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Prevalence of Bacillus Species and Fungal Species Isolated from Cooked and Raw Rice

Rabia Qureshi, Shahbaz Ahmad Zakki, Areeba Hussain

Department of Microbiology, IMBB, The University of Lahore, Pakistan

ABSTRACT

Rice is delightful food used extensively all over the Asian countries including Pakistan and consumption rate is very high. The aim of the present observation was to estimate the microbial load of the rice in the Lahore city of Pakistan. A total of 168 rice samples of raw and cooked categories were collected from different food-stalls and karyana stores from Raiwind, Lahore. Highest average of mean Viable Count (6.19 log10CFUg-1) of uncooked rice samples while in the cooked samples the highest mean Viable Count (3.84 log10CFUg-1) was recorded. It was also found that the overall percentage of Bacillus cereus and Bacillus subtilis in raw rice 38% and 52% while in cooked rice samples were 46% and 25% respectively. Fungal species isolated cooked rice samples were found in the order of Aspergillus niger (14%), Rhizophus stolonifer (12%), Pencillium chrysogenum (9%), Aspergillus flavus (8%), Fusarium equiseti (8%), Aspergillus fumigatus (0%), Fusarium avenaceum (0%), and Alternaria alternata (0%). Aspergillus species were found to be predominant in fungal isolates. The data was analyzed statistically by One Way ANOVA and found significant (p<0.05). It was concluded from the study, Bacillus cereus breakouts can be controlled by quick ingestion of cooked rice.

Keywords: Prevalence of Bacillus cereus, Bacillus subtilis and fungus in Raw rice and Cooked rice.

NTRODUCTION

Rice is an important foodstuff with Bacillus cereus food poisoning [1]. Bacillus spp. is a usually present in soil and rhizosphere dweller [2]. Bacillus cereus comprises 10% of the soil microflora in rice fields [3, 4, 5].

Genus Bacillus is complex in physiological and genetic properties [6]. As Bacillus cereus is present everywhere in nature, anaerobic, Gram-positive, motile, endospores forming (central, ellipsoid) with granular internal structure, rod shaped bacteria, which manifest numerous pathogenic properties. The primary domain of Bacillus cereus is soil. It is found frequently in foods such as milk, cereals, meats, poultry, starches, herbs, and spices [7].

Foodborne breakouts caused by Bacillus cereus [8] have been associated with more or less all the groups of foodstuffs [9] also further more Bacillus species, just as Bacillus licheniformis, Bacillus subtilis, Bacillus pumilus [10] Bacillus thuringiensis [11], and along with others, have been fix and accountable for diseases in human, including gastroenteritis, meningitis, endophthalmitis, and

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*Corresponding author: dr_rabiaqureshi@yahoo.com cutaneous infection.

In Norway, diarrhoeal type of Bacillus cereus food poisoning was first investigated after an investigation of a hospital outbreak, on the other hand Bacillus cereus emetic syndrome was first observed in the 1970s after consumption of cooked rice in Chinese cuisine in the United Kingdom [12, 13] Between the molds, bacteria and fungi that can spread on brown rice, Aspergillus flavus is between the most dangerous, it is also known as an aflatoxin, likely to cause cancer if consumed. Aspergillus flavus grow on both cooked and uncooked rice [14].

MATERIAL AND METHODS

Sample collection

A total of 168 rice samples included of two main categories, raw and cooked rice. The raw rice contained seven varieties whereas, cooked rice comprised of three varieties were collected randomly from different karyana stores and food-stalls from Raiwind, Lahore city, Pakistan and packed separately in the sterile polythene bags aseptically, and then transported to the laboratory of the Institute of Molecular Biology and Biotechnology (IMBB), The university of Lahore, for immediate of sample collection.

