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Diversity of pectinolytic bacteria causing soft rot disease of vegetables in Ibadan, Nigeria

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ABSTRACT

Abstract: Pectinolytic organisms are a constant threat with regard to spoilage of vegetables because of their extensive host range and widespread distribution. It is estimated that between 10 and 30% of fresh vegetables are wasted, mainly due to three factors: mechanical injuries, physiological decays and microbial spoilage. The role of microorganisms in this wastage is significant, hence the need to better understand the microbial diversity responsible for soft rot spoilage in vegetables.

Methodology and results: Mineral medium described by Jayasankar and Graham (1970) was used to isolate and enumerate pectinolytic organisms. Shannon-Weaver indices of general biodiversity and equitability were used to analyze their diversity and evenness. A total of seventy-six (76) strains of bacteria were isolated from carrot, tomato, onion, pepper and potato. They were identified as *Erwinia carotovora, E. chrysanthemi, E. herbicola, E. atroseptica, E. amylovora, Pseudomonas flourescens, P. syringae, P. cichorii* and *Xanthomonas campestris* isolates. Most of the organisms grew well on the mineral medium and showed pectinolytic activity at pH 6.0. *Erwinia* species were the most isolated (64.44%) with *E carotovora* having the highest frequency of occurrence (21) and the greatest diversity index of 0.36 among the samples analyzed. This was closely followed by *Pseudomonas* species (31.58%) with *P. syringae* having an occurrence frequency of 14 and index of 0.31. The least occurrence (3) and diversity (0.13) was recorded for *X. campestris.* Among the samples, onion had the greatest diversity of organisms with a total index of 0.98, followed by tomato (0.91) and the least was from carrot (0.69). Ojoo market had the greatest diversity among the sampled locations. Statistical analysis of the biodiversity index indicated that carrot, tomato and pepper (from Bodija), onion and potato (from Ojoo) as well as carrot and onion (from Oja oba) had no significant differences ($p \geq 0.05$) in the diversity of the organisms isolated. Also, no significant difference ($p \geq 0.05$) was recorded in onion and potato (from Bodija), carrot, tomato and pepper (from Ojoo) as well as tomato and pepper (from (Oja oba).

Conclusion and application of results: The results of this work showed that diverse pectinolytic organisms cause soft rot in various vegetables and pectinolytic organisms varied with different vegetables and locations of purchase. Therefore, there is the need for good hygiene, proper handling and sorting during transportation of retail vegetables to ensure good quality and safe vegetable.

Key words: Vegetables, soft rot, pectinolytic organisms, biodiversity index

INTRODUCTION

In recent years, consumers have been demanding convenience and high quality vegetable products for which they are willing to pay a premium. One of the responses of the food industry has been to produce vegetables that do not need intense cooking or processing. The consumption of fresh vegetables is increasing as consumers strive to eat healthy diets and benefit from the year-round availability of these products that up until recently were considered to be seasonal. Global trade in vegetables and changing horticultural practices have enabled this year-round availability to be possible, as well as adding new varieties of fresh produce to the market .

During the last few decades pre-prepared, minimally processed vegetables have become popular among consumers. These vegetables combine their fresh-like and healthy characteristics (preserved during storage by a natural packaging system) with a minimal time of preparation before consumption both at the consumer and catering levels (Ahvenainen, 1996; Watada *et al.,* 1996). This has led to a rapid increase in the type of minimally processed vegetables offered and consequently to high sales.

The appeal and demand of these vegetables are due to consumer perceptions that they are healthy, tasty, convenient and fresh (Sloan 2000; Buck *et al.,* 2003; Garrett *et al.,* 2003; Mehrotra, 2004; Pivarnik *et al.,* 2005). The shelf-life of vegetables is generally about 7-14 days at 5 °C, being limited by endogenous biochemical and physiological changes in the produce, as well as the growth of spoilage microorganisms (Garcia-Gimeno and Zurera-Cosano, 1997; Heard, 1999 & 2002). Vegetables consist mainly of water, resulting in a high water activity (>0.99). The intracellular pH, being another important intrinsic factor, ranges from 4.9 to 6.5 for most vegetables (Lund, 1992). These properties allow the growth of microorganisms from the moment that nutrients become available. Vegetables carry a natural non-pathogenic epiphytic microflora. During growth, harvest, transportation, handling and further processing, the produce can, however, be physically damaged. Wounded areas on plant tissue provide a better substrate for microbiological growth by providing nutrients (King *et al*., 1991; Zagory, 1999), and the properties of the tissue determine which microorganisms will be active.

