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Review

A review of cashew (*Anacardium occidentale* L.) apple: Effects of processing techniques, properties and quality of juice

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A review including the processing techniques, properties and uses of cashew apple juice is reported. Cashew apple has multi-purpose; it can be processed to obtain human food. The process of cashew apple into several by-products can affect its nutritional, microbiological, and sensorial quality attributes. Therefore, clarification methods, thermal treatment and high hydrostatic pressure modify nutritional, microbiological, and sensorial attributes of cashew apple products. Moreover, the storage stability of cashew apple juice depends on the preservative methods used. Cashew apple is usually used in the fortification of the nutritional quality of some tropical foods, because of its high percentage of vitamin C. Cashew apple juice has great potential for bioprocess to obtain fermented products. Cashew apple contains phenolic compounds generally related to antioxidant. The valorization of cashew apples in developing countries by the improvement of the process of cashew apples available in these countries can contribute to cover the nutritional needs of the populations.

Key words: Cashew apple, physico-chemical, processing, juice quality, storage.

INTRODUCTION

Cashew (*Anacardium occidentale* L.) is a tropical fruit native from Brazil, principally grown in the North and Northeast regions. The pseudo-fruit, known as the cashew apple, is the part of the tree that connects it to the cashew nut, the real fruit, a well-known product worldwide (Zepka and Mercadante, 2009). The cashew nuts represent only 10% of the total fruit weight, and large amounts of cashew apples are left in the field after the removal of the nut (Honorato et al., 2007a).

The cashew tree grows even on poor soils with low

rainfall and is cultivated in 32 countries around the world, with Brazil, India, Vietnam, and Nigeria as the main producers (Rabelo et al., 2009). Cashew apple is the peduncle of the cashew fruit, which is rich in reducing sugars (fructose and glucose), vitamins, minerals, and some amino acids, carotenoids, phenolics, organic acids and antioxidants, and also considered as a source of energy (Oliveira et al., 2002; Campos et al., 2002; Trevisan et al., 2006; Carvalho et al., 2007; Honorato et al., 2007a). It can be processed to obtain juice, ice

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cream, and other foodstuffs (Dèdèhou et al., 2015a).

Astringency of cashew apple undertakes consumption, due to polyphenols, tannins (0.35%), and unknown oily substances (3%) present in the waxy layer of the skin (Cormier, 2008; Michodjehoun-Mestres et al., 2009a). Many factors, such as the seasonal nature of the cashew trees produce, the extreme perishable character of apples hindering its full utilization (Bidaisee and Badrie, 2001).

Thermal processing has a negative effect on the sensory and nutritional characteristics of the juice as the compounds responsible for aroma and flavor are volatile and some vitamins are thermosensitive (Polydera et al., 2003).

On the other hand, the biological composition of cashew can be influenced by variety, geographic locality and ripening stage (Lowor and Agyente-Badu, 2009; Sivagurunathan et al., 2010; Adou et al., 2011a; Gordon et al., 2012).

Some studies focused on the physico-chemical characteristics of cashew apple (Assuncao and Mercadante, 2003; Lavinias et al., 2006; Brito et al., 2007; Silva et al., 2008; Michodjehoun-Mestres et al., 2009a; Adou et al., 2011a,b, 2012) and also on the effects of postharvest process on the physico-chemical quality attributes of cashew (Souza et al., 1999, 2009; Falade et al., 2003; Figueiredo et al., 2007; Marques et al., 2007; Martins, et al., 2008; Lima et al., 2010); the effects of processing methods, such as clarification by membrane and enzymatic methods or the use of clarifying agents on the nutritional quality of cashew apple juice have also been investigated. Furthermore, the effect of thermal treatment and high hydrostatic pressure on cashew apple juice have been reported by various workers (Campos et al., 2002; Couri et al., 2003; Jayalekshmy and John, 2004; Abreu et al., 2005; Cianci et al., 2005; Castro et al., 2007; Damasceno et al., 2008; Zepka and Mercadante, 2009; Sampaio et al., 2011; Gyedu-Akoto, 2011; Talasila et al., 2011). Other studies on the storage stability of cashew apple juice by using artificial preservative or microfiltration (Talasila et al., 2012) and the effect of storage conditions on cashew apple juice stability were reported (Lavinias et al., 2006; Queiroz et al., 2008). On the other hand, cashew apple was used in the fortification of the nutritional quality of some tropical foods by mixing the apple juice or powder with other tropical food to increase its vitamins and minerals level for example (Akinwale, 2000; de Carvalho et al., 2006; Silva et al., 2008; Queiroz et al., 2008; Gyedu-Akoto, 2011; Talasila et al., 2011; Gao and Rupasinghe, 2012; Talasila et al., 2012) and in the processing of added value fermented products because of its high content of reducing sugars (Osho, 1995; Melo and Macedo, 2008; Giro et al., 2009; Venkatesh et al., 2009; Honorato and Rodrigues, 2010; Lima et al., 2010; Vergara et al., 2010; Kuila et al., 2011; Silveira et al., 2012).