Sample preparation

Samples were prepared according to the method of with few modifications; 5g of each rice sample were introduced in 45ml of 0.1percent (weight/volume) sterile buffered peptone water and homogenized (mixed) for 2 mints in the sterile Motar and Pestle while raw rice samples were grinded in the sterile Grinder Machine. One milliliter of the homogenate was introduced into the test tube containing 9ml buffered peptone water, labeled 1:10 (10-1) dilution and then serially diluted into two more test tubes, labeled 10-2, and 10-3 [15].

Culturing of sample and bacterial count

From 10-3 dilution of the each diluted sample, 0.1ml was swabbed over the Nutrient Agar plate for aerobic incubation at the temperature at 37oC for 24-48 hours. After the incubation total plate count (TPC) was done with the help of digital colony counter [16].

Preservation of isolated strains

The isolated bacteria were preserved in the solution of 40% Nutrient broth with 60% Normal Saline in 1.5ml eppendorfs tube and kept in the freezer at a temperature of -18oC for later use according to the method of [17].

Purification of isolated colonies

All the isolated colonies obtained from Nutrient Agar were streaked on Bacillus cereus specfic Agar i.e Polymyxin Egg Yolk Mannitol- Bromothymol Agar (PEMBA). The plates were incubated at 37oC for 24 to 36 hours with an additional 24 hours at room temperature to make possible the growth of turquoise to peacock blue colonies that is classic of Bacillus cereus group [18].

Microscopic staining

The microscopic morphology and arrangement of bacteria were examined using Gram staining and Spore staining [19].

Biochemical test

The biochemical tests that were used for the identification of Bacillus cereus group included: catalase test, hydrolysis of gelatin, hydrolysis of starch, and casein hydrolysis [19].

Statistical analysis

The samples were analyzed through the SPSS v16.0 by One Way ANOVA test to evaluate the significance of the data

RESULTS

Mean Viable Count of Rice samples

A total of 168 rice samples (Raw and Cooked rice) were collected randomly from food-stalls and karyana stores in Lahore, Pakistan. The raw rice samples were of seven varieties which are

Table I: Mean Viable Count (Log10Cfug-1) And Percentage Of Bacillus Cereus And B. Subtilis Examined From Cooked And Raw Rice Samples

Categories	**Varieties of Rice**	**No. of Samples**	**Mean $\log_{10}$CFUg^{-1}**	**Std. Deviation**	**P-value**	***B. cereus***		***B. subtilis***	
						No. of Positive Samples	**% prevalence**	**No. of Positive Samples**	**% prevalence**
RAW RICE (n= 84)	Saila Super Fine	12	6.19	0.85	0.00	4	38%	7	52%
	Saila Kainat	12	5.8	0.78		6		6	
	Super Karnel	12	6.13	0.79		4		6	
	86 Basmati	12	5.77	0.75		5		8	
	Super	12	5.93	0.98		4		6	
	Supri	12	6.15	0.83		4		6	
	Super Basmati	12	6.11	0.84		5		5	
COOEKD RICE (n=84)	Pulawo	28	3.84	0.58		19	46%	7	25%
	Biryani	28	3.64	0.58		11		8	
	Boiled	28	3.33	0.44		9		6	

as follows, (Saila Super fine, Saila Kainat, Super Karnel, 86 Basmati, Super, Supri, and Super Basmati). The cooked rice samples were of three varieties as follows: (pulawo, Biryani and Boiled rice) they were collected from food-stalls of Raiwind in Lahore, Pakistan. The Mean Viable Count and Prevalence of B. cereus and B. subtilis were noted from rice samples are mentioned in Table I. In the above table, it is found that “Saila Super Fine” contained the highest “Mean viable Count” 6.19 as compared to the other raw rice samples whereas in cooked rice samples “Pulawo” contained highest “Mean Viable Count” 3.84. The data of the samples is analyzed through the SPSS by One Way ANOVA method to evaluate the data significance P-value (0.00). It is also observed that raw rice contained highest load of B.subtilis 52% and least load of B.cereus 38% against to the cooked rice. It is found that cooked rice contained highest load of B.cereus46% and least load of B.subtilis 25%.

