IDENTIFICATION of *Bacillus* SPECIES ISOLATED FROM ROPEY BREADS BOTH with CLASSICAL METHODS and API IDENTIFICATION KITS[1]

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Abstract

In this study, white and whole meal breads baked at laboratory conditions were stored at 37 ºC for 7 days to isolate and identify the *Bacillus* species responsible for the development of rope spoilage which is the most important bacterial problem in breads. The *Bacillus* species which were isolated from ropey breads were identified by using classical methods and API identification kits. It was understood that definite identification of some of the isolates using these methods was not possible and these strains could only be identified as being most likely a certain species. Isolates from white bread were identified as *B. subtilis, B. megaterium, B. licheniformis, B. coagulans* and *B. pumilus*; and isolates from whole meal bread were identified as *B. subtilis, B. megaterium, B. licheniformis* using biochemical tests. Using the API identification kits, on the other hand, resulted in the identification of *B. licheniformis, B. pumilus, B. subtilis/amyloliquefaciens,* 'most likely' *B. megaterium* in white breads and *B. licheniformis, B. subtilis/amyloliquefaciens,* 'most likely' *B. megaterium,* 'most likely' *B. thuringiensis* and *Bacillus* spp. in whole meal breads. According to the results of the classical methods *B. subtilis* was the most abundant species in both white and whole meal breads. API kits, on the other hand, confirmed *B. licheniformis* as the predominant species.

Keywords: *Bacillus*, Ropey Bread, Isolation, Identification

Sünmüş Ekmekten İzole Edilen *Bacillus* Türlerinin Klasik Yöntemler ve API Kitleri ile Tanılanması

Özet

Bu çalışmada, ekmeklerdeki bakteriyel bozulmaların en önemlisi olan sünme hastalığından sorumlu *Bacillus* türlerini izole etmek ve tanılamak için normal ve kepekli ekmekler, laboratuvar koşullarında pişirilmiş ve 37 ºC'de 7 gün boyunca muhafaza edilmiştir. Sünmüş ekmeklerden izole edilen *Bacillus* türleri klasik yöntemler ve API kitleri kullanılarak tanılanmıştır. İzole edilen bazı suşların kesin olarak tanılanmasının bu yöntemlerle mümkün olmadığı, bu suşların ancak muhtemelen tanılanabildiği anlaşılmıştır. Biyokimyasal testler ile normal ekmeklerden izole edilen suşlar *B. subtilis, B. megaterium, B. licheniformis, B. coagulans* ve *B. pumilus*; kepekli ekmeklerden izole edilen suşlar *B. subtilis, B. megaterium, B. licheniformis* olarak tanılanmıştır. API tanılama kitleri kullanıldığında ise tanılama, normal ekmekler için *B. licheniformis, B. pumilus, B. subtilis/amyloliquefaciens,* muhtemelen *B. megaterium;* kepekli ekmekler için *B. licheniformis, B. subtilis/amyloliquefaciens,* muhtemelen *B. megaterium,* muhtemelen *B. thuringiensis* ve *Bacillus* spp. şeklinde sonuçlanmıştır. Biyokimyasal test sonuçlarına göre hem normal hem de kepekli ekmeklerde *B. subtilis* en fazla bulunan tür olarak belirlenirken; API kitleri ile *B. licheniformis*'in baskın tür olduğu saptanmıştır.

Anahtar Kelimeler: *Bacillus*, Sünmüş Ekmek, İzolasyon, Tanılama

1. Introduction

Bacillus species are Gram-positive, aerobic, rod-shaped and endospore forming bacteria commonly found in nature. By means of their spores they are resistant to adverse environmental conditions and may cause food spoilage (Bailey and von Holy, 1993). Rope which is one of the most important diseases in breads, results in

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economic losses as well as possible food-borne illness risks, and is caused by *Bacillus* species (Bailey and von Holy, 1993; Kirschner and von Holy, 1989; Thompson et al., 1998; Volavsek et al., 1992).

Rope is a type of spoilage seen in bread especially in regions where the climate is warm and moist. This spoilage is considered to be a bread disease. The disease factor is generally *B. subtilis* (formerly referred to as *B. mesentericus*), however *B. licheniformis*, *B. megaterium*, *B. pumilus* and *B. cereus* can also be the causative agents (Collins et al., 1991; Kirschner and von Holy, 1989). These bacteria are of soil origin and may contaminate bread through the raw materials and bakery equipments used (Bailey and von Holy, 1993), proliferate under inappropriate production conditions and retain their viability by sporulating during baking. The spores which have survived the baking process, where the temperature in the center of the crumb may not rise above 100 °C, germinate and cause the rope disease under conditions of storage which are not suitable for the storage of bread. Stacking the loaves of bread closely together after baking is an example of unsuitable storage conditions of bread, thus delaying the cooling process, encourages the formation of rope disease (Kirschner and von Holy, 1989; Smith et al., 2004).

