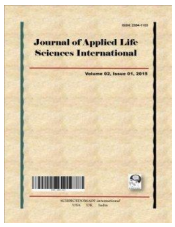

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Characterization of *Bacillus* Species from Convenience Foods with Conventional and API Kit Method: A Comparative Analysis

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Authors' contributions

This work was carried out in collaboration between both authors. Author CEA designed the study, wrote the protocol and wrote the first draft of the manuscript. Author CEA managed the literature searches, analyses of the study; authors CEA and SOAO managed the experimental process and both authors identified the species of Bacillus. Both authors read and approved the final manuscript.

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ABSTRACT

Identification of microorganisms is central to the study of microbiology at all levels/strata of research. The methods employed are also important. It is however pertinent that scientists need to improve on the method of microbial identification for greater efficiency. A study was conducted to isolate and identify *Bacillus* species from some ready-to-eat food (RTEs) samples. The *Bacillus* species isolated were identified by using the classical method. Major groups were further identified using the API kits. It was observed that definite identification of some bacilli isolates using these methods was not possible. API system combination of the 50 CHB and 20E was able to identify about 80.0% of the bacilli isolates (16 of 20). The API *Bacillus* identification system failed to identify 4 isolates of *Bacillus thuringiensis*. Isolates were identified as *B. cereus*, *B. subtilis, B. megaterium, B. licheniformis, B. sphaericus* and *B. polymyxa* using biochemical tests. On the other hand, use of the API kits showed the identification of *B. cereus*, *B. subtilis/amyloliquefaciens, B. licheniformis/ B. subtilis, B. subtilis/B. pumilus, B. licheniformis, B. megaterium* and *B. sphaericus*. 'Most likely' *B. thuringiensis* from classical identification were identified as other closely related members of the *B.*

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cereus group (*B. cereus, B. anthracis*, *B. mycoides* and *B. pseudomycoides*). According to the results of the classical methods *B. subtilis* was the most abundant species. API kits confirmed *B. amyloliquefaciens* as the predominant species.

Keywords: Identification; classical; API; Bacillus; RTEs.

1. INTRODUCTION

Gram positive and aerobic spore-forming bacilli belonging to the genus *Bacillus* and other related species play important roles in food poisoning and spoilage. There is however some difficulty due to the lack of standard methods for identification of members of *Bacillus* species in food testing laboratories [1]. Species differentiation of the genus is complex and in some instances in routine laboratories, a combination of Gram stain and colonial appearance may be regarded as sufficient indication of a *Bacillus* species being present in a clinical sample. Although the use of morphological and physiological tests have provided the best means available for laboratories to identify organisms, these methods have proven to be quite laborious, inconsistent and generally unreliable for this group of microorganisms (ibid). As a result of the absence of reliable and standardized methods for the identification of this group of organisms, investigators have generally focused on the isolation and identification of *Bacillus cereus* as a causative agent of food related illnesses. However, numerous other investigations have demonstrated that a considerably larger range of species can cause food related illness [2,3]. The API *Bacillus* identification system comprising the API 50 CHB and API 20E identification systems from BioMerieux (France) has been reported as being able to identify organisms to the strain level. Some of the major mesophilic *Bacillus* spp. and related species causing food poisoning or other problems in food include *B. cereus*, *B. subtilis*, *B. mycoides*, *B. pumilus*, *B. weihenstephanensis*, *B. thuringiensis*, *B. coagulans*, *B. sphaericus* and *B. licheniformis*. *B. cereus* group consists of *B. cereus, B. thuringiensis, B. mycoides* and *B. weihenstephanensis.* The *B. subtilis* group consists of *B. subtilis* and *B. amyloliquefaciens* [1].