The valorization of cashew products especially cashew

apple in developing countries is a relevant topic. In order to improve the valorization of cashew products, it is necessary to find out what is already done in this respect. This review aims to give information on the physico-chemical characteristics of cashew (*A. occidentale*) apple and the effects of some processing methods on the quality of cashew apple juice.

PHYSICO-CHEMICAL CHARACTERISTICS OF CASHEW (*A. OCCIDENTALE*) APPLE

Geographical and varietal effects

Biochemical profile of the apples of different species of cashew grown in some area of Ivory Coast on specific soils and climate in the various producing regions were evaluated (Adou et al., 2011a). The analyses of the juices found 10 minerals of which seven were macro-minerals and three were trace elements. The macro-minerals in order of occurrence were K>P>Mg>S>Na>Si>Cl; the distribution of the three trace elements was not uniform in all the analyzed juice samples. In addition, the minerals were not free, but in the oxidized state with oxide contents in the apples. In Ghana, the juices, from both the red and yellow cashew apples from three agro ecological zones, mineral composition (mg/100 ml) showed potassium (76.0) to be the highest, followed by calcium (43.0), magnesium (10.92), phosphorous (0.79), and sodium (0.41). While, zinc, copper, and iron concentrations were lower and ranged from 0.05 to 0.08 mg/100 ml. Phenol and tannin contents in the juice showed significant ($p<0.05$) variation among the ecological zones (Agostini-Costa et al., 2002; Lowor and Agyente-Badu, 2009). Moreover, protein, reducing sugars, total sugars showed significant ($p<0.05$) variation among the ecological zones except the pH value (Adou et al., 2011a). The variations in the physico-chemical characteristics of cashew apple juices from the different locations is associated with changes in soil conditions, cultural practices and other climatic conditions such as temperature and humidity (Egbekun and Otiri, 2010).

The results of vitamin C content, total sugars, concentrations of glucose, fructose and sucrose, level of organic acids, citric acid, tartaric acid, acetic acid, oxalic acid, fumaric acid, pH, titratable acidity, total soluble solid content, dry matter, ash, protein content, and amino acids showed that except pH, the color of apples influenced significantly ($p<0.05$) the parameters analyzed (Adou et al., 2012). In general, elongated red variety showed higher carotenoid levels than the yellow one. In contrast, ascorbic acid values were higher in the yellow variety from both regions (Assuncao and Mercadante, 2003).

Different clones of early dwarf cashew tree (*A. occidentale*, L.) CCP-06, CCP-1001, and CCP-76, developed by Embrapa Agroindústria Tropical (Brazilian Agricultural Research Corporation) located in Fortaleza,

Ceará, Brazil were characterized. Physical-chemical determinations were done in cashew apples, randomly at interval period of 15 days (during 75 days). Chemical analysis evaluated the total acidity, reduced content, not reduced and total sugars, vitamin C, phenolic compounds (tannins), pH, soluble solids, moisture, ash, protein, fiber, iron, calcium, and phosphorus (Maia et al., 2004). The authors concluded that the stems of different cashew clones differ in acidity, moisture, and tannin content, being a good source of sugar and excellent vitamin C.

Effect of ripening stage

Cashew apples at three different maturity stages (Unripe fruits grew for 33 to 36 days, medium-ripe fruits 45 to 50 days and ripe fruits 52 days) were examined according to their ascorbic acid content, phenolic compounds and antioxidant capacity (Gordon et al., 2012). The results showed that the quantities of phenolic compounds were higher in immature cashews and decreased during the ripening process. Myricetin-3-O-rhamnoside, 3-O-galactoside quercetin, and quercetin 3-O-rhamnoside were the main flavonoid present in all phases. The antioxidant capacity and the concentration of ascorbic acid increased in the course of ripening. The antioxidant activity was significantly ($p < 0.05$) influenced by ascorbic acid, more than the content of phenolic compounds.

Different early dwarf clones: CCP 76, CCP 09, BRS 189, and BRS 265 during seven ripening stages were analyzed for vitamin C, total carotenoid, total anthocyanin, yellow flavonoids and polyphenol contents, and total antioxidant capacity. Clone BRS 265 ripe cashew apple presented the highest vitamin C content (279.37 mg/100 g). The ripe BRS 189 cashew apple is colored bright red, and its total anthocyanin content was the highest (21.16 mg/100 g). Yellow flavonoid content was higher in ripe CCP 76 and BRS 189 cashew apples with 56.32 and 50.75 mg/100 g, respectively. The highest levels of extracts of polyphenols and antioxidant capacity were observed in CCP 09 during the first five ripening stages. The antioxidant activity of cashew apples (*A. occidentale* L.) was mainly attributed to polyphenol content ($r = 0.90$; $p < 0.01$) (Almeida Lopes et al., 2012).