Initial symptoms of the disease are an unpleasant odor similar to that of rotting melons or pineapples and patchy discoloration of the central portions of the loaf. Then the crumb becomes very soft and when broken open, shows fine web-like strands which gives the condition its name (Kirschner and von Holy, 1989; Thompson et al., 1993; Voysey, 1989). These distinctive characteristics occur by degradation of starch and proteins in the bread crumb, caused by microbial amylases and proteases secreted by the vegetative cell and its sticky nature is due to the extracellular slimy polysaccharides formed by certain rope-producing strains of *Bacillus* (Bailey and von Holy, 1993; Rosenkvist and Hansen, 1995; Volavsek, 1992).

Microbial flora of the wheat, consequently, the flora of bread changes depending on the regional diversity. Thus, *Bacillus* species, the causative agents of the rope spoilage, show discrepancies according to the region (Kirschner and von Holy, 1989).

Several studies were made for the inhibition of rope spoilage including usage of antimicrobials such as organic acids (Pattison et al., 2004), sourdough, nisin (Rosenquist and Hansen, 1998), lactic acid bacteria (Katina et al., 2002; Menteş et al., 2007; Pepe et al., 2003) and propionic acid-producing bacteria (Marshall and Odame-Darkwah, 1994; Odame-Darkwah and Marshall, 1993).

Due to the phenotypic similarities between the strains of *Bacillus* species and the need for stringently controlled conditions during the identification, it is difficult to characterize the closely related species with classical methods. The use of API identification strips have been shown to give more reliable and reproducible results than classical methods (Collins et al., 1991; Logan and Berkeley, 1984; Thompson et al., 1993).

The aim of this paper was to identify the rope-producing strains of *Bacillus* species isolated from ropey white and whole meal breads baked at laboratory conditions by using classical methods and API identification kits. The isolated strains were tested to confirm their rope-producing ability. The main goal was to compare the results of both identification methods as well as to determine whether there were differences in prevalent strains depending on the type of bread.

2. Materials and Methods

2.1. Bacillus strains

Bacillus subtilis ATCC 6633 used as the reference strain was purchased from Refik Saydam Hygiene Center, Ankara. *Bacillus* strains were isolated from ropey white and whole meal breads. Isolated and reference strains of *Bacillus* were grown in nutrient broth or nutrient agar (Merck, Germany) at 37 °C for 18–24 hours, as required by the identification method.

2.2. Preparation of the breads

Test bakes were carried out at laboratory conditions. Two hours after baking, the white and whole meal breads sprayed with a mixture of 40% sodium propionate/propionic acid to prevent mold growth were individually wrapped in polyethylene bags and stored at 37 ºC for 7 days.

2.3. Isolation of Bacillus species

When the symptoms of the disease were apparent, approximately 10 g of crumb from the center of the loaves were sampled into a stomacher bag aseptically and homogenized using a stomacher (Seward 80, England) with 90 mL sterile saline for 5 minutes. Then, 10 fold dilutions were prepared and plated on nutrient agar (Merck, Germany) by the pour plate method. Plates were incubated at 37 ºC for 24 h.

Morphologically different colonies were selected from the agar plates, streaked on nutrient agar plates to purify and plates were incubated at 37 ºC for 24 h. At the end of this incubation period, a total of 22 isolates from white breads and 19 isolates from whole meal breads were isolated. The isolates were inoculated into nutrient broth (Merck, Germany) and stored in 50% glycerol at -80 °C for further analysis.

2.4. Identification of Bacillus species

Identification was performed using classical methods and API identification kits, API 20E and API CHB50 (Biomerieux, France). Biochemical tests (Gram staining, determination of incubation temperature, catalase, growth in NaCl, anaerobic growth, Voges-Proskauer and Methyl-Red test, growth at pH 5.7, fermentation of carbohydrate, hydrolysis of starch, utilization of citrate, formation of indole, formation of dihydroxyacetone, deamination of phenylalanine, hydrolysis of casein, degradation of tyrosine, hydrolysis of gelatin, egg yolk lecithinase, growth with lysozyme present) were executed according to methods described by Sneath (1984). Identification according to the biochemical tests were based on comparison of the test results with dichotomous keys. API kits were used according to manufacturer's instructions and identification was done with API-web program. To identify an organism API software compares the profiles obtained with the profiles of taxa in the database and assigns a positivity percentage to each test which is then interpreted as 'excellent identification', 'very good identification', 'good identification' or 'acceptable profile'. In cases where the percentage is low, it is possible to obtain comments such as 'not reliable identification', 'doubtful identification' or 'unacceptable profile'.