The prevailing neglect of *Bacillus* identification may be attributed to two factors. Firstly, the diagnostic tests used; many of the classical tests for *Bacillus* described by Gordon et al. [4] require special, selective/differential media. These are very time consuming and expensive to prepare. Many of these media have short shelf lives resulting in considerable wastage if their use is infrequent. The requirement for media containing unusual ingredients increases the familiar problems of test standardization [5] and inconsistent results may be obtained in consequence. Any new scheme for *Bacillus* identification should therefore use widely available and standardized materials for performing a good number of rapid tests which give reproducible results. The second factor leading to neglect of *Bacillus* identification is the character of the genus. *Bacillus* is an unusually wide taxon which contains most aerobic endospores-forming rods. In terms of DNA base ratios it is the equivalent of some bacterial families [6]. Furthermore, some species are ill-defined, existing with closely related species as complexes or in which the boundary of a particular species is difficult or impossible to identify. Even in well established species there is considerable variation between strains. Thus, classical test schemes using few characters often do not permit identification of atypical and intermediate strains and in spite of the excellent work of Gordon and her colleagues [4], as well as others, it is widely agreed that there is considerable room for improvement in the taxonomy of the genus and that a study of new isolates, particularly, is important.

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 [7]. The aim of this paper was to identify strains of *Bacillus* species using classical methods and API identification kits and proceed to compare results from both methods. This paper also aimed at providing initial data which would encourage the use of other identification methods in the assay for *Bacillus* species especially in developing countries. It is also the hope that this class of microorganisms would be included in routine food test, especially in Nigeria. No matter how

expensive the identification method, it is believed that no expense should be spared to ensure our foods meet quality standards and are safe for consumption.

2. MATERIALS AND METHODS

2.1 Isolation of *Bacillus* Species

Food Samples were collected according to the methods of Cheesbrough [8] and Fawole and Oso [9]. A total of sixty RTE food samples [10 samples each of 3 different types of pastry products (Buns, Meat pie and Egg roll) and 10 each of different rice products (White rice, Fried rice and Jollof rice)] were purchased from different food vending sites and cafeterias within a period of ten weeks. Food samples purchased were appropriately labelled and transferred to the Microbiology laboratory for immediate analysis.

Microbiological analysis for *Bacillus* species was done using serial dilution technique with spread plating (0.1 ml inoculum) unto HiCrome *Bacillus* agar (HiMedia). This is a selective/differential isolation media, for assessment of *Bacillus* species. The fourth dilution was used for plating unto the media. Culture medium was prepared according to manufacturer's specification (49.22 g/L) and sterilization of materials was done in an autoclave at 121ºC for 15 minutes [10].

2.2 Identification of *Bacillus* Species

Identification was performed using classical methods [10,11] and API identification kits [12] [API 20E and API CHB 50 (Biomerieux, France)]. Of all isolates, 20 *Bacillus* species were selected and subjected to the API identification kit.

Classical method: Biochemical tests carried out in the conventional method include the following-catalase, spore staining, growth in sodium chloride (NaCl), Voges-Proskauer and Methyl-Red test, fermentation of carbohydrate and hydrolysis of starch among others, were executed according Olutiola et al. [10].

API kit method: Sufficient growth of each isolate being identified was inoculated into the suspension medium supplied with the API *Bacillus* kit so as to produce an inoculum density equivalent to a MacFarland 2 standard. Two separate suspensions were prepared for use with the API systems, one for the API 20E, the other for the API 50 CHB. Both were prepared at a density equivalent to a MacFarland 2 standard. Using sterile Pasteur pipettes, approximately l ml of the organism suspension was inoculated into each microtubule of the incubation tray.

The API systems were inoculated in accordance with manufacturer's instructions. After inoculation, all identification systems were incubated at 36ºC for 24-48 hrs. Each system was read after 24 hrs incubation, with a final reading made after 48 hrs incubation. All results were recorded on the work sheets provided and interpreted using the API Web database systems. To identify an organism, the APIweb software compares the profiles obtained with the profiles of taxa in the database and assigns a positivity percentage to each test [12].