Soft rot is one of the significant spoilage diseases of vegetables caused by both pectinolytic bacteria and fungi that break down the pectic substances (pectin) of the middle lamella which is a mechanical protector, constituting a glue between the cells and giving rigidity (Brummel, 2006).The capacity to degrade pectin, a major constituent of the primary plant cell wall and middle lamella, is a feature of many plant-associated bacteria, especially pectinolytic organisms.

A review of the currently available literature reveals little quantitative information about the diversity of pectinolytic organisms on vegetables. Although various bacterial species such as *Pseudomonas*, *Serratia, Erwinia, Enterobacter, Pantoea, Burkholderia* and *Stenotrophomonas* are frequently isolated, their populations are rarely quantified (Riser *et al.,* 1984; Magnuson *et al.,* 1990; King *et al.,* 1991; Khan *et al.,* 1992; Freire & Robbs, 2000; Hamilton-Miller & Shah, 2001). Population data are particularly important in determining which species will mostly impact on produce quality.

Tomato, onion, carrot, pepper and potato were chosen for this study because they are minimally processed, thus are referred to as ready-to-eat and also have a physical structure which is susceptible to microbial invasion. The focus on the microbiology of these products has increased because of their growing popularity and associated public health risks. From a marketing perspective, there has been a goal to enhance their shelf-life and sensory acceptability, considering their characteristics such as neutral pH and a high water activity which makes them susceptible to microbial attack.

The objective of this study was to determine the diversity of pectinolytic organisms causing soft rot disease of five different vegetables marketed in Ibadan, Oyo state, Nigeria.

MATERIALS AND METHODS

Sample collection and isolation of microorganisms: Spoilt vegetable samples were collected aseptically in sterile polythene bags from three different retail markets namely, Ojoo, Bodija and Oja oba, all in Ibadan, Oyo state. Mineral medium, a chemically defined medium described by Jayasankar and Graham

(1970), was used for the isolation and enumeration of pectinolytic organisms by pour plate technique of Harrigan and McCance (1976).

Pectinolytic test: This test was done to detect the organism that has the ability to degrade pectin which constitutes the cell wall in vegetables. Pure cultures of the bacterial isolates were inoculated onto the Mineral medium plates and incubated for 48 h at 30±2 ^{o}C. Plates were flooded with 1% solution of hexadecyltriymethylammoniumbromide precipitant (Jayasankar and Graham, 1970). Clear zone around colonies within 15 minutes indicated the breakdown of pectin, against a rather opaque medium.

Effect of pH on pectinolytic activity: The mineral medium was adjusted to different pH, ranging from 5.5 to 7.5 using phosphate buffer and a pH meter. Pour plate method was used for inoculation and growth on each plate was counted after 48 hours of incubation at 30±2 ^{o}C.

Characterization of isolates: Isolates were characterized and identified on the basis of their cultural, morphological, physiological and biochemical properties using Bergeys Manual of Systematic Bacteriology (Sneath *et al.,* 1986).

General Biodiversity (H) and Equitability (J) index: The Shannon Weaver index of general diversity and equitability was used (Shannon and Weaver, 1963). (**H =** $-\sum P_i(\ln P_i)$**, J =** $H/\ln R$).

Statistical analysis: The statistical analysis of the diversity was carried out using SPSS software (version 10.0). One way ANOVA with Waller- Duncan significant difference ($p<0.05$) was used to separate the means.

RESULTS

The result of microbial load of each vegetable sample plated on the mineral medium was as shown in table 1. The highest count of pectinolytic bacteria recorded in carrot and potato was 2.0×10^8cfu/g and 7×10^7cfu/g, respectively, with an average pectinolytic zone of 7mm, both from Bodija market (table 1). The highest count for tomato, pepper and onion was 2.7×10^6 cfu/g, 9×10^7 cfu/g and 9×10^7 cfu/g, respectively, with average zone size of 3, 10 and 18mm, respectively, all from Ojoo market. A total of seventy six (76) strains of different bacteria were randomly picked from the various organisms isolated from the spoilt vegetable samples. They were subjected to morphological, physiological and biochemical test after showing pectinolytic clear zones on plates flooded with hexadecyltrimethylammoniumbromide precipitant (Plate 1) and were identified on the basis of the results of the characterization test.