2.5. Confirmation tests for the determination of rope producing ability of identified Bacillus strains

Confirmatory tests were done in order to determine the rope producing ability of isolates identified as *B. subtilis, B. licheniformis, B. megaterium, B. pumilus and B. thuringiensis*. For this purpose, *Bacillus* strains were grown overnight at 30 °C in bread extract broth (BEB) for 24 hours. The medium was prepared according to Pepe et al. (2003). The individual cultures of potential rope producing strains and their factorial combinations were homogeneously distributed on autoclaved (121 °C, 15 min) slices of white bread. The amounts distributed totaled 5 mL culture volume. The slices were incubated at 37 °C and examined daily for rope spoilage.

3. Results

3.1. Identification of Bacillus isolates

A total of 41 isolates, 22 from white bread and the remaining 19 from whole meal bread were identified by using classical methods and API identification kits. All of the strains isolated were determined as Gram-positive and rod-shaped bacteria.

In this study, some of the isolates

were determined as the same species with both methods while some of the identification results were contradictory. Identification results of both methods were shown in Table 1. Both methods provided the same identification for 13 of the 41 strains. Of these 13 strains, six were *B. licheniformis*, four were *B. megaterium*, two were *B. subtilis* and one was *B. pumilus*. Isolates confirmed as *B. megaterium* by biochemical tests were identified as most likely being *B. megaterium* with the API kits. Identification of 18 isolates could be done only by biochemical tests. Of these isolates, 11 were *B. subtilis*, 6 were *B. megaterium* and one was *B. coagulans*. Strains K1 and K7, identified respectively as *B. subtilis* and *B. licheniformis* by biochemical tests could only be confirmed as *Bacillu*s spp. by the API software. It was not possible to identify strain K3 by either of the two methods. Two of the isolates, strains N3 and N6, were identified by the biochemical methods as *B. subtilis*, whereas using the API method the same strains were classified as *B. pumilus*. As seen in Table 1, strains N2, K6 and K15 were identified using the API kits as being either *B. subtilis* or *B. amyloliquefaciens*. Using the API kits, strain N9 could not be identified and strain N13 was identified as *B. licheniformis*, although both strains were identified as *B. coagulans* by biochemical tests.

Figure 1 shows the percentage distribution of 22 isolates of *Bacillus* isolated from white breads identified using (a) classical methods and (b) the API kits. It can be seen from this figure that there are obviously considerable discrepancies between the results of the two methods. One distinct feature of the API kits is that identification of about 55% of the strains found in white breads was not possible due to unacceptable profile results. In this study 50% of the isolates were identified as *B. subtilis* by the biochemical tests, whereas using the API kits only 4.55% of the same isolates were identified as *B. subtilis* / *B. amyloliquefaciens*. This situation can be attributed to the fact that approximately 55% of the isolates could not be identified by API kits at all. Using the biochemical tests however, most of the same isolates were identified as *B. subtilis* (see Table 1).

A similar situation was evident for *B. megaterium*. In white bread, 22.73% of the total isolates were identified as *B. megaterium* by classical tests, whereas the percentage was only 4.55% when the API kits were used.

Differentiation of *B. coagulans* from *B. licheniformis* was difficult using classical methods. Only 3 of the tests applied provide differentiation for these two organisms. *B. coagulans* is only present in Figure 1a. *B. licheniformis* and *B. pumilus*, on the other hand, the situation is the opposite. Use of API kits resulted in identification of higher numbers of strains as *B. licheniformis* and *B.*