3. RESULTS AND DISCUSSION

Details of some morphological and biochemical characteristics of these selected test bacilli are shown in Table 1. Table 2 shows the percentage identity of the five (5) test bacilli selected for further study, inclusive of their identity using the conventional and Analytical Profile Index (API) kit method. A summary of the identifications made for each method employed and showing the percentage of identifications are depicted in Table 3.

Both classical method and API identification kits were used in order to make suitable comparison and determine whether there was conformity between them. According to Collins et al. [13] both of the methods were reliable in most cases, as is the case with the results from this study. It is however important to state that 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 [4,12]. The fact that most *Bacillus* species only differ in one biochemical property makes classical biochemical identification at the species level very difficult. According to Logan and Berkeley [12] 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. [13] noted that the API identification kits were more reliable in these circumstances.

Table 1. Some morphological and biochemical characteristics of isolates using the classical method

Gram reaction	Morph.	Catalase	Oxidase	MR.	VP.	Citrate	Urease	Starch	Nitrate.	Spore	Glucose	Xylose	Lactose	Sucrose	Maltose	Mannitol	Galactose	Probable Identity
+	R	+	+	-	+	+	-	+	+	+	+	-	+	+	+	-	+	*Bacillus cereus*
+	R	+	+	-	+	+	-	+	+	+	+	-	+	+	-	+	+	*Bacillus subtilis*
+	R	+	+	-	-	+	-	+	-	+	+	+	-	+	-	+	-	*Bacillus megaterium*
+	R	+	-	-	+	+	-	+	+	+	+	-	-	+	-	-	-	*Bacillus thuringiensis*
+	R	+	-	-	+	-	-	+	-	+	+	-	-	+	+	-	-	*Bacillus stearothermophilus*
+	R	+	-	-	+	+	-	+	+	+	+	-	-	+	+	+	-	*Bacillus licheniformis*
+	R	+	+	-	+	+	-	+	+	+	+	-	+	+	-	-	+	*Bacillus amyloliquefaciens*
+	R	+	+	-	+	-	-	+	+	+	+	-	+	+	-	+	+	*Bacillus mycoides*
+	R	+	+	+	-	+	-	+	-	+	+	+	-	-	-	+	+	*Bacillus brevis*
+	R	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	*Bacillus polymyxa*
+	R	+	+	-	-	-	-	+	+	+	+	-	+	+	-	+	-	*Bacillus laterosporus*

Key: - = negative reaction, + = positive reaction, morph = cell morphology, coag. = coagulase, MR. = methyl red, VP. = voges proskauer, R = rods

Table 2. Identification of *Bacillus* using both classical and API methods

Isolate code	Conventional identification	API identification (% ID)
A0	*Bacillus cereus*	*Bacillus cereus* (99.6%)
A1	*Bacillus cereus*	*Bacillus cereus* (90.1%)
A2	*Bacillus cereus*	*Bacillus cereus* (86.0%)
A3	*Bacillus cereus*	*Bacillus cereus* (89.6%)
A4	*Bacillus cereus*	*Bacillus cereus* (99.5%)
B0	*Bacillus subtilis*	*Bacillus amyloliquefaciens* (65.3%)
		Bacillus subtilis (34.1%)
B1	*Bacillus subtilis*	*Bacillus amyloliquefaciens* (88.8%)
B2	*Bacillus subtilis*	*Bacillus pumilus* (66.3%)
		Bacillus subtilis (32.4%)
B3	*Bacillus subtilis*	*Bacillus subtilis* (83.4%)
B4	*Bacillus subtilis*	*Bacillus amyloliquefaciens* (98.3%)
D0	*Bacillus thuringiensis*	*Bacillus mycoides* (57.2%)
		Bacillus cereus (41.9%)
D1	*Bacillus thuringiensis*	*Bacillus cereus* (57.2%)
		Bacillus pseudomycoides (41.9%)
D2	*Bacillus thuringiensis*	*Bacillus mycoides* (57.2%)
		Bacillus cereus (41.9%)
D3	*Bacillus thuringiensis*	*Bacillus cereus* (67.2%)
		Bacillus anthracis (31.6%)
E0	*Bacillus licheniformis*	*Bacillus subtilis* (95.6%)
E1	*Bacillus licheniformis*	*Bacillus licheniformis* (99.0%)
E2	*Bacillus licheniformis*	*Bacillus licheniformis* (89.1%)
F0	*Bacillus polymyxa*	*Bacillus polymyxa* (61.2%)
		Bacillus macerans (38.6%)
F1	*Bacillus polymyxa*	*Bacillus polymyxa* (89.8%)
G0	*Bacillus sphaericus*	*Bacillus sphaericus* (96.6%)
F0	*Bacillus stearothermophilus*	NA

