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CHARACTERIZATION OF *BACILLUS* SPP. FROM SOME SPICES AND ASSESSMENT OF THEIR SPOILAGE POTENTIAL IN VARIOUS TRADITIONAL ETHIOPIAN SAUCES

Feleke Moges[1] and Mogessie Ashenafi[2,*]

[1] Department of Biology, Faculty of Science

[2] Department of Microbiology and Immunology, Institute of Pathobiology
Addis Ababa University, PO Box 1176, Addis Ababa, Ethiopia

ABSTRACT: One hundred twenty five samples from five different Ethiopian sauce spices were examined for the incidence and level of contamination of *Bacillus* species. The spices consisted of fenugreek (*Trigenella foenum-graecum*), black cumin (*Nigella sativa*), Ethiopian caraway (*Trachyspermum ammi*), ginger (*Zingiber officinale*) and korarim cardamon (*Aframomum corrorima*). Spore counts ranged from log 1.63 cfu(g)$^{-1}$ in cumin to log 8.32 cfu(g)$^{-1}$ in ginger. Of the 781 *Bacillus* isolates obtained, the most frequently encountered species was *Bacillus pumilus* (43.7%) followed by *B. subtilis* (16.6%), *B. circulans* (11.2%), *B. licheniformis* (8.2%) and *B. cereus* (4.9%). The *B. pumilus* and *B. subtilis* isolates were active in proteolysis and lipolysis, whereas *B. cereus* isolates were more proteolytic and amylolytic. All test strains grew well in three different types of traditional Ethiopian sauces, but growth was markedly lower in vegetable-based sauces. Spoilage was manifested only in the form of foul odour, and was noted faster in legume-based sauces (< 24 hours) than in meat-based (24–36 hours) and vegetable-based (48–60 hours) sauces.

Key words/phrases: *Bacillus spp.*, Ethiopia, sauces, spices, spoilage

INTRODUCTION

Spices are known to be contaminated with bacteria, yeast and mould, causing spoilage of foods (Krishnaswamy *et al.*, 1971). This is because spice bearing plants are exposed to a wide variety of microorganisms from the environment

* Author to whom all correspondence should be addressed.

in which they are grown and harvested. Numerous species of bacteria, yeasts and moulds constitute the normal microflora of dried spices (Christensen *et al.*, 1967), although aerobic spore forming bacteria usually predominate (Kovacs-Domjan, 1988). However, the incidence and number of amylolytic and proteolytic organisms, thermophilic spore formers, yeasts and mould, and total microorganisms vary among the different spices as well as within each type of spices (Julseth and Deibel, 1974).

The level of consumption of spices by man is low when compared to the consumption of staple cereals like corn. However, some spices are considered to be high risk commodities in supporting fungal growth, aflatoxin and heavy loads of bacteria (Tjaberg *et al.*, 1972; Madhyastha and Bahat, 1985). They are also considered as sources of spoilage microorganisms to food products (Julseth and Deibel, 1974).

Although spices are not produced in large quantities, their significance in Ethiopia can hardly be over-estimated (Jansen, 1981). Spices are needed every day in considerable amounts for the preparation of the main dish of the day (Goettsch, 1991). Ethiopian sauces are usually hot-spiced made of a variety of ingredients. Different sauces have different flavours depending on the type and amount of spices and other constituents, the extent of cooking and other factors. The sauces are basically legume-based, vegetable-based or meat-based. Legume-based sauces are frequented in low income families and during fasting periods, whereas meat based sauces are mostly luxuries for most families. In most households, sauces are usually prepared early in the day and are supposed to last until dinner or kept over night at ambient temperatures.

Although post-cooking contamination of Ethiopian sauces could contribute to spoilage (Mogessie Ashenafi, 1996), *Bacillus* spp. were found to be the dominant members of the spoilage flora in such sauces (Mogessie Ashenafi, 1997). The aerobic spore forming bacilli are among the most important groups of microorganisms occurring in food due to their versatile metabolism, strong saccharolytic and proteolytic activity as well as the high resistance of their spores to heat (Priest, 1977). Furthermore, it would be evident that with increasing degrees of heat treatment, spores become increasingly important, because they are likely to survive while competing species are eliminated (Ingram, 1969).

The objectives of this study was, thus, to characterize the common aerobic spore formers from spices used in the preparation of some traditional sauces. The proteolytic, lipolytic and amylolytic activities of some of the dominant *Bacillus* isolates were determined to assess their potential as spoilers. Time and type of spoilage of some common sauces were also evaluated using the frequently isolated *Bacillus* spp.