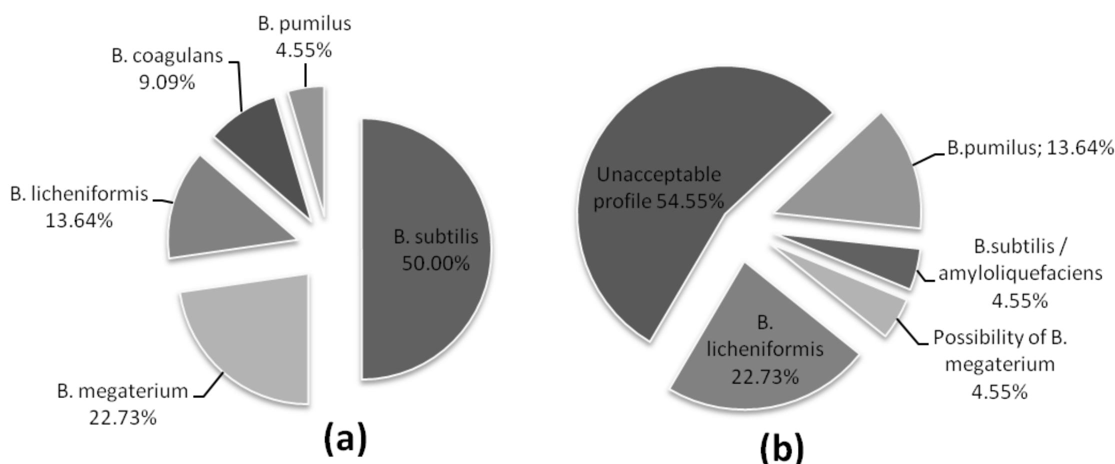

Figure 1. Percentage Distribution of 22 Isolates of *Bacillus* Isolated from White Breads Identified by Using (a) Classical Methods and (b) API Kits

Table 1. Identification Results of Both Methods

Isolates	Biochemical tests	API CH
N1	*Bacillus licheniformis*	*Bacillus licheniformis*
N2	*Bacillus subtilis*	*Bacillus subtilis / Bacillus amyloliquefaciens*
N3	*Bacillus subtilis*	*Bacillus pumilus*
N4	*Bacillus subtilis*	UP[a]
N5	*Bacillus megaterium*	UP
N6	*Bacillus subtilis*	*Bacillus pumilus*
N7	*Bacillus megaterium*	Possibility of *Bacillus megaterium*
N8	*Bacillus megaterium*	*Bacillus licheniformis*
N9	*Bacillus coagulans*	UP
N10	*Bacillus licheniformis*	*Bacillus licheniformis*
N11	*Bacillus subtilis*	UP
N12	*Bacillus subtilis*	UP
N13	*Bacillus coagulans*	*Bacillus licheniformis*
N14	*Bacillus subtilis*	UP
N15	*Bacillus subtilis*	UP
N16	*Bacillus subtilis*	UP
N17	*Bacillus megaterium*	UP
N18	*Bacillus megaterium*	UP
N19	*Bacillus licheniformis*	*Bacillus licheniformis*
N20	*Bacillus pumilus*	*Bacillus pumilus*
N21	*Bacillus subtilis*	UP
N22	*Bacillus subtilis*	UP
K1	*Bacillus subtilis*	*Bacillus* sp.
K2	U[b]	*Bacillus licheniformis*
K3	U[b]	UP
K4	*Bacillus subtilis*	UP
K5	*Bacillus subtilis*	UP
K6	*Bacillus subtilis*	*Bacillus subtilis /Bacillus amyloliquefaciens*
K7	*Bacillus licheniformis*	*Bacillus* sp.
K8	*Bacillus megaterium*	Possibility of *Bacillus megaterium*
K9	*Bacillus licheniformis*	*Bacillus licheniformis*
K10	*Bacillus licheniformis*	*Bacillus licheniformis*
K11	*Bacillus megaterium*	Possibility of *Bacillus megaterium*
K12	*Bacillus megaterium*	Possibility of *Bacillus megaterium*
K13	*Bacillus megaterium*	UP
K14	*Bacillus megaterium*	UP
K15	*Bacillus subtilis*	*Bacillus subtilis /Bacillus amyloliquefaciens*
K16	*Bacillus subtilis*	Possibility of *Bacillus thuringiensis*
K17	*Bacillus licheniformis*	*Bacillus licheniformis*
K18	*Bacillus subtilis*	UP
K19	*Bacillus megaterium*	UP

[a] UP: unacceptable profile [b] U: unidentifiable

pumilus. The percentage distributions of both were higher in Figure 1b. This could be attributed to the higher specificity of the API kits towards the identification of these two species in contrast to the classical biochemical tests where most of the same strains were identified as *B. subtilis*. Classical biochemical tests were not sufficient in distinguishing *B. subtilis* from *B. licheniformis* and *B. pumilus*. In Figure 2, percentage distribution of 19 isolates of *Bacillus* isolated from whole meal breads

identified by using (a) classical methods and (b) API kits are presented. According to the results of the classical methods *B. subtilis* (36.84%) was the most abundant species in whole meal breads as it was in white breads. Other abundant strains were *B. megaterium* (31.58%) and *B. licheniformis* (21.05%). Of the 19 isolates, 10.53% could not be identified at all by classical biochemical methods due to their irregular profiles (Figure 2a). A similar profile mismatch problem resulted in even a greater percentage (42.11%) of unidentifiable strains with the API kits (Figure 2b). It can be observed from this chart that *B. licheniformis* (21.05%) was the predominant species and 15.79% of the isolates were identified as 'most likely' being *B. megaterium*. Bacteria identified as *B.subtilis / amyloliquefaciens* and 'most likely' *B. thuringiensis* had equal percentages (5.26%) and 10.53% of the isolates could only be identified at the genus level.