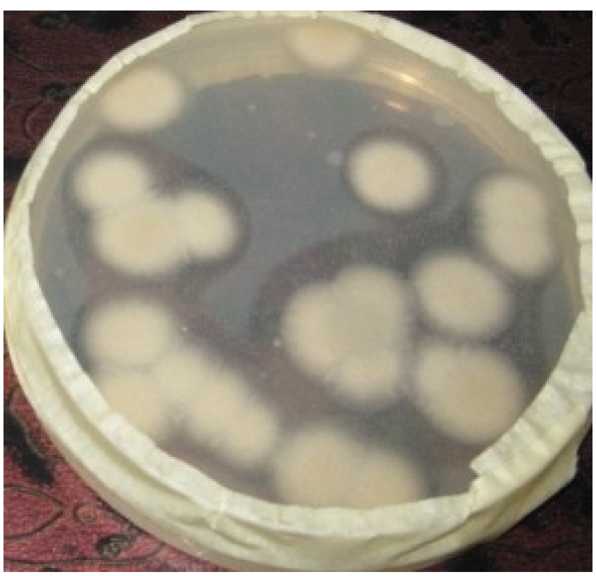

Plate 1: Isolates showing pectinolytic clear zones after flooding

Erwinia, Pseudomonas and Xanthomonas species identified included *Erwinia carotovora, E. amylovora, E. chrysanthemi, E. herbicola and E. artroseptica, Pseudomonas syringae, P. cichorii, P. marginalis* and *Xanthomonas campestris. E. carotovora* occurred most frequently and with the highest percentage of occurrence (27.63%). *P. syringae* constituted 18.42%, *E. herbicola* (11.84%), *E. chrysanthemi* (10.5%), *E.*

artroseptica (9.21%), *P. cichorii* and *P. marginalis* (6.6%) *each, E. amylovora* (5.3%) *and X. campestris* (3.95%) (table2).

Table3 shows the effect of pH on pectinolytic activity of isolates when pH was varied from 5.0 to 7.5. Isolates from Bodija market grew at 5.0, 6.0 and 6.5 while isolates from Ojoo and Oja oba markets grew at 6.0 and 6.5. Generally, the highest growth was at pH 6.0 for isolates from all the markets, but none grew at 7.0 and 7.5.

Shannon-Weaver indices of diversity and equitability showed that the highest diversity within samples was in onion with an index of 0.98 and species evenness of 0.43, followed by tomato 0.91 with 0.35 evenness while the least was from carrot with index 0.69 and evenness of 0.89 (Table4). Statistical analysis showed no significant difference in pectinolytic organisms in the carrot and tomato purchased from Bodija, while the onion, pepper and potato showed significant difference ($p<0.05$) in the diversity of the pectinolytic organisms isolated. Also, carrot and potato as well as onion and pepper from Ojoo showed no significant differences ($p>0.05$) in the diversity of their organisms. There was significant difference among the vegetables within each market except for carrot and potato purchased from Ojoo and Oja oba markets (Table5).

General diversity among the three markets indicated Ojoo market as having the highest diversity of pectinolytic organisms with an index of 1.51 and species evenness of 0.37 while the least was from Bodija market, having an index of 1.28 and evenness of 0.35 (Fig1). Also, statistical analysis indicated that carrot, tomato and pepper (from 'Bodija'), onion and potato (from 'Ojoo') as well as carrot and onion (from 'Oja oba') showed no significant differences ($p>0.05$) in the diversity of the organisms. There was no significant difference ($p>0.05$) in onion and potato (from 'Bodija'), carrot, tomato and pepper (from Ojoo) as well as tomato and pepper (from 'Oja oba') (Table6).

Figure 2 shows the diversity of the pectinolytic organisms with *E. carotovora* having the greatest diversity with an index of 0.36, followed by *P. syringae* with an index of 0.31. *X. campestris* was the least diverse and has an index of 0.13.

Table 1: Total count of pectinolytic organisms isolated from spoilt vegetable samples from three markets at Ibadan city, Nigeria.

