinin is widespread, particularly in temperate climates such as Food product like rice, wheat, corn, rye, barley are widely contaminated by citrinin in North America and even in India (Reddy *et al.*, 2010). Buckle (1983) [33, 3]. Reported the incidence of citrinin cereals and animal feedstuffs from the European nation. Citrinin is an important nephrotoxic, hepatotoxic cytotoxic, mycotoxin and reported to be the most contaminant of animal and poultry feed. Its presence in feed causes serious concern to the livestock industry reported by Gentles and Smith (1999) [11]. Besides, *Aspergillus*, *Penicillium* species are reported to be the most producers of citrinin. Apart from this, citrinin is additionally considered to be teratogenic and immunotoxic. Present investigations revealed the high incidence of *Aspergillus* species in animal and poultry feed which is probably going to pose a significant ill health problem to chicken and animal in turn to human beings. The present study is aimed toward investigating the efficacy of food preservatives and volatile compounds within the control of growth and citrinin production also on understand the character and role of those fungi in mycotoxin contamination of animal and poultry feed and consequent health problems.

### Material and Methods

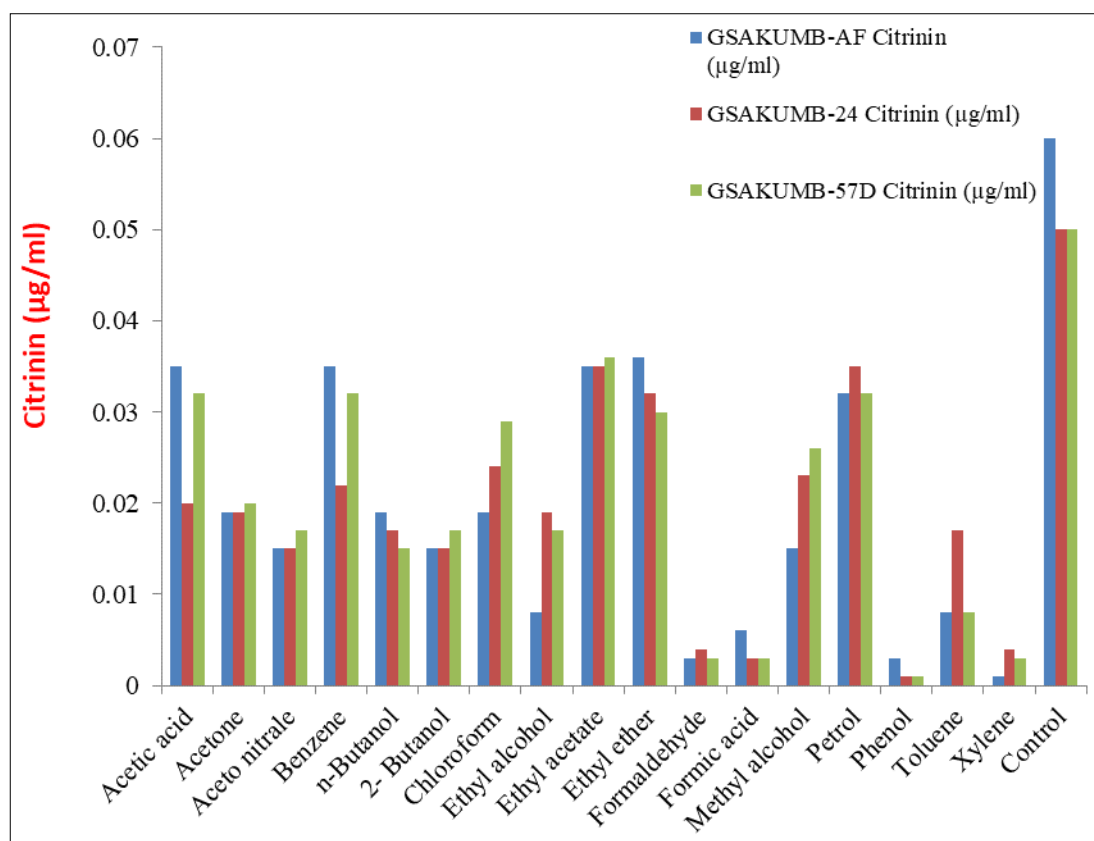
The citrinin producing strains of *Aspergillus terreus* isolated from different food and feed used in the present investigation were gifted by Rao *et al.* (2011) [19]. The growth of these were done Czepak Yeast Autolysate (CYA) medium. The extraction and cleanup of food preservatives and volatile compounds on growth and citrinin production was similar to that of Rao *et al.* (2011) [19]. The possible production of mycotoxins of all isolates of *Aspergillus* species was seen in CYA broth of 7 days old cultures. The spore suspension of 10<sup>-5</sup> dilution was inoculated for 15 days at 27 ± 2°C into 50 ml of CYA broth and incubated in dark for on shaker for 150 rpm. At the end of the incubation