According to the identification results of classical tests, the occurrence of *B. megaterium* and *B. licheniformis* in white breads was 22.73% and 13.64% respectively, whereas for whole meal breads it was 31.58% and 21.05% respectively. API kits, on the other hand, showed the proportions of *B. megaterium* and *B. licheniformis* in white breads as 4.55% and 22.73% respectively and in whole meal breads as 15.79% and 21.05% respectively.

3.2. Rope-producing ability of identified Bacillus species

Results of confirmation tests that was done to determine which *Bacillus* species, isolated from white and whole meal breads really have the ability of causing rope spoilage are given in Table 2. In this table, each additional "+" sign represents the development of rope spoilage. No visual symptoms of disease were observed within the first 24 hours. There was, on the other hand, a noticeable level of bad odor which is one of the characteristic properties of the disease. Observation of rope spoilage by day 2 in all of the breads indicated that all *Bacillus* species isolated from ropey breads could cause the disease.

4. Discussion

4.1. Identification of Bacillus species

In this study, the reason for using both, classical methods and API identification kits was to compare the results of both methods and to determine whether there was conformity between them. According to Collins et al. (1991) both of the methods were reliable in most cases. However, in some instances either of them could give incorrect results because of the use of non-standardized diagnostic tests and the heterogeneity of *Bacillus* genus itself. As a consequence inconsistent results may be obtained (Gordon et al., 1973; Logan and

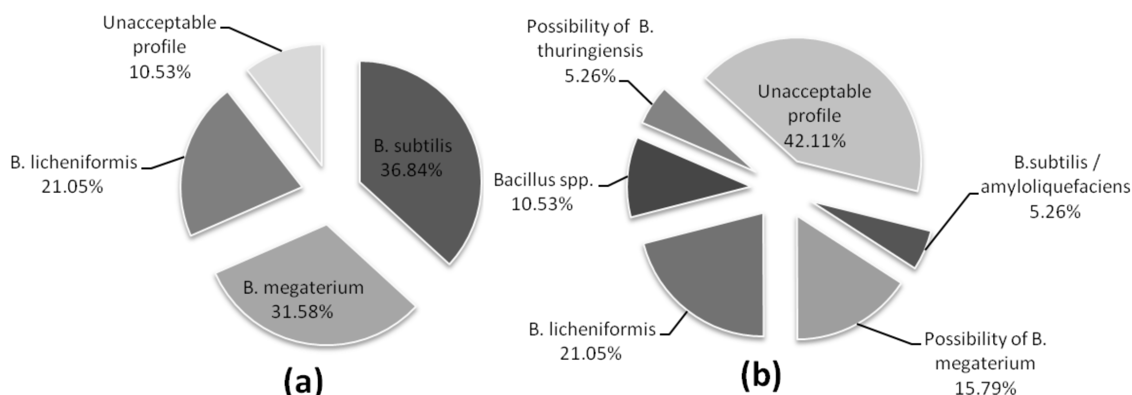

Figure 2. Percentage Distribution of 19 Isolates of *Bacillus* Isolated from Whole Meal Breads Identified by Using (a) Classical Methods and (b) API Kits

Table 2. Results of The Confirmation Tests for The Rope-Producing Ability of Identified *Bacillus* Species

Bacillus species	1st day	2nd day	3rd day	4th day	5th day	6th day
S	-	++++	++++	++++	+++++	+++++
L	-	++	++++	++++	+++++	+++++
M	-	++++	+++++	+++++	+++++	+++++
P	-	++++	++++	++++	++++	++++
T	-	+++++	+++++	+++++	+++++	+++++
S + L	-	++	+++	+++	++++	++++
S + M	-	++++	++++	++++	++++	++++
S + P	-	++++	++++	++++	+++++	+++++
S + T	-	+++	+++	+++	+++	+++
L + M	-	++++	+++++	+++++	+++++	+++++
L + P	-	+++	+++	+++	+++	+++
L + T	-	++	+++	++++	+++++	+++++
M + P	-	++++	++++	++++	+++++	+++++
M + T	-	+++++	+++++	+++++	+++++	+++++
P + T	-	++++	++++	++++	+++++	+++++
S + L + M	-	++++	++++	++++	+++++	+++++
S + L + P	-	++++	++++	++++	+++++	+++++
S + L + T	-	+	++	+++	++++	++++
S + M + P	-	++	++	+++	++++	++++
S + M + T	-	++++	++++	+++++	+++++	+++++
S + P + T	-	+++	+++	+++	++++	++++
L + M + P	-	++	++++	+++	++++	++++
L + M + T	-	+++	++++	++++	+++++	+++++
L + P + T	-	+++	++++	++++	+++++	+++++
M + P + T	-	++++	+++++	+++++	+++++	+++++
S + L + M + P	-	++++	++++	++++	+++++	+++++
S + L + M + T	-	++	++	+++	++++	++++
S + M + P + T	-	++++	++++	++++	+++++	+++++
S + L + P + T	-	++	++	+++	++++	++++
L + M + P + T	-	++++	+++++	+++++	+++++	+++++
S + L + M + P + T	-	+++	+++	++++	+++++	+++++
Control	-	-	-	-	-	-