Prevalence of fungus isolates

Prevalence of fungus isolates were recorded from raw and cooked rice samples. It was found that Aspergillus niger (21%) found highest prevalence as compare to the other fungus species. It was found to be the most prevalent species obtained from both raw and cooked rice samples. Rhizophus stolonifer was the second prevalent fungus species in both varieties of rice samples 16% in raw and 12% in cooked samples as shown below in the Table II.

Table II: Percentage Of Fungus Isolates Examined From Rice Sample

Sr. No.	Fungus Isolates	Raw rice (n=84)	Cooled rice (n=84)
1	*Aspergillus nigar*	21%	*14%*
2	*Aspergillus flavus*	13%	*8%*
3	*Aspergillus fumigatus*	8%	*0%*
4	*Rhizophus stolonifer*	16%	*12%*
5	*Pencillium chrusogenum*	13%	*9%*
6	*Fusarium equiseti*	5%	*8%*
7	*fusarium avenaceum*	2%	*0%*
8	*Alternaria alternate*	3%	*0%*
9	*Yeast*	3%	*3%*
10	*Unknown fungus*	13%	*7%*

Table III: Antagonistic potential of Bacillus cereus isolates by Spot and Overlay Assay.

	zone of inhibition (mm)						
Indicator strains	*E.coli*	*Satmonella spp*	*Shigella spp*	*S.aureus*	*S.epidermis*	*M.luteus*	*Psudomonas spp*
7.1	-	-	-	-	-	-	-
8.1	14	-	-	-	-	-	-
11.1	12	-	-	-	-	-	-
11.2	13	-	-	-	-	-	-
11.3	11	-	-	-	-	-	-
11.4	9	-	-	-	-	-	-
12.1	-	-	-	-	-	-	-
12.2	10	-	-	-	-	-	-
12.3	-	-	-	-	-	-	-
12.4	-	-	-	-	-	-	-
14.1	9	-	-	-	-	-	-
14.2	-	-	-	-	-	-	-

7.1=isolate #1 from sample #10, 8.1=isolate#2 from sample #12, 11.1=isolate #3 from sample #21, 11.2=isolate #4 from sample #23, 11.3=isolate #5 from sample#28, 11.4=isolate #6 from sample # 34, 12.1=Isolate #7 from sample #36, 12.2=isolate #8 from sample #40, 12.3 =isolate#8 from sample #70.12.4=isolate#9 from sample37. 14.1=isolate#10 from sample 68.14.2=isolate#11 from sample #61.

Confirmation of extracellular antibacterial metabolites production by Bacillus cereus isolates

Bacillus cereus isolates Cell Free Culture Supernatant (100µl) were (significantly = 10mm) active against Escherichia coli. Bacillus cereus cultures were added to each well and noted as zone of inhibition (mm) after overnight incubation at 37oC as shown in the Table V.

Table IV: Agar Well Diffusion Assay as confirmatory test for antibacterial metabolite by test Bacillus cereus isolates.

	zone of inhibition (mm)						
Indicator strains	*E.coli*	*Satmonella spp*	*Shigella spp*	*S.aureus*	*S.epidermis*	*M.luteus*	*Psudomonas spp*
7.1	-	-	-	-	-	-	-
8.1	-	-	-	-	-	-	-
11.1	15	-	-	-	-	-	-
11.2	14	-	-	-	-	-	-
11.3	-	-	-	-	-	-	-
11.4	-	-	-	-	-	-	-
12.1	-	-	-	-	-	-	-
12.2	-	-	-	-	-	-	-
12.3	-	-	-	-	-	-	-
12.4	-	-	-	-	-	-	-
14.1	12	-	-	-	-	-	-
14.2	-	-	-	-	-	-	-
10.3	10	-	-	-	-	-	-
2.3	-	-	-	-	-	-	-
2.5	9	-	-	-	-	-	-

*Bacillus cereus isolate; **-** = no activity.