period, 25 ml of the broth was filtered through Whatman No.42 filter paper. The filtered samples were acidified with 6 N O-Phosphoric acid. Added twenty-five ml of ethyl acetate to 25 ml culture filtrate of *Aspergillus* species in 100 ml of the conical flask and shaken thoroughly and extracted twice with 25 ml of ethyl acetate. The ethyl acetate extract was evaporated by a water bath and the obtained compound was resuspended in 500  $\mu$ l of methanol and preserve at 10<sup>0</sup> C until use. Analysis and quantification of citrinin was done as per the earlier reported method of Rao *et al* (2015) [18]. Using a HPLC (JASCO-975, Japan) C18 isocratic reverse phase Luna column with mobile phase Methanol: water (20:80, v/v). The statistical analysis was performed using Graph Pad Software.

## Results and Discussion

*Aspergillus* species isolated from seeds, foods and feeds were primarily identified by standard protocols and manuals based on phenotypic characters. The identified isolates were confirmed by molecular methods by targeting ITS. The ITS

positive isolates were subjected to RFLP analysis and were restriction digestion with *Eco*-RI enzyme and the resultant bands were sequenced and three strains *Aspergillus terreus* (*Aspergillus terreus* GSAKUMB-24; *Aspergillus terreus* GSKUMB-57 D; *Aspergillus terreus* GSAKUMB-AF) were identified based on nucleotide homology and phylogenetic analysis. The identified *Aspergillus* species were deposited in the National Centre for Biotechnology Information (NCBI) their accession number MN326724.1, MN326737.1 and MN326736.1. The present plan to screen different volatile compounds in checking the citrinin production by three strains of *Aspergillus terreus* reveals the inhibitory effect on citrinin production by three strains of *Aspergillus terreus* (Table.1 and 1A). The use of volatile compounds for mycotoxin control have been suggested in the protection of food grains (Narsimha Rao *et al.*, 2009) [25]. Feeds and fodders (Rafiyuddin, 1999) [29]. Because of the above facts, it was felt necessary to screen some of the common volatile compounds against three strains of *Aspergillus terreus* and the results are detailed in Table 1, and Text Fig. 1B.



**Fig 1:** Effect of volatile compounds on citrinin production by three strains of *Aspergillus terreus*.

**Table 1:** Effect of volatile compounds on growth and citrinin production by three strains of *Aspergillus terreus*

Name of the	Gsakumb-af			Gsakumb-24			Gsakumb-57d		
	Final ph	Dry weight (mg/ml)	Citrinin (µg/ml)	Final ph	Dry weight (mg/ml)	Citrinin (µg/ml)	Final ph	Dry weight (mg/ml)	Citrinin (µg/ml)
Acetic acid	7.4	0.78	0.035	7.3	0.72	0.020	6.9	0.73	0.032
Acetone	7.6	0.72	0.019	7.5	0.72	0.019	8.9	0.73	0.020
Acetonitrile	8.6	0.65	0.015	8.7	0.63	0.015	7.6	0.66	0.017
Benzene	8.2	0.72	0.035	8.6	0.63	0.022	6.8	0.73	0.032
n-Butanol	6.3	0.93	0.019	6.9	0.57	0.017	7.5	0.95	0.015
2-Butanol	7.5	0.75	0.015	7.9	0.72	0.015	7.3	0.73	0.017
Chloroform	4.9	0.85	0.019	4.2	0.57	0.024	6.4	0.83	0.029
Ethyl alcohol	7.5	0.82	0.008	7.3	0.76	0.019	9.1	0.74	0.017
Ethyl acetate	7.7	0.99	0.035	7.2	1.1	0.035	9.3	0.83	0.036

Ethyl ether	8.8	0.63	0.036	6.3	0.59	0.032	8.2	0.58	0.030
Formaldehyde	8.1	0.65	0.003	8.3	0.84	0.004	6.3	0.92	0.003
Formic acid	7.6	0.73	0.006	5.3	1.06	0.003	4.2	0.84	0.003
Methyl alcohol	7.7	0.66	0.015	7.5	0.85	0.023	8.1	0.63	0.026
Petrol	8.3	0.69	0.032	8.2	0.74	0.035	8.6	0.74	0.032
Phenol	8.4	0.59	0.003	8.3	0.52	0.001	8.2	0.57	0.001
Toluene	8.9	0.75	0.008	8.3	0.86	0.017	8.3	0.72	0.008
Xylene	8.4	0.58	0.001	6.4	0.63	0.004	6.1	0.63	0.003
Control	6.7	1.03	0.060	6.8	1.10	0.050	7.6	0.89	0.050