MATERIALS AND METHODS

Collection and processing of samples

A total of 125 samples of unprocessed dry spices comprising of Black kumen (*Nigella sativa*), "tikur azmud"; Ethiopian caraway (*Trachyspermum ammi*), "nech azmud"; Fenugreek (*Trigon ellafoenum-graecum*), "abish"; Ginger (*Zingiber officinale*) "zingibel" and Korarima cardamom (*Aframomum corrorima*) "korarima" were collected using sterile containers from different local markets in Addis Ababa, Ethiopia. Twenty five grams of dried and ground spices (moisture content, <10%) were homogenized in 225 ml sterile 1% peptone water.

Appropriate dilution of the sample was immersed in a water bath and heated at 80° C for 10 minutes. A volume of 0.1 ml was surface plated in duplicate on Plate Count agar (OXOID) plates and incubated at 32° C for 24–48 h to count germinated spores. Ten to twenty colonies were picked at random from countable plates, purified by repeated plating and transferred to nutrient agar slants for further identification to the species level.

Identification of Bacillus *spp.*

Isolates were tentatively identified to the species level using the simplified identification scheme of Berkeley *et al.* (1984). Anaerobic growth was assessed in anaerobic agar according to (Dea'k and Timar, 1988). Production of acetyl methyl carbinol (acetoin) was determined on MR-VP medium (OXOID) following the methods of Collins and Lyne (1976). Nitrate reduction was evaluated as in Edwards and Ewing (1972). Enzymatic hydrolysis of starch by amylases was detected according to Claus and Berkeley (1986). The method of Aneja (1993) was used to see acid and gas production from glucose. Growth in 7% NaCl, at

50° C and 65° C was determined as in Claus and Berkley (1986). Utilization of citrate as a sole source of carbon was conducted on Simmon's citrate agar (OXOID).

Assessment of growth potential of test strains and their growth potential in traditional sauces

Legume-based, vegetable-based and Meat-based sauces were prepared following traditional methods (Mogessie Ashenafi, 1996) and the sauces were filled in test tubes in 20 ml amounts and sterilized at 121° C for 15 minutes. For the determination of growth and spoilage potential of *Bacillus* spp. in various sauces the most frequently isolated spp. and the known food poisoning spp. were selected. Ten isolates of each of *B. pumilus*, *B subtilis* and *B. cereus* were, thus, considered for this study. A loopful of the test strains was inoculated in to a sterile Brain Heart Infusion broth (OXOID). After incubation for 24 h at 32°C, the broth was separately inoculated into the various sauces in duplicates to give a final inoculum level of 10^2–10^3 cfu(ml)$^{-1}$. The inoculated sauces were mixed thoroughly and left at ambient temperatures for 72 hours.

About 0.1 ml of appropriate dilution from freshly inoculated sauces were surface plated on Nutrient agar in duplicate to determine the initial inoculum level. The various sauces were then sampled at 6 hour intervals. Inoculated plates were incubated at 37° C for 24–48 hours for colony counting. Inoculated sauces were kept at ambient temperature and checked periodically at 6 h intervals for signs of food spoilage (foul odour or gas production) (Mogessie Ashenafi 1996; 1997). The signs of food spoilage were checked by three different individuals aseptically in disinfected hood.

Detection of proteolytic, lipolytic and amylolytic activities of some* Bacillus *species isolated from spices

The three test species considered in the spoilage study were also studied for their enzymatic activities. Proteolytic activity was detected on Calcium Caseinate Agar. Lipolytic activity was determined using Tributyrin agar. Amylolytic activity was tested on Starch Agar as in Claus and Berkeley (1986).

To see if there was significant variation in counts among samples of the same spice, coefficient of variation (CV) was calculated by dividing the standard deviation by the mean.

RESULTS

The highest average spore count [8.32 log $cfu(g)^{-1}$] was noted in Ginger and this was not significantly different within samples of the same spice (coefficient of variation, CV, < 10%) (Table 1). Although Black Cumin yielded the smallest average count of spores [1.63 log $cfu(g)^{-1}$], the counts in 13 of the 25 samples was below detectable levels [< 1 log $cfu(g)^{-1}$], resulting in quite significant variation within samples (CV, 117%). The average spore counts in the other three spices ranged between 4 log and 5 log $cfu(g)^{-1}$ and the coefficient of variation ranged between 15 and 25%.

Table 1. Average counts [log $cfu(g)^{-1}$] of aerobic spore formers in five different spices.