Antimicrobial potential of Chloroform solvent

The middle chloroform-insoluble interface (CL) layer obtained from the CFCS Bacillus cereus isolates had significantly inhibited the growth of Escherichia coli. The upper aqueous layer (CW) and chloroform layer (CCL) from the CFCS Bacillus cereus isolates examined low levels of antimicrobial activity. The upper aqueous layer (CW) and chloroform layer (CCL) layers found minor antimicrobial activity against Staphylococcus aureus and Micrococcus luteus as shown in the Table V.

Table V: Antagonistic potential of chloroform fraction of CFCS Bacillus cereus isolates

		zone of inhibition (mm)						
	Indicator strains	*E.coli*	*Satmonella spp*	*Shigella spp*	*S.aureus*	*S.epidermis*	*M.luteus*	*Psudomonas spp*
CCL	10.3	-	-	-	9	-	-	-
	11.1	-	-	-	12	-	-	-
	11.2	-	-	-	11	-	-	-
	14.1	-	-	-	10	-	-	-
CL	10.3	14	-	-	-	-	-	-
	11.1	16	-	-	-	-	-	-
	11.2	12	-	-	-	-	-	-
	14.1	10	-	-	-	-	-	-
CW	10.3	-	-	-	-	-	9	-
	11.1	-	-	-	-	-	8	-
	11.2	-	-	-	-	-	10	-
	14.1	-	-	-	-	-	11	-

CW= upper aqueous layer, CL= middle chloroform -insoluble interface, CCL= chloroform layer.

Thin Layer Chromatography

Six specific antagonistic Bacillus cereus isolates (13.1, 10.1, 5.1, 15.1, 3.1, and 6.1) were subjected to Thin layer chromatography; produced different

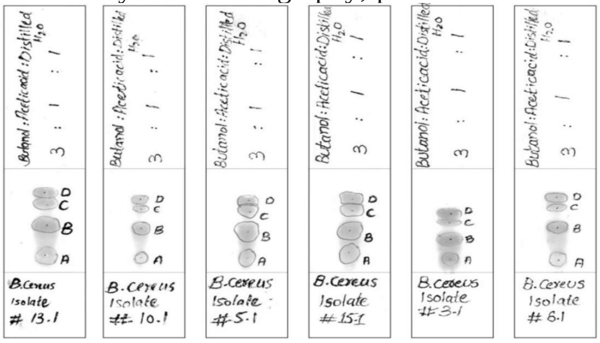

Figure I: Different number of components of Bacillus cereus isolate

Table VI: Retention factor Rf-value of Bacillus cereus isolates.

Sr. No	*Bacillus cereus* isolates	Chroma-togram	Solvent front (cm)	Compon-ents	Distance covered by components (cm)	Retention factor value	Average (R_f-value) of components
1	*B. cereus # 13.1*	Red-yellow	3.1	A	0.5	0.16	A= 0.14
				B	1.4	0.45	
				C	2	0.64	
				D	2.3	0.74	
2	*B. cereus # 10.1*	Red-yellow	3.1	A	0.5	0.16	
				B	1.4	0.45	
				C	2	0.64	B= 0.40
				D	2.4	0.77	
3	*B. cereus # 5.1*	Red-yellow	3.1	A	0.4	0.12	
				B	1.2	0.38	
				C	1.8	0.58	
				D	2.2	0.7	
4	*B. cereus # 15.1*	Red-yellow	3.1	A	0.5	0.16	C= 0.58
				B	1.2	0.38	
				C	1.8	0.58	
				D	2.2	0.7	
5	*B. cereus # 3.1*	Red-yellow	3.1	A	0.4	0.12	
				B	0.1	0.32	
				C	1.5	0.48	
				D	1.8	0.58	D= 0.70
6	*B. cereus # 6.1*	Red-yellow	3.1	A	0.4	0.12	
				B	1.4	0.45	
				C	1.9	0.61	
				D	2.3	0.74	

DISCUSSION

Examined that Total Viable Count (1.69×105CFU/mL) in uncooked rice and Total Viable Count (4.13×105CFU/mL) in cooked rice respectively, these findings agree with the present study in which Total Viable Count (2.74×105CFU/mL) in uncooked rice while the Total Viable Count (1.54×105CFU/ml) in cooked rice were similar [3].