Ethyl acetate, ethyl ether petrol were responsible for total inhibition of citrinin production followed by acetic acid and chloroform, methyl alcohol wherein 30–35% of inhibition of citrinin production by strain of *Aspergillus terreus* GSAKUMB-24 was observed Rest of the volatiles exhibited moderate toxicity on citrinin production by three strains of

*Aspergillus terreus*. Statistically analysis results are presented in Table 1A. The mean citrinin production by three strains of *Aspergillus terreus* GSAKUMB-AF (3.13 µg/ml), GSAKUMB-24 (5.45µg/ ml) and GSAKUMB-57D (7.08µg/ml) was significantly recorded.

**Table 1A:** ANOVA of the effect of volatile on citrinin production by three strains of *Aspergillus terreus*

Source of variation	Sum of squares	Df	Mean Square	F- value	P-value	R <sup>2</sup>	Result
GSAKUMB-AF							
Between columns	2.533e-005	2	1.267e-005	40.37	< 0.0001	0.7037	S
Between rows	0.01253	17	0.0007368				
Random	1.067e-005	34	3.137e-007				
Total	0.01256	53					
GSAKUMB-24							
Between columns	1.744e-005	2	8.722e-006	15.98	< 0.0001	0.4846	S
Between rows	0.008261	17	0.0004859				
Random	1.856e-005	34	5.458e-007				
Total	0.008297	53					
GSAKUMB-57D							
Between columns	3.226e-005	2	1.613e-005	227.8	< 0.0001	0.9306	S
Between rows	0.009706	17	0.0005710				
Random	2.407e-006	34	7.081e-008				
Total	0.009741	53					

P > 0.05 - Not Significant (NS); P < 0.05 Significant (S); R<sup>2</sup>- Coefficient of determination.

A positive correlation between GSAKUMB-AF (0.7037), GSAKUMB-24 (0.4846) and GSAKUMB-57D (0.9306) ( $P < 0.001$ ) was observed on citrinin production by three strains of *Aspergillus terreus* towards the toxicity of volatile compounds assayed. Among the citrinin production, three strains of *Aspergillus terreus* proved to be more resistant in all volatiles tried. Utama *et al.* (2002) [35]. have reported that acetaldehyde, benzaldehyde, ethanol, benzyl alcohol were lethal to fungal sheath and from the present investigations, it is clear that formaldehyde, formic acid, phenol and xylene can be of use in checking the mycotoxin production. Volatile compounds are reported to be effective inhibitors of mould growth and mycotoxins contamination and at the same time lack residual effect. The present investigations revealed that Formaldehyde, Formic acid, Phenol were responsible for all most total inhibition of citrinin production. Volatile compounds have an added advantage of non-residual nature. However, the economies and other adverse effects if any have to be critically assessed. Francisco *et al.* (2007) [10]. Observed that volatile compounds naturally liberated by ripening strawberry fruit were highly fungicidal.

From Table.1 the toxic effect on the growth of GSAKUMB-AF was recorded with the least percentage of 20% to 30% in

ethyl acetate and n-Butanol followed by ethyl alcohol and chloroform, and acetic acid. The highest toxic effect on the growth of biomass was recorded was 90 % to 100% in xylene and phenol followed by ethyl ether, acetonitrile. In GSAKUMB- 24 the least toxic effects on the growth of biomass was about 35% to 40% with Ethyl acetate and Formic acid followed by Toulene, formaldehyde was seen. The highest toxic effect on the growth of biomass of *A.terreus* strain recorded was 85%-90% in phenol and n-butanol, followed by chloroform, ethyl ether acetonitrile, and benzene. Rest of the volatile compounds are responsible for mild inhibition of biomass yield in *A. terreus* GSAKUMB- 24. In GSAKUMB-57D the highest growth of biomass recorded was 95-100% with n-butanol, formaldehyde followed by formic acid, ethyl acetate and chloroform. Least growth was recorded with 35-40% in phenol, ethyl ether, methyl alcohol followed by xylene and acetonitrile.