Spices	Count				Standard deviation	Coefficient of variation (%)
	No.	Max	Min	Mean		
Fenugreek	25	7.68	3.30	4.55	1.15	25
Black Cumin	25	4.98	0	1.63	1.91	117
Ethiopian Caraway	25	5.90	3.38	4.33	0.67	15
Ginger	25	8.32	6.30	7.53	0.48	6.3
False Cardamom	25	7.80	3.68	5.33	1.07	20

A total of seven hundred eighty one isolates of *Bacillus* spp. were isolated from the above mentioned spices. The *Bacillus* flora was dominated by *B. pumilus* (43.7%), *B. subtilis* (16.7%), *B. circulans* (11.3%), *B. licheniformis* (8.8%) and *B. cereus* (4.9%). The other ten *Bacillus* species constituted < 10% of the total isolates (Table 2).

Table 2. Frequency distribution of *Bacillus* isolates from five different spices.

Isolated spp.	FG	BK	ET	GG	KO	Total	
						No.	%
B. Pumilus	77	11	51	103	99	341	43.7
B. subtilis	33	3	41	24	29	130	16.7
B. circulans	19	10	28	19	12	88	11.3
B. licheniformis	12	12	15	16	14	69	8.8
B. cereus	14	6	9	1	8	38	4.9
B. megaterium	9	2	11	8	4	34	4.4
B. sphaericus	12	3	7	3	8	33	4.2
B. alvei	1	2	7	4	-	14	1.8
B. brevis	2	-	1	2	3	8	1.0
B. firmus	1	1	4	1	-	7	0.9
B. stearothermophilus	-	1	3	1	-	5	0.6
B. coagulans	-	-	1	-	3	4	0.5
B. larvae	-	1	-	1	2	4	0.5
B. lentimorbus	1	-	1	2	-	4	0.5
B. papilliae	-	-	-	1	1	2	0.2
Total	181	52	179	186	183	781	100.0
(%)	23.2	6.7	22.9	23.8	23.4		

FG, Fenugreek; BK, Black Cumin; ET, Ethiopian Caraway; GG, Ginger; and KO, Korarima cardamom.

All spice types yielded seven major *Bacillus* species at varying frequencies (Table 2). *B. pumilus* and *B. subtilis* were the most frequently isolated species from all spice types except from Black Cumin. The extent of distribution of the test strains in various spices showed that, although *B. pumilus* was dominant in all spices, the highest number was observed in Ginger and the lowest in Black Cumin (Table 2). For *B. subtilis* the highest and lowest counts were obtained in Ethiopian Caraway and Black Cumin, respectively. The *B. cereus* count in all spices was lower than that for the above mentioned test strains. However, relatively higher number of *B. cereus* isolates were obtained from Black Cumin and the lowest from Ginger.

Of the 15 different *Bacillus* species encountered in this study, two of the most frequently isolated species, *B. pumilus* and *B. subtilis*, and the potentially food poisoning species, *B. cereus*, were selected and their growth pattern and spoilage potential in legume-based, vegetable-based and meat-based sauces was determined. *B. pumilus* showed almost a similar growth pattern in all sauce types, but counts in legume-based sauces were higher than those in the other sauces (Fig. 1). *B. subtilis* increased by about 3 log units between 6 h and 12 h in legume- and meat-based sauces and reached counts as high as 7 log $cfu(ml)^{-1}$ at 24 h. Its growth was, however, markedly low in vegetable-based sauce and the count at 24 h was slightly higher than 5 log $cfu(ml)^{-1}$ (Fig. 2). *B. cereus* increased steadily in legume- and meat-based sauces and reached maximum counts at 18 h. In vegetable-based sauce, however, it increased by slightly over 1 log unit within 24 h (Fig. 3).