The recommended limit of bacterial contamination for foods by International microbiological standards is 105 cfu/g for Total bacterial plate count [20,21,22] Bacillus cereus was not present in ready-to-eat vinegar rice samples, which was amazing since this pathogen has been usually; exist in rice and sushi [1, 23].

By analyzing, the chief Bacillus species were develop in cooked rice were Bacillus cereus 22%, contrary to these findings with [24] they reported that Bacillus cereus were 4.08 log CFU/g in cooked rice.

From scrutinizing the report [25], the prevalence of Bacillus cereus in cooked rice was found 12% contrary via the findings of [24], 100% in India, 92.9% and [26] 91.7% in the United States, [27], 10%–93% in the Europe, and [28] 93.9% in the England.

In this study, the Percentage of Bacillus cereus in pulawo was 12%. Contrary to the findings of other investigators reported that widespread of Bacillus cereus in pan-fried rice, [27] 12%–86% in the Netherlands, [25] 85.7% in the US, and [29] 33% in the UK.

In present study, fungal species isolated from uncooked and cooked rice were found followed by Aspergillus niger (27%), Rhizophus stolonifer (19%), Pencillium chrysogenum (13%), Aspergillus flavus (12%), Fusarium equiseti (9%), Aspergillus fumigatus (4%), Fusarium avenaceum (1%), Alternaria alternata (1%) respectively.

Contrary to the findings of the present studie different fungal species, were found in the uncooked and cooked rice followed by Rhizophus spp. (76%), Aspergillus flavus (42%), Mucor species (64%), and penicillium species (31%) respectively [30].

In the present studies, different fungi were found in raw rice were Aspergillus niger, Rhizophus stolonifer, Pencillium chrysogenum, Aspergillus flavus, Fusarium equiseti, Aspergillus fumigatus, Fusarium avenaceum, Alternaria alternata respectively.

Described the following fungi in Japanese raw rice: Aspergillus, Penicillium, Fusarium, Phoma, Curvularia, Helminthosporium, Cladosporium Arthrinium and Alternaria [31] that is contrary to the present studies. Species of Rhizopus, Alternaria, Aspergillus, Fusarium and Penicillium were the most frequently encountered fungi in raw rice [32].

CONCLUSION

This study indicates the presence of Bacillus species in stuffy food need not be insensitive, because of the endospores-forming species may inhabit in the outside of rice. Food-handlers must be command on secure implementation which include care cooked rice each of two at > 60oC (hot steaming) or chill quickly and shift to a refrigerator within 4 hours WHO (World Health Organization). Bacillus species linked with both vomiting and lose stool syndromes. The raw rice becomes contaminated due to poor preservation and handling.

REFERENCES

1. Adams, MH. In: Bacteriophages Hershey, A.D., ed., Interscience, New York, pp. 388-389 (1959).

2. Reinoso Pozo, Y Casades´us Romero, L Garc´ia Su´arez, A Guti´errez P´erez, J Pazos Alvarez-Rivera, V. de Pectobacterium carotovorum. Fitosanidad 10: 187–91 (2006).