The rest of the volatile compounds are responsible for mild inhibition of biomass yield *A.terreus* GSAKUMB-57D. From Table 1 a positive correlation was found on growth and citrinin production by three strains of the *A.terreus* species in their sensitivity towards volatile compounds tried. Table: 2,

**Table 2:** Effect of food preservatives on growth and citrinin production by three strains of *Aspergillus terreus*

Name of the compound	Concen-trations (µg/ml)	Gsakumb-af			Gsakumb-24			Gsakumb-57d		
		Final pH	Dry weight (mg/ml)	Citrinin produ-ction (µg/ml)	Final pH	Dry weight (mg/ml)	Citrinin produ-ction (µg/ml)	Final pH	Dry weight (mg/ml)	Citrinin produ-ction (µg/ml)
Acetone	100	6.9	9.2	0.027	7.7	9.1	0.028	7.7	9.1	0.019
	150	7.0	7.3	0.006	7.9	6.2	0.011	7.9	6.2	0.016
	200	7.6	4.5	0.000	7.6	5.5	0.001	7.5	5.2	0.006
Boric acid	100	7.7	8.6	0.022	8.9	8.5	0.028	8.6	9.3	0.014
	150	7.4	8.1	0.01	9.0	8.2	0.015	8.4	9.1	0.002
	200	7.1	6.1	0.005	7.5	7.1	0.004	8.5	5.5	0.000
Benzoic acid	100	6.1	8.4	0.038	6.2	9.2	0.039	6.2	9.1	0.032
	150	5.7	6.3	0.022	5.5	6.4	0.017	5.8	6.6	0.020
	200	5.1	0.0	0.000	5.2	6.2	0.005	5.2	6.3	0.009
Citric acid	100	6.3	8.6	0.017	6.2	7.2	0.014	6.4	9.2	0.018
	150	5.7	7.3	0.010	5.6	5.1	0.008	5.8	7.7	0.008
	200	5.1	4.1	0.003	5.2	4.3	0.003	5.2	5.2	0.005
Crystal violet	100	6.5	8.3	0.010	6.6	7.6	0.013	6.4	6.8	0.017
	150	6.4	4.9	0.006	6.3	6.2	0.005	6.1	6.5	0.017
	200	5.4	0.0	0.000	5.3	0.0	0.000	5.6	5.6	0.008
Sodium acetate	100	8.6	9.2	0.057	8.3	9.1	0.033	8.7	9.3	0.053
	150	8.1	8.4	0.030	9.1	8.5	0.030	8.5	8.7	0.042
	200	8.2	6.3	0.017	9.1	5.5	0.014	8.1	5.1	0.008
Sodium chloride	100	7.9	6.1	0.019	8.6	6.2	0.013	8.4	6.5	0.018
	150	7.6	5.7	0.008	8.4	5.9	0.015	8.5	6.1	0.019
	200	8.1	5.5	0.003	8.4	5.2	0.013	8.6	5.3	0.002
Sodium meta bisulphate	100	8.2	9.9	0.030	9.5	8.6	0.042	9.0	9.3	0.028
	150	8.1	7.7	0.016	9.6	8.1	0.032	9.0	8.5	0.030
	200	8.1	6.6	0.010	9.5	6.5	0.017	8.4	6.3	0.010
Control	-	6.6	6.8	0.044	6.7	9.8	0.034	6.9	7.3	0.045

**Table 2A:** ANOVA of the effect of food preservatives on citrinin production by three strains of *Aspergillus terreus*

Source of variation	Sum of squares	df	Mean Square	F- value	P-value	R <sup>2</sup>	Result
GSAKUMB-AF							
Between columns	0.0001313	2	6.564e-005	85.80	< 0.0001	0.7814	S
Between rows	0.01645	24	0.0006854				
Random	3.672e-005	48	7.650e-007				
Total	0.01662	74					
GSAKUMB-24							
Between columns	0.0001446	2	7.228e-005	136.4	< 0.0001	0.8504	S
Between rows	0.01093	24	0.0004555				
Random	2.544e-005	48	5.300e-007				
Total	0.01110	74					
GSAKUMB-57D							
Between columns	0.0001584	2	7.921e-005	208.5	< 0.0001	0.8968	S
Between rows	0.01336	24	0.0005567				
Random	1.824e-005	48	3.800e-007				
Total	0.01354	74					

2A and Text Fig.2B reveals that as the concentration of each food preservative increases, the growth and citrinin production was decreased gradually. Acetone, benzoic acid, boric acid and crystal violet were found to be highly toxic and did not allow the growth of GSAKUMB-AF at 200 µg/ml concentration. Ghosh *et al.* (1996) [12]. Reported that propionic acid, sodium bisulphate and sodium hydroxide could check aflatoxin production in groundnut cake. Crystal violet at 200 µg/ml concentration inhibited the growth and citrinin production by GSAKUMB-24, while acetone, citric acid, boric acid, benzoic acid, sodium acetate, sodium chloride and sodium metabisulphate showed decreased growth and citrinin production as the concentration of these preservatives increases. On the other hand, boric acid at 200 µg/ml concentration inhibited the growth and citrinin production by GSAKUMB-57, whereas sodium chloride, crystal violet, sodium acetate and benzoic acid supported the

least growth at higher concentration of these food preservatives.