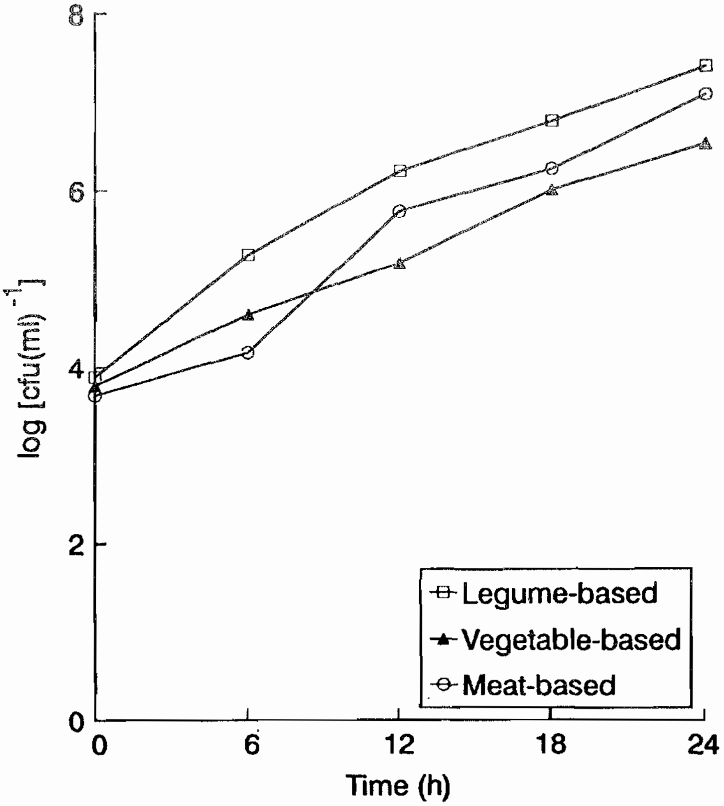

Fig. 1. Growth pattern of *Bacillus pumilus* in legume-based, meat-based and vegetable-based sauces.

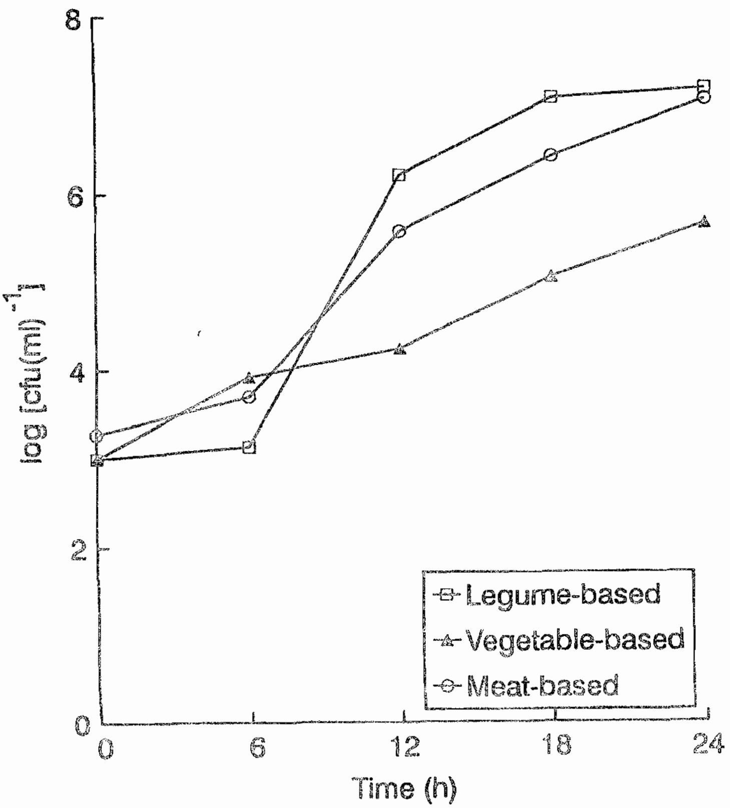

Fig. 2. Growth pattern of *Bacillus subtilis* in legume-based, meat-based and vegetable-based sauces.

Spoilage of the various traditional sauces by our test organisms was observed at 6 h intervals for 72 h. Spoilage was manifested mainly through production of foul odour. Gas production was not detected in all cases (Table 3). Twenty-six of the thirty test organisms resulted in foul odour in legume-based sauces within 24 h. Foul odour in meat-based sauces was detected between 24–48 h. In vegetable-based sauces 26 of the 30 test organisms caused foul odour between 48 and 60 h.