S: *B. subtilis* L: *B. licheniformis* M: *B. megaterium* P: *B. pumilus* T: *B. thuringiensis*

Berkeley, 1984). According to Berkeley et al. (1984) it was easy to identify the typical strains of common species by the dichotomous key but difficulty was encountered with atypical or intermediate strains. For these reasons, Collins et al. (1991) noted that the API identification kits were more reliable in these circumstances.

In this study, there were difficulties distinguishing *B. subtilis* from *B. licheniformis* and *B. pumilus* by the classical biochemical tests. The fact that most *Bacillus* species only differ in one biochemical property makes classical biochemical identification at the species level very difficult. For instance *B. subtilis* and *B. pumilus* are only distinguished by the hydrolysis of starch. *B. subtilis* can hydrolyse starch while *B. pumilus* cannot, that being the case, an error in the interpretation of this test can therefore alter the identification of an isolate (Collins et al.,

1991; Thompson et al., 1993). Similarly, anaerobic growth is the single character that can be used to distinguish *B. subtilis* and *B. licheniformis.* Experimental error in this test that could be due to air introduced into the medium during inoculation could result in aerobic growth and hence a positive result could be incorrectly obtained. Furthermore, in connection with mutation and plasmid loss, a strain could lose or gain the ability to grow on anaerobic agar (Collins et al., 1991). In the same way, Collins et al. (1991) have identified nine of the strains they isolated from ropey bread, bakery equipment and raw materials as *B. subtilis* using the dichotomous key and as *B. pumilus* with the API kits. They also identified their ten isolates as *B. subtilis* by the key, but as *B. licheniformis* by API kits. A further ten isolates of theirs were identified as *B. licheniformis* by the key and as *B. subtilis* by API kits.

There were also differences between the results of identification methods distinguishing *B. coagulans* and *B. licheniformis* in this study. This may be due to the fact that most of the biochemical characteristics tested were not determinative because a proportion of positives to negatives (i.e. for some biochemical characteristics of *B. coagulans* a great range of 11-89%) is an acceptable result in classifying using the dichotomous key. This can be seen in a differential characteristic table for the *Bacillus* species given by Sneath (1984). Therefore, using the key, it is extremely difficult to distinguish between two species such as *B. coagulans* and *B. licheniformis* even though the test has a certain positive result for the organism in question. Similarly, Thompson et al. (1993) made reference to an unpublished paper where *B. licheniformis and B. coagulans* could not be distinguished from the obtained profiles.

B. amyloliquefaciens and *B. subtilis* are basically indistinguishable from each other using classical biochemical techniques. It was revealed that the presence of intermediate strains which obscured the distinction so that even in using the API kits, it is virtually impossible to distinguish between these two species clearly (Logan and Berkeley, 1984). According to Logan and Berkeley (1984) there are only two test, acid production from inulin and chains of cells, that are of value separating the two species. Fritze (2002) on the other hand, has expressed that *B. amyloliquefaciens* is much faster than *B. subtilis* in acid production from lactose and slower in gluconate usage. Hence one can make use of these two characteristics in telling the two species apart, but for a clear, unquestionable identification molecular techniques must be used. Therefore, based on the API-web results of N2, K6 and K15 isolates for the lactose and gluconate tests, it was concluded that strains N2 and K15 are *B. amyloliquefaciens* and strain K6 is *B. subtilis* in this study.