Sample	Market	Total Plate Count (Cfu/Ml)	Pectinolytic Count (Cfu/Ml)	Percentage of Pectinolytic (%)	Average Colony Size (Mm)	Average Zone Size (Mm)
Carrot	Bodija	2.6×10^8	**2.0×10^8**	76.9	12	7
	Ojoo	1.8×10^8	1.2×10^8	66.7	7	5
	Oja Oba	2.0×10^8	1.5×10^8	75	11	9
Tomato	Bodija	6.2×10^8	5.0×10^7	8.06	13	3
	Ojoo	5.6×10^6	**2.7×10^6**	48.2	13	3
	Oja Oba	4.7×10^8	2.2×10^8	46.8	7	9
Onions	Bodija	2.0×10^7	1.0×10^7	50	14	4
	Ojoo	1.5×10^8	**9.0×10^7**	60	1	18
	Oja Oba	1.5×10^8	3.0×10^7	20	12	5
Pepper	Bodija	3.1×10^8	3.0×10^7	9.7	7	8
	Ojoo	5.2×10^8	**9.0×10^7**	17.3	12	10
	Oja Oba	2.1×10^8	3.0×10^7	14.3	12	6
Potato	Bodija	1.2×10^8	**7.0×10^7**	58.3	5	7
	Ojoo	1.4×10^6	2.0×10^5	14.3	6	25
	Oja Oba	1.0×10^8	1.0×10^7	10	3	2

Table 2: Frequency of occurrence of pectinolytic organisms isolated from vegetables from three markets in Ibadan city, Nigeria.

Isolate	Frequency of occurrence	Percentage occurrence (%)
Erwinia amylovora	4	5.30
Erwinia chrysanthemi	8	10.5
Erwinia carotovora	21	27.63
Erwinia artroseptica	7	9.21
Erwinia herbicola	9	11.84
Pseudomonas syringae	14	18.42
Pseudomonas cichorii	5	6.60
Pseudomonas fluorescens	5	6.60
Xanthomonas campestris	3	3.95
Total	**76**	**100**

DISCUSSION

The high number of pectinolytic bacteria in all the vegetables; carrot (8.3log$_{10}$ cfu/g), potato (7.8 log$_{10}$ cfu/g) with an average pectinolytic zone size of 7mm; tomato(6.4 log$_{10}$ cfu/g), pepper and onion (7.9 log$_{10}$ cfu/g) with average zone sizes of 3, 10 and 18mm, respectively, was within the range reported by Meir *et al*. (1995) who reported organisms in wrapped and unwrapped fruits stored at ambient temperature to average 8.9 log$_{10}$ cfu/g and 7.5 log$_{10}$ cfu/g, respectively. Most of the organisms showed pectinolytic activity at pH 6, with few exhibiting at 6.5 and 5.5. The ability of the organisms to grow best and mostly at pH of 6 indicated that greater portion of the enzymes produced by the test organisms are polygalacturonase, as reported by Albersheim and Killias (1962), Bateman and Miller (1966) and Moran *et al.* (1968). Although it has been reported that a few strains of *P. fluorescens* produce polygalacturonase (Zucker and Hankin,1970), pectin methylesterase (Nasumo and Starr,1966) and pectin lyase (Schlemmer *et al*.,1987), almost all strains of soft rotting Pseudomonads so far examined produce pectate lyase. Hankin and Anagnostakis (1975) described a plate technique with agar adjusted to pH 7.5 for best activity, but this was not supported by our work as no activity was recorded by any organism at this pH.

Erwinia, Pseudomonas and Xanthomonas species were identified in this work agreeing with the reports of Liao *et al*. (1987) which indicated pectolytic *Pseudomonas*, mainly strains of *P flourescens (*or *P maginalis)* and *P viridiflava* to have accounted for over 40% of bacterial rot of fruits and vegetables in storage and transit. The soft rot group comprises several bacteria strains, of which *Erwinia carotovora* subspecies *carotovora, atroseptica* and *chrysanthemi* are the major soft rot-causing bacteria (Toth *et al*., 2001). Brocklehurst *et al*. (1981) further showed that the Pseudomonads are unique among post harvest pathogens in that they are able to grow under refrigerated conditions and to use a wide variety of simple compounds as carbon and energy sources.