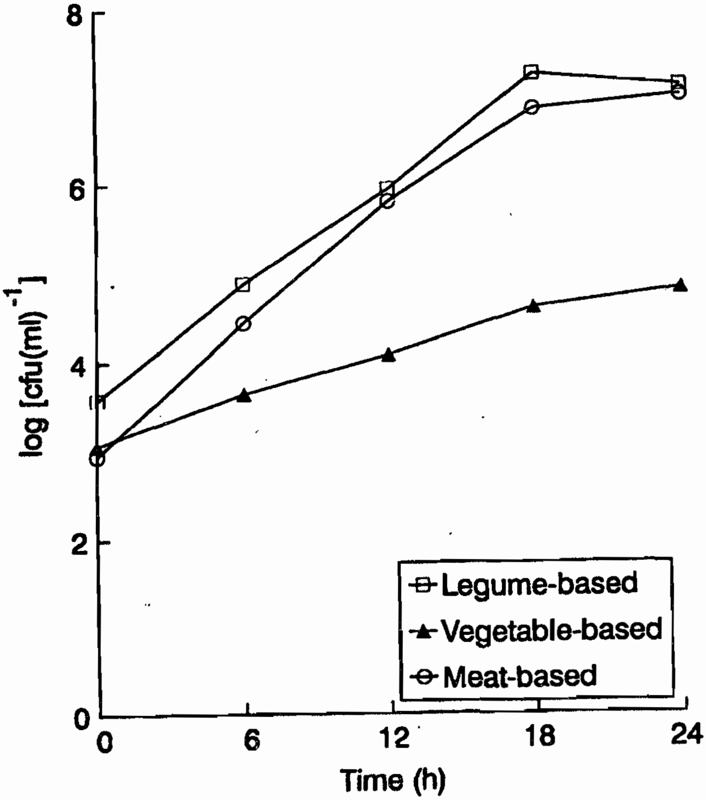

Fig. 3. Growth pattern of *Bacillus cereus* in legume-based, meat-based and vegetable-based sauces.

Half of the *B. pumilus* isolates used in the test showed strong proteolytic activity and almost all showed strong lipolytic activity. None manifested amylolytic activity (Table 4). *B. subtilis* also showed strong proteolytic and lipolytic activity, but most were more important as proteolytic than lipolytic strains. Strong amylolytic activity was noticed in few. Although about half of *B. cereus* test strains exhibited strong proteolytic and lipolytic activity, all were also able to manifest a degree of amylolytic activity (Table 4).

Table 3. Spoilage pattern of the various sauces by test organisms.

Test Organisms	Sample number	Attribute	Number of samples showing the attributes: Legume-based 12h	24h	36h	48h	60h	Vegetable-based 12h	24h	36h	48h	60h	Meat-based 12h	24h	36h	48h	60h
B. pumilus	10	Foul odour	-	8	2	-	-	-	-	2	5	3	-	2	5	3	-
B. subtilis	10	Foul odour	-	10	-	-	-	-	1	1	5	3	-	7	3	-	-
B. cereus	10	Foul odour	-	8	2	-	-	-	-	-	4	6	-	5	2	2	1

Table 4. Proteolytic, lipolytic and amylolytic activities of some *Bacillus* species isolated from spices. (++, Strong positive; +, Positive; -, No odour).

Test organisms	Number of isolates	Proteolysis	Lipolysis	Amylolysis
Bacillus pumilus	25	13++ 12+	22++ 3+	25 -
Bacillus subtilis	25	20++ 5+	12++ 13+	20+ 5++
Bacillus cereus	21	9++ 12+	10++ 8+ 3 -	21+

DISCUSSION

The dominant role in the microbiology of spices is played by aerobic spore formers (Kovacs-Domijan, 1988). Other vegetative forms are usually absent in spices mainly due to their susceptibility to desiccation and toxicity of spices (Christensen *et al.*, 1967; Julseth and Deibel, 1974).

Of the spices considered in this study, Ginger had the highest count of spore formers and this was consistently observed in all samples as also reported by other workers (Tjaberg *et al.*, 1972). This may be due to the underground nature of the spice part of the plant, thus resulting in close contact with soil. In addition to the fact that Ginger contains sufficient moisture that can allow the survival of certain groups of microorganisms, the essential oils of Ginger are reported to be less effective against microorganisms (Meena and Sethi, 1994). According to Madhyastha and Baht (1985), Ginger and Black Pepper are better substrates for fungal growth and aflatoxin production. Ginger is commonly added in mild-type Ethiopian sauces and, in certain cases, in some hot meat-based sauces (e.g., chicken sauce) as an important condiment. It may, thus, be an important source of spoilage microorganisms to sauces.

False Cardamon, Ethiopian Caraway and Fenugreek also contained substantial amount of spore formers in all samples and may be considered as possible sources of spoilage microorganisms to the various sauce types. The fact that no spore formers were detected in half of the Black Cumin samples makes this spice not a major source of spoilage microorganisms to sauces. The low count may be due to the heat processing of the samples. According to Blank *et al.* (1988), heat treatment at 80° C for 10 minutes in certain spice mixtures resulted in a great reduction of the initial count. The high frequency of isolation of *B. cereus* in black cumin than in the other spices could be due to the nature of the spore which is reported to have great resistance to essential oils of some spices (Meena and Sethi, 1994).