In our study, according to the results of the classical methods *B. subtilis* was the most abundant species in both white and whole meal breads. API kits, on the other hand, confirmed *B. licheniformis* as the predominant species. Previous studies on the identification of bacteria causing rope spoilage in bread have revealed that different *Bacillus* spp. may be more prevalent in ropey breads. Pepe et al. (2003) identified a total of 61 cultures of gram-positive spore-forming rods, isolated from ropey breads, as *B. subtilis* by using dichotomous key but they characterized other strains of *Bacillus* by molecular methods. Sorokulova et al. (2003) examined ropey breads and identified 50% of the strains as *B. licheniformis* according to phenotypic characteristics and found that only one spoiled loaf to have *B. megaterium*. They confirmed the identification results by 16S rDNA sequencing and found that the results were in accordance except for one strain resembling *B. licheniformis* phenotypically. Rosenkvist and Hansen (1995) isolated *Bacillus* strains from wheat, raw materials for bread production, normal and ropey breads and verified the isolates as *Bacillus* spp. by examining classical tests and confirmed using the API kits that *B. subtilis* was the only species in ropey bread. Bailey and von Holy (1993) have isolated *Bacillus* species from raw materials, dough, brown bread and food contact surfaces and identified the species from brown bread as

B. subtilis (58.7%), *B. licheniformis* (31%), *B. pumilus* (6.8%) and *B. megaterium* (3.5%). So they confirmed the predominance of *B. subtilis* in rope spoilage. Collins et al. (1991) determined that *B. subtilis* was the most abundant species both by using biochemical tests (63.7%) and by using API identification kits (40.8%) and 17.4% of the isolates were identified as *B. amyloliquefaciens.*

Comparison between the identification results of white and whole meal breads showed that white breads did not contain the strain *B. thuringiensis*, in contrast, whole meal breads do not contain *B. pumilus*. Leuschner et al. (1998) isolated *B. pumilus* in addition to *B. subtilis* and *B. licheniformis* in brown bread. Pepe et al. (2003) also isolated *B. thuringiensis* from ropey bread but could identify this strain only by molecular techniques.

4.2. Results of the confirmation tests for the rope-producing ability of identified Bacillus species

In this study, it was determined that all of the *Bacillus* species isolated from both white bread and whole meal bread were the causative agent of the rope spoilage. Contrary to the results of this study, Rosenkvist and Hansen (1995) and Leuschner et al. (1998) found that *B. subtilis* is the only species associated with ropiness, on the other hand, Sorokulova et al. (2003) determined that *B. subtilis* and *B. licheniformis* were responsible for the disease. Collins et al. (1991) did not make confirmation tests but they suggested that *B. subtilis* and *B. licheniformis* whose percentage distributions on ropey breads were the highest, were the most important species that can cause rope spoilage. Similar to the results obtained from our study, Thompson et al. (1998) demonstrated that *B. subtilis, B. licheniformis, B. megaterium* and *B. pumilus* were responsible for ropiness. In addition to this, they found that *B. polymyxa* had a low level of rope-producing capacity. Although, *B. thuringiensis* was found to be one of the causative agents, no literature was available citing the incidence of such. It could be taken into consideration that identifying this species as 'most likely *B. thuringiensis*' (Table 1) is in fact a tentative approach to the identification and that *B. cereus* being closely related to this organism (Pepe et al., 2003) may have been misidentified as *B. thuringiensis*.

The development of rope spoilage increased throughout the storage period of breads. In the second day, rope-producing capacity of *B. licheniformis* was low when present in bread as a single species. However, when it was present in bread along with other *Bacillus* species synergy was observed and the symptoms of ropiness were more obvious. This may be due to two reasons. One reason why one strain of bacteria may cause less of the symptoms of rope spoilage than another could be that the bacteria may require a longer period of time for germination and/or may have a longer lag phase during vegetative growth. Another reason might be a lower enzyme production/secretion capacity, thus resulting in less ropiness. As can be seen from Table 2, the case was opposite for *B. subtilis*. When present as a single strain its rope-producing capacity was high, however when it was combined with *B. licheniformis*, a decrease was observed in the rope-producing ability. This gives rise to the thought that *B. licheniformis* may hinder the activity of *B. subtilis*.

It can be seen in Table 2 that when *B. licheniformis* was present in the bacteria combinations inoculated to the breads, lesser degree of rope formation was observed. However, when it present with *B. megaterium*, there was an increase in rope formation. Therefore it might be said that there is a synergistic effect between these species.

5. Conclusion

The results obtained from this study show a clear difference in differentiating between strains using the biochemical tests and API kits. It can be concluded that

biomolecular methods may provide to be more helpful in obtaining a more exact and credible identification for some of the isolates studied. Therefore one should associate the biochemical test results (classical biochemical tests and API system) with molecular methods in order to confirm the identification obtained.

References

Bailey, C. P. and von Holy, A. 1993. *Bacillus* spore contamination associated with commercial bread manufacture. Food Microbiology 10, 287-294.

Berkeley, R. C. W., Logan, N. A., Shute, L. A. and Capey, A. G. 1984. In: Berkeley, R. C. W. (Ed.), Methods in Microbiology, vol 16, chap. 12, Academic Press, London, pp 292-323.