Table 3: Effect of pH variation on growth of pectinolytic organisms isolated from spoilt vegetables sampled from three markets in Ibadan city, Nigeria.

SAMPLE	pH					
	5	**5.5**	**6**	**6.5**	**7**	**7.5**
			BODIJA market			
Carrot	1.8×10^8	ND	2.0×10^8	8.0×10^7	ND	ND
Tomato	ND	ND	5.0×10^7	ND	ND	ND
Onion	ND	ND	1.0×10^7	ND	ND	ND
Pepper	ND	ND	ND	2.0×10^7	ND	ND
Potato	ND	ND	3.0×10^7	ND	ND	ND
			OJOO market			
Carrot	ND	ND	1.2×10^8	ND	ND	ND
Tomato	ND	ND	2.5×10^8	1.0×10^7	ND	ND

Onion	ND	ND	6.0×10^{7}	ND	ND	ND
Pepper	ND	ND	ND	9.0×10^{7}	ND	ND
Potato	ND	ND	2.0×10^{7}	ND	ND	ND
			OJA OBA market			
Carrot	ND	ND	1.5×10^{8}	ND	ND	ND
Tomato	ND	ND	ND	2.2×10^{8}	ND	ND
Onion	ND	ND	3.0×10^{7}	ND	ND	ND
Pepper	ND	ND	3.0×10^{7}	ND	ND	ND
Potato	ND	1.0×10^{7}	ND	ND	ND	ND

ND – Not detected.

The high frequency of occurrence of *Erwinia* species could be due to the fact that they differ in their pathological capacity from non-*Erwinia* soft rot bacteria in that the former attack plants both in the field and in storage (Perombelon, 1980). Liao and Wells (1986, 1987) and Lund (1983) indicated that the latter usually cause spoilage of detached plant products after harvest. Davis *et al.* (1984) also proposed that soft rot pathogens must produce large quantities or multiple forms of pectate lyase to overcome the host defense mechanism triggered by pectic enzymes or other elicitors. The correlation between the low pectate lyase activity and the inability to infect field plants, as observed in non-*Erwinia* soft rot bacteria, seems to provide indirect evidence supporting the high frequency of *Erwinia* species. In other studies, Schwan *et al.* (1997) isolated four pectinolytic yeast species, which produce endopolygalacturonases from cocoa fermentation and *Klueveromyces marxianus* was reported to be the best enzyme producer. Moreover, Kakiomenou *et al*. (1996) reported that microbial spoilage was dominated by lactic acid bacteria and that type of spoilage has less decomposition character compared to Gram negative bacteria. *Clostridium flavum* and *C. laniganii* have also been reported to ferment pectin, thus their use in retting process of major textile fibres as reported by Lanigan (1951). Grajek (1987) reported other organisms determined to be pectinolytic included *Bacillus subtilis, Paenibacillus polymyxa, Pantoea agglomerans, Serratia fonticolar* and *Pseudomonas putida*. Pectinolytic activity has also been reported in non-phytopathogenic species such as *Rhizobium* species (Hubbell *et al*., 1978).

Table 4: Shannon-Weaver Indices of Diversity (H') and Equitability (J) of pectinolytic organisms isolated from spoilt vegetables sampled from three markets in Ibadan City, Nigeria.

SAMPLE	**H'**	**J**	**H'**	**J**	**H'**	**J**
	BODIJA		**OJOO**		**OJA OBA**	
Carrot	0.2	0.7	0.27	0.11	0.22	0.08
Tomato	0.2	0.12	**0.35**	0.11	**0.36**	0.12
Onion	**0.35**	0	**0.31**	0.14	**0.32**	0.29
Pepper	0.22	0.2	0.3	0.13	0.28	0.26
Potato	**0.31**	0.16	0.28	0.41	0.23	0

Table 5: Diversity index of pectinolytic organisms isolated from spoilt vegetables sampled from three markets in Ibadan city, Nigeria.