Final pH was found to be alkaline in the GSAKUMB-AF and *A. terreus* growing media containing sodium acetate, sodium metabisulphate and sodium chloride individually. Rest of the preservatives containing media showed neutral final pH. Similarly, sodium metabisulphate, sodium chloride, sodium acetate and boric acid supplemented GSAKUMB-57 cultured media showed alkaline pH, while rest of the preservatives showed neutral pH. Krishna Reddy and Reddy (1997) [20]. reported that propionic acid and benzoic acid can be a good antifungal agents for control of mycotoxin contamination. Marin *et al.* (2000) [43]. Reported the efficacy of propionates in checking mould infestation and fumonisin B<sub>1</sub> production in maize. Sreenivasa *et al.* (2009) [34]. Also reported the effectiveness of food preservatives such as boric acid against the fungal

infestation of maize and sorghum and on seed germination. Rao *et al.* (2009) [25]. Have reported the efficacy of naphthalene and boric acid in reducing fumonisins contamination. Rao *et al.* (2015) [18]. have reported sodium acetate and sodium metabisulphate were responsible for inhibition of ochratoxins by *P. verrucosum* and *P. nordicum*. A positive correlation coefficient was observed between growth and citrinin production by all the three strains of *A. terreus*. The mean citrinin production by three strains of *Aspergillus terreus* GSAKUMB-AF (7.65 µg/ml), GSAKUMB-24 (5.30µg/ ml) and GSAKUMB-57D

(3.80µg/ml) was significantly recorded. A positive correlation GSAKUMB-AF (0.7814), GSAKUMB-24 (0.8504) and GSAKUMB-57D (0.8968) ( $P < 0.001$ ) was observed on citrinin production by three strains of *Aspergillus terreus* toward the toxicity of food preservatives assayed. Among the citrinin producing strains, three strains of *Aspergillus terreus* proved to be more resistant in all food preservatives. From the knowledge acquired within the current investigation, it's clear that the growth of biomass of *Aspergillus terreus* and Citrinin production can be checked by the utilization of food additives and volatile compounds.

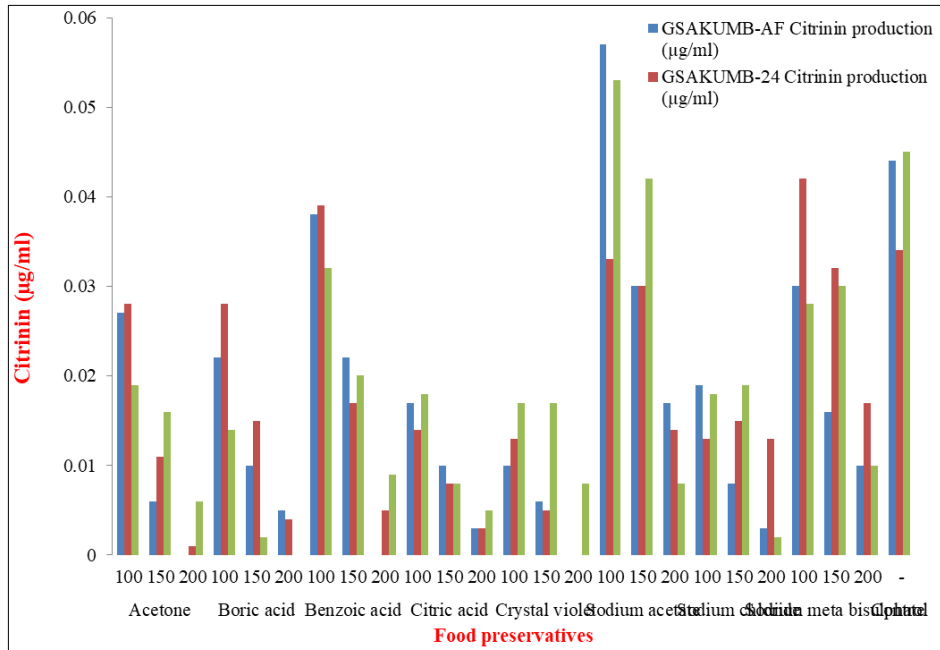


Fig 2: Effect of food preservatives on Citrinin Production by three strains of *Aspergillus terreus*

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