Collins, N. E., Kirschner, L. A. M. and von Holy, A. 1991. Characterization of *Bacillus* isolates from ropey bread, bakery equipment and raw materials. South African Journal of Science 87, 62-66.

Fritze, D. 2002. *Bacillus* Identification-Traditional Approaches. In: Berkeley, R., Heyndrickx, M., Logan, N. and Vos, P. (Eds.), Applications and Systematics of *Bacillus* and Relatives, Blackwell Publishing, Cambridge, pp 100-123.

Gordon, R. E., Haynes, W. C. and Pang, C. H.-N. 1973. The genus *Bacillus*. United States Department of Agriculture, Agricultural Handbook No. 427, Washington, DC.

Katina, K., Sauri, M., Alakomi, H. L. and Mattila-Sandholm, T. 2002. Potential of lactic acid bacteria to inhibit rope spoilage in wheat sourdough bread. Lebensmittel-Wissenschaft und Technologie*,* 35, 38-45.

Kirschner, L. A. M. and von Holy, A. 1989. Rope spoilage of bread. South African Journal of Science 85, 425-427.

Leuschner, R. G. K., O'Callaghan, M. J. A. and Arendt, E. K. 1998. *Bacilli* spoilage in part-baked and rebaked brown soda bread. Journal of Food Science 63, 915-918.

Logan, N. A. and Berkeley, R. C. W. 1984. Identification of *Bacillus* strains using the API system. Journal of General Microbiology 130, 1871-1882.

Marshall, D. G. and Odame-Darkwah, J. K. 1994. Mechanism of inhibited growth of *Bacillus pumilus* by *Propionibacterium freudenreichii* subsp. *shermanii.* International Journal of Food Microbiology 22, 11-22.

Menteş, Ö., Ercan, R. and Akçelik, M. 2007. Inhibitor activities of two *Lactobacillus* strains, isolated from sourdough, against rope-forming *Bacillus* strains. Food Control 18, 359-363.

Odame-Darkwah, J. K. and Marshall D. L. 1993. Interactive behavior of *Saccharomyces cerevisiae*, *Bacillus pumilus* and *Propionibacterium freudenreichii* subsp. *shermanii*. International Journal of Food Microbiology 19, 259-269.

Pattison, T. L., Lindsay, D. and von Holy, A. 2004. Natural antimicrobials as potential replacements for calcium propionate in bread. South African Journal of Science 100, 342-348.

Pepe, O., Blaiotta, G., Moschetti, G., Greco, T. and Villani, F. 2003. Rope-producing strains of *Bacillus* spp. from wheat bread and strategy for their control by lactic acid bacteria. Applied and Environmental Microbiology 69, 2321-2329.

Rosenkvist, H. and Hansen, A. 1995. Contamination profiles and characterization of *Bacillus* species in wheat bread and raw materials for bread production. International Journal of Food Microbiology 26, 353-363.

Rosenquist, H. and Hansen, A. 1998. The antimicrobial effect of organic acids, sour dough and nisin against *Bacillus subtilis* and *B. licheniformis* isolated from wheat bread. Journal of Applied Microbiology 85, 621-631.

Smith, J. P., Dafias, D. P., El-Khoury, W., Koukoutsis, J. and El-Khoury, A. 2004. Shelf life and safety concerns of bakery products – a review. Critical Reviews in Food Science and Nutrition 44, 19-55.

Sneath, P. H. A. (1984). Endospore forming Gram-Positive Rods and Cocci. In: Sneath, P. H. A (Editor), Bergey's Manual of Systematic Bacteriology Volume 2. Williams&Wilkins. Baltimore, pp 1104-1139.

Sorokulova, I. B., Reva, O. N., Smirnov, V. V., Lapa, S. V. and Urdaci, M. C. 2003. Genetic diversity and involvement in bread spoilage of *Bacillus* strains isolated from flour and ropy bread. Letters in Applied Microbiology 37, 169-173.

Thompson, J. M., Dodd, C. E. R. and Waites, W. M. 1993. Spoilage of bread by *Bacillus*. International Biodeterioration and Biodegredation 32, 55-66.

Thompson, J. M., Waites, W. M. and Dodd, C. E. R. 1998. Detection of rope spoilage in bread caused by *Bacillus* species. Journal of Applied Microbiology 85, 481-486.

Volavsek, P. J. A., Kirschner, L. A. M. and von Holy, A. 1992. Accelerated methods to predict the rope-inducing potential of bread raw materials. South African Journal of Science 88, 99-102.

Voysey, P. A. 1989. Rope: a problem for bakers. Journal of Applied Bacteriology 67, xxv-xxvi.