Sample	**Market**		
	Bodija	**Ojoo**	**Oja oba**
Carrot	0.2050^{d}	0.2750^{c}	0.2250^{d}
Tomato	0.2050^{d}	0.3550^{a}	0.3650^{a}
Onion	0.3550^{a}	0.3150^{b}	0.3250^{b}
Pepper	0.2250^{c}	0.3050^{b}	0.2850^{c}
Potato	0.3150^{b}	0.2850^{c}	0.2350^{d}

Enzyme production activities have been detected previously in culture supernatants of diverse groups of soft rot bacteria including *Erwinia spp*. (Willis *et al*.,1987), *Pseudomonas flourescens* (Hagar *et al*.,1972), *Pseudomonas viridiflava* (Hildebrand,1971), *Xanthomonas campestris* (Nasuno *et al*.,1967) and *Cytophaga johnsonae* (Liao *et al*.,1986). In the past decade, the genetics of bacterial pectinase biosynthesis has been extensively studied in phytopathogens, especially in soft-rotting *Erwinia* species which were found to produce a set of pectin-depolymerizing activities such as pectate lyases, polygalacturonases, pectin methyl esterases, and a pectin acetyl esterase (Barras *et al*., 1994; Pissavin *et al*., 1996).

The Shannon-Weaver index of biodiversity indicated diversity among the organisms. The diversity measure came from information theory and measures the order (or disorder) observed within a particular system (Shannon-Weaver, 1963). *Erwinia carotovora*, showing the greatest diversity, and also with a high frequency would be as a result of it having a wider host range as earlier reported by Van Gijsegem (1986) and Lund (1983). *Pseudomonas* strains have also been reported to cause spoilage of various vegetables like carrot (Cuppels and Kelman, 1980), onion (Sitarama and Bener, 1980), potato (Sampson and Hayward, 1971), broccoli, lettuce, spinach, tomato and asparagus (Liao and Wells, 1987) which explains their high diversity. Although pseudomonads are often considered to be ubiquitous, there are also many reports of niche specialization and diversity. Schroth *et al*. (1981) reported that *P. syringae* isolates are generally only found in association with live plants or propagative material, and in these niches they appear as virtually homogeneous populations. *P. aeruginosa* was reported by Bradbury (1986) and Palleroni (1984) to be widely distributed in soil and water, but it is occasionally isolated from both healthy and diseased plants. Green *et al*. (1974) also observed lettuce and bean being colonized by *P. aeruginosa* under varying conditions of temperature and humidity (27ºC, 80-95% humidity). Ormrod and Jarvis (1994) described *P. flourescens* to be an opportunistic pathogen causing soft rot in plants. However, Hildebrand (1989) and Brock *et al*. (1994) considered it to be actively pectinolytic, causing soft rot of various plants, thus referring to it as a plant pathogen, as also observed in the present work.

Table 6: Diversity index of pectinolytic organisms among three sampled markets in Ibadan city, Nigeria.

Market	Carrot	Tomato	Onion	Pepper	Potato
Bodija	0.2050[b]	0.2050[b]	0.3600[a]	0.2250[b]	0.3150[a]
Ojoo	0.2750[a]	0.3550[a]	0.3150[b]	0.3050[a]	0.2850[b]
Oja oba	0.2250[b]	0.3650[a]	0.3250[b]	0.2850[a]	0.2350[c]

Means followed by the same letter along the column indicate no significant difference at 0.05% level of probability.