3. Arifa Tahir, Isbah Hameed, Madiha Aftab And bushra Mateen. Pakistan Journal of Botany. 44: 267-270 (2012).

4. Sarrias, JA M Valero and MC Salmeron. Food Microbiology, 19: 589-595 (2002).

5. Varnam, AH and MG Evans. Institute Foodborne pathogens. (Eds.): Wolfe Publishing Ltd. pp. 267 (1991).

6. Drobniewski, FA. Clinical Microbiology 6: 324-338 (1993).

7. Euzeby, JP. International Journal of Systemetic Bacteriology. 47: 590-592. Last full update: January 26, 2008 (1997).

8. Auth, J. EFSA Journal. 175: 1–48 (2005).

9. Salkinoja-Salonen, MS Vuorio, R Andersson, MA Kampfer, P Food Poisoning and Applied Environmental Microbiology. 65: 4637–45 (1999).

10. Tena, D Mart´inez-Torres, JA P´erez- Pomata, MT S´aez-Nieto, JA Rubio, V Bisquert, J. report of 3 cases. Clinical Infectious Diseases. 44:40–42 (2007).

11. Jackson, SG Good brand, RB Ahmed, R Kasatiya . Applied Microbiology. 21: 103–5 (1995).

12. Hauge, S. Journal of Applied Bacteriology. 18: 591–595 (1955).

13. Sanla-Ead, N Jangchud, A Chonhenchob, V and Suppakul, P. The 15th IAPRI world conference on packaging (2006).

14. Cary, JW et al. Section Flavi (March-April 2005).

15. Kramer, JM PC Turnbull, G Munshi, and Rj Gilbert. In isolation and identification Methods for food poisoning Organisms (1982).

16. Parry, JM PC Turnbull and JR Gibson. Farbatlas der Bazillusarten: Anleitung zur Diagnose. Sschober Verlags-GmbH. D- 8355, Hengerberg (1983)

17. Sanla-Ead, N Jangchud, A Chonhenchob, V and Suppakul, P. The 15th IAPRI world conference on packaging (2006).

18. Buchanan, RE and NE Gibbons. Bergey's Manual of Determinative Bacteriology.9th ed. Williams and Wilkins Comp., Baltimore (1984).

19. Cappuccino, JG and Sherman, N. Microbiology laboratory manual, seventh edition. ISBN 978-81-317-1437-9 (2005).

20. Amon. Biological specifications for foods principles and specific applications (1974)
21. Refai, MK. Manual of Food quality control Microbiological Analysis (1979).

22. Andersson, MA Mikkola, R Helin, J Andersson, MC and Salkinoja Salonen, M. Applied Environmental Microbiology. 64: 1338–13 (1998).

23. Chen, Y Tenover, F and Koehler, TM. Antimicrobial Agents Chemotherapy. 48: 4873–4877 (2004).

24. Kramer, JM PC Turnbull, G Munshi, and Rj Gilbert. In isolation and identification Methods for food poisoning Organisms (1982).

25. Bryan, FL Bartleson, CA and Christopherson, N. Journal of Food Protection. 44: 500–512 (1981).

26. Harmon, SM and Kautter, DA. Journal of Food Protection. 54: 372–374 (1991).

27. Notermans, S and Batt, CA. Journal of Applied Microbiology. 84: 51S–61S. 29 (1998).

28. Nichols, GL Little, CL Mithani, V, and de Louvois, J. Journal of Food Protection. 62: 877–882 (1999).

29. 29 . Schiemann, DA. Journal of Food Protection. 41: 450–454 (1978) .

30. Reddy, CS K RN Reddy, N Raja, GSL Kumar and K Muralidharan. Journal of Mycology and Plant Pathology, 34: 816-820 (2004).

31. Uraguchi, K and Yamazaki, M. Toxicology: biochemistry and pathology of mycotoxins. Halsted press, Japan. pp. 1-278 (1978). 33).

32. Hussaini, A. Makun1*, Timothy, A. Gbodi1, Olufunmilayo, H. Akanya1, Ezekiel, A. Salako2 and Godwin, H. Journal. Biotechnol. Volume. 6 (2), pp. 099-108, 18 January 2007.