Of the 15 different *Bacillus* spp. encountered in this study, *B. pumilus, B. subtilis, B. licheniformis* and *B. cereus* constituted about 85% of the total isolates. These were isolated from all types of spices at varying frequencies and may be considered as important sources of spoilage microorganisms to various sauces. Similar studies from other countries showed that *B. subtilis* and *B. pumilus* were the most frequently isolated species from spices in Malaysia (Mohammed *et al.*, 1986) and these species were also the dominant isolates in spices and other foods in Hungary (Dea'k and Timar, 1988). In Nigeria, however, *B. polymyxa* and *B. coagulans* were part of the dominant flora in addition to *B. subtilis* and *B. cereus* (Antai, 1988). In Canada, the dominant isolate was *B. cereus* (Seenapa and Kempton, 1981).

Eight other *Bacillus* spp., although encountered in the various spices, constituted a very small proportion of the *Bacillus* flora. These may not, thus, be considered as spoilage species in sauces where these spices are included. The number of thermophilic 'flat sour' groups are limited in all spices as reported by Krishawamy *et al.* (1971) and Dea'k and Timar (1988).

All our test strains effected spoilage through production of foul odour and no gas production, as sign of spoilage, was noted. In other studies on spoilage, only a very small proportion of *Bacillus* spp. were able to produce gas (Al-Diejaili and Anderson, 1991; Mogessie Ashenafi, 1997). In Ethiopian sauces, the major gas producing spoilers belonged to the Enterobacteriaceae (Mogessie Ashenafi, 1997), which are usually post-cooking contaminants. The spoilage pattern of the most dominant isolate, *B. pumilus*, was mainly lipolytic and this may be indicative of the spoilage role of *B. pumilus* in sauces with sufficient fat. As most *B. subtilis* test strains showed quite strong proteolytic activity, *B. subtilis* may rather be considered as a very strong spoiler by producing foul odour. This could be noted in the production of foul odour in the protein-rich sauces (legume-based and meat-based), where all or most of our *B. subtilis* test strains showed foul odour within 24 hours. Production of foul odour in vegetable-based sauces was mostly detected after 48 hours.

A good proportion of the *B. cereus* test strains could produce foul odour in legume-based sauces within 24 hours. Similar pattern was also observed in meat-based sauces but to a lesser extent.

In most households in Ethiopia, sauces are usually consumed the same day they are prepared (Mogessie Ashenafi, 1996). Therefore, spore-formers may not play a major role as spoilers in traditional sauces. However in compelling situations where sauces have to be kept longer at ambient temperatures, legume-based and meat-based sauces seem to be susceptible to spoilage by such microorganisms. Meat and legumes contain sufficient proteins to be acted upon by proteolytic organisms and to produce foul odour. Production of foul odour in vegetable-based sauces by all test strains was mainly detected after 48 hours. Vegetable-based sauces were able to retard the rate of production of foul odour mainly due to the absence of sufficient degradable proteins or fats and also due to the presence of certain antimicrobial substances that retard microbial growth (Marchetti *et al.*, 1992).

The growth pattern of the test strains in the various sauces supported this argument. Growth was steady in legume-based and meat-based sauces in most cases and the number reached at 24 h [log 7 $cfu(ml)^{-1}$] was high enough to result in sufficient amounts of proteolytic or lipolytic enzymes to effect spoilage through foul odour production. The delay of spoilage in vegetable-based sauces could, however, be due to the low rate of growth of the various test strains in the sauces and the resulting low count at 24 h.

Although *B. cereus* was shown to produce spoilage at 24 h in legume-based and meat-based sauces, the count reached at this time could be high enough to produce sufficient toxins that could result in food poisoning.

It may also be noted that, although spices may be considered as sources of spoilage microorganisms, they may also exhibit some inhibitory effect on certain microorganisms (Aureli *et al.*, 1992).

The *Bacillus* species would not be killed by the temperature that most traditional sauces are normally cooked (Mogessie Ashenafi, 1996). This temperature may rather serve as a heat shock to activate spores to germinate. Therefore, methods to avoid spoilage should include adequate cooking followed by immediate consumption or continued cold storage. It the case of Ethiopia, however, where cold storage is not affordable by most households, longer keeping for more than 12 hours at ambient temperatures should be avoided.

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