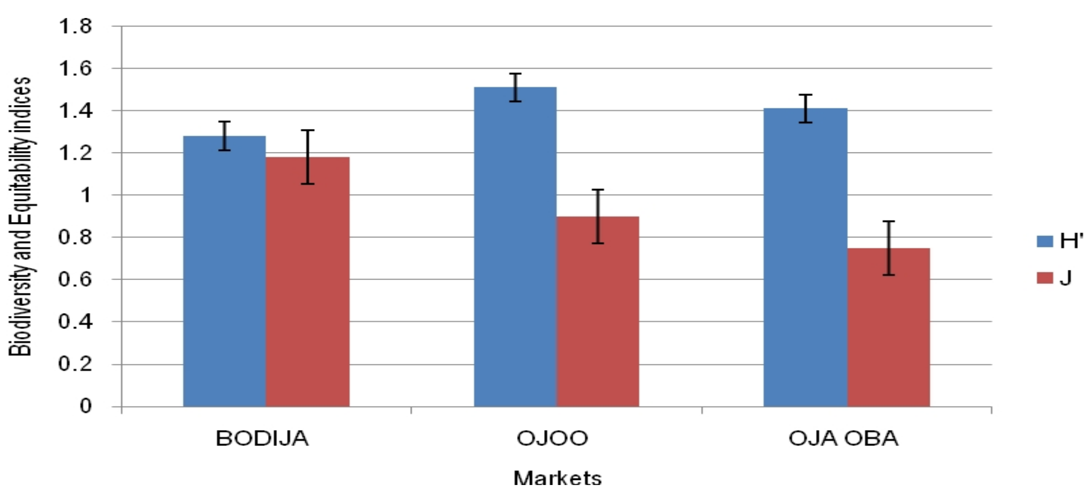

Figure 1: Shannon-Weaver Indices of diversity (H') and Equitability (J) of pectinolytic organisms isolated from spoilt vegetables sampled from three markets in Ibadan city, Nigeria. Error bars on the columns indicate the standard error.

Onion had the highest diversity of organisms with a total index of 0.98; closely followed by tomato with 0.91, potato 0.82, pepper 0.80 and carrot 0.69. High water activity and presence of different soluble sugars are important factors that contribute to the establishment and distribution of spoilage in fresh produce. Tomato and onion, showing greater diversity of organisms have these characteristics in abundance as reported by Aboaba and Ekundayo (2000) and Frazier and Westehoff (1978). Collectively, Ojoo market has the greatest diversity of pectinolytic organisms which could be as a result of cross contamination between different crops due to the same handler or polluted wash water. Earlier reports also indicated it could be as a result of contamination from the field (Perombelon, 1982).

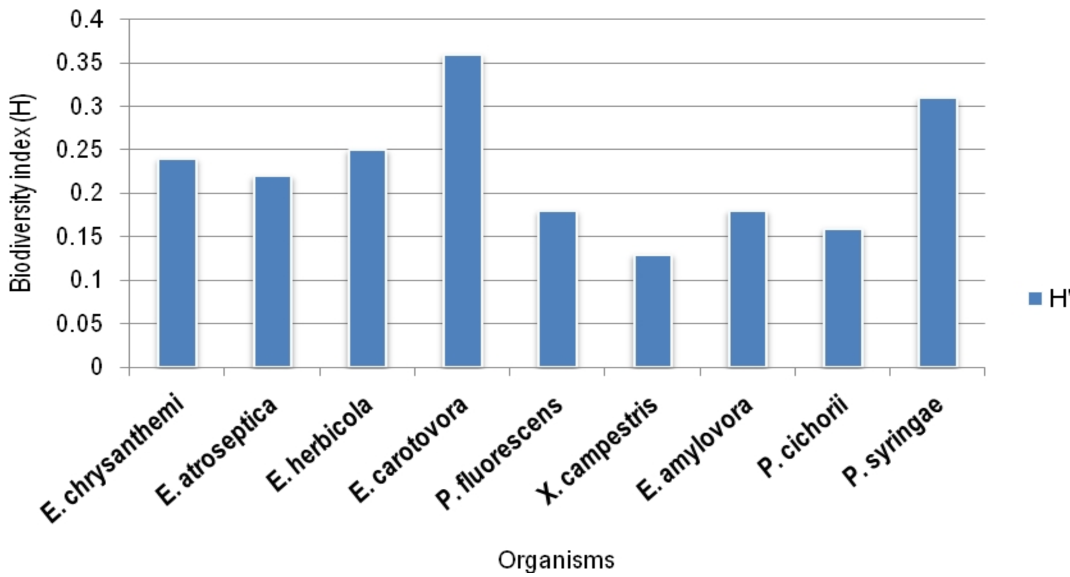

Figure 2: Shannon-Weaver index of diversity (H') of pectinolytic organisms isolated from spoilt vegetables sampled from three markets in Ibadan city, Nigeria.

CONCLUSION

The results indicated that soft rot of vegetables is caused by a wide range of pectinolytic organisms that vary with different vegetables and locations of purchase. *Erwinia* species were the dominating organisms, although, *Pseudomonads* and *Xanthomonas* strains were also reported. Vegetables have high water activity, thin skin, soluble sugars and other nutrients which makes them vulnerable to microbial spoilage right from the farm to the consumer. It is therefore recommended that optimal conditions of hygiene, transportation and storage should be observed, so that the produce gets to the consumer at the best quality and safe from microbial induced spoilage. .

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