Key: API = analytical profile index, % ID = percentage identification, NA = not applicable

Table 3. Classical and API identification summary

***Bacillus* microorganism**	**Classical**	**API**
Bacillus cereus	5	5
Bacillus subtilis	5	5
Bacillus thuringiensis	4	0
Bacillus licheniformis	3	3
Bacillus polymyxa	2	2
Bacillus sphaericus	1	1
Bacillus stearothermophilus	1	NA
Total	20	16
	100%	80%

Identification of the test *Bacillus* species using the Analytical Profile Index (API) kit revealed identities similar to those obtained using conventional methods. The API kit is however able to identify the microorganisms to the strain level and is considered by some authors as a molecular method for identification for microbes. The API is based on the principle that microorganisms breakdown various substrates during metabolism to release by products and metabolites which can be detected by a change in colour and on addition of specific reagents. Both the API 20E and the API 50 CHB, have been adapted to the identification of this group of organisms. While the API was able to identify the various test *Bacillus* spp. to the strain level, it failed to identify *Bacillus thuringiensis*. Similar observation was made by Mugg et al. [1]. This microorganism was not included in the API database and hence, was identified as a member of the *B. cereus* group. *B. thuringiensis* had been placed in the *B. cereus* group according to current taxonomy [2] (Kramer and Gilbert). API system combination of the 50 CHB and 20E was able to identify 80.0% of the isolates (16 of 20). The API *Bacillus* identification system failed to identify 4 isolates of *Bacillus thuringiensis*. As posited by Mugg et al. [1], the combination of the two API identification systems require the inoculation and software interpretation of seventy (70) separate substrates many of which are duplicated between the two systems.

B. amyloliquefaciens and *B. subtilis* have been considered to be 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 [12]. According to Logan and Berkeley [12] there are only two test, acid production from inulin and chains of cells, that are of value separating the two species. Fritze [14] on the other hand, had 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.

Based on the APIweb software results for *Bacillus subtilis* isolates; strains B0, B1 and B4 were considered as *B. amyloliquefaciens* and B3 as *Bacillus subtilis* and B2 as *Bacillus pumilus*. Results from the classical methods show *Bacillus subtilis* as the most abundant species, followed by *Bacillus cereus*. API kits, on the other hand, was able to confirm the identities of *B. subtilis* either as *B. subtilis* or other closely related members of the *B. subtilis* group; likewise in the case of *B. cereus* [15]. It has however been opined that members of the *Bacillus* group such as the *B. thuringiensis* should be tagged as 'most likely *B. thuringiensis*' from classical method use, prior to identification by other methods, API or molecular and even more so as *B. cereus* is closely related to this organism [16]. Some *B. cereus* may have been misidentified as *B. thuringiensis*. It is humbly put forward here that classical methods do not suffice for identification of members of the *Bacillus* group of microorganism. Hence, additional methods such as the API kit or other efficient kit methods, as well as DNA/molecular method are needed for optimal identification.

4. CONCLUSION

Results obtained from this study show clear differentiation between strains of *Bacillus* species using the biochemical tests and API kits. Hence, biomolecular methods may prove to be more helpful and reliable in obtaining more credible identification for some of the isolates studied. At this juncture it is opined that biochemical test results (classical/conventional biochemical tests and API system) in combination with molecular methods would provide the best confirmation of the identification of food microflora that may be obtained.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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