Tannin contents and flavonoid components of cashew apple (*A. occidentale*)

The interference of the genetic and climatic variations in cashew apple tannin contents was evaluated during 2000 and 2001 harvest. Cashew apples were harvested from microcarpum cashew tree and from seven clones of early cashew tree (var. nanum) in the Experimental Stations of the Embrapa in Pacajus and Paraipaba (CE) (Brazil) and the Pimenteiras Farm in Aracati (CE) (Brazil). Cashew apples from CP-076 early cashew tree, the most popular commercial clone at Northeastern Brazil, were used as

Table 1. Tannin contents of peduncle of microcarpum cashew tree from seven clones of early cashew tree (var. nanum) (Agostini-Costa et al., 2002).

Clones	Harvest	Origin	Tannins (mg/100 g)
<i>Microcarpum</i>	2001	Pacajus	116
Embrapa-50	2001	Paraipaba	134
Embrapa-51	2001	Pacajus	162
Embrapa-51	2001	Paraipaba	134
Embrapa-51	2000	Aracati	211
Embrapa-51	2000	Pacajus	196
END-157	2001	Paraipaba	108
END-157	2001	Pacajus	105
END-157	2000	Aracati	168
END-189	2000	Aracati	125
END-183	2000	Aracati	183
CP-1001	2001	Pacajus	84
CP-1001	2001	Paraipaba	85
CP-1001	2000	Paraipaba	124
CP-1001	2000	Pacajus	166
CP-076	2001	Pacajus	121
CP-076	2001	Paraipaba	104
CP-076	2000	Pacajus	132
CP-076	2000	Paraipaba	133
CP-076	2000	Aracati	154
CP-09	2001	Pacajus	274
CP-09	2001	Paraipaba	237
CP-09	2000	Pacajus	181
CP-09	2000	Paraipaba	304

control. Table 1 shows that tannin contents in cashew apples from CP-09 clone were significantly higher than other analyzed clones and the cashew apples from CP-1001 clone presented the lowest tannin values. For 2001-harvest, the tannin values of CP-09 cashew apples from Pacajus, CE, were 14% higher than tannin values of Paraipaba, CE. This difference is certainly due to climatic variations. The harvest year also significantly influenced tannin. For example, tannin values obtained in 2000 were about 25% higher than tannin values obtained in 2001 for CP-09 cashew apple. This difference was probably induced by rainfall which was more intensive at Pacajus in 2000. Indeed, a significantly positive correlation between rainfall intensity and tannin content was demonstrated, mainly during ripening period of cashew apples, when these phenolic compounds were metabolized (Agostini-Costa et al., 2002).

In addition, it was reported that the various types of tannins were unequally distributed in the skin and the flesh of cashew apples (Michodjehoun-Mestres et al., 2009b). Results showed that both skin and flesh tannins contained high percentages of (-)-epigallocatechin and (-)-epigallocatechin-O-gallate, followed by low quantities of (-)-epicatechin and (-)-epicatechin-3-O-gallate; 100% of

Table 2. Flavonoids components detected in cashew apple (Brito et al., 2007).

Compound	Cashew apple (mg/g)
Myricetin 3-O-galactoside	0.0532
Myricetin 3-O-glucoside	0.0274
Myricetin 3-O-xylopyranoside	0.0124
Myricetin 3-O-arabinopyrannoside	0.0104
Myricetin 3-O-arabinofuranoside	0.0097
Myricetin 3-O-rhamnoside	0.0400
Total myricetin glycosides	0.1511
Quercetin 3-O-galactoside	0.0465
Quercetin 3-O-glucoside	0.0144
Quercetin 3-O-xylopyranoside	0.0116
Quercetin 3-O-arabinopyrannoside	0.0108
Quercetin 3-O-arabinofuranoside	0.0079
Quercetin 3-O-rhamnoside	0.0227
Total quercetin glycosides	0.1139
Kaempferol 3-O-glucoside	Trace amount
5-Methylcyanidin 3-O-hexoside	0.0197
Total glycosylatedflavonoids	0.2847

the compounds were 2,3-cis configuration. Skin tannins were half as galloylated (20%) than flesh tannins (40%).

The 14 flavonoids determined in cashew apple by Brito et al. (2007) are shown in Table 2. The results showed that one anthocyanin and thirteen glycosylated flavonols were detected in cashew apple methanol-water extract. This study demonstrated that cashew apple is a good source of flavonoids. Indeed, flavonoid of food plants has been reported to offer biological benefits, such as reduced risk of cancer and cardiovascular disease.

FLAVOR CHEMISTRY OF CASHEW JUICE

The evaluation of the volatile flavor compounds from cashew juice by the Osme gas chromatography/olfactometry technique showed that ethyl 3-methyl butanoate (16.70%), *trans*-2-hexenal (14.27%), methyl 3-methyl butanoate (9.72%), 2-methyl-2-pentenal (9.27%), ethyl butanoate (8.47%), hexanal (7.68%), 2-butoxyethanol (3.35%), 3-methyl-1-butanol (3.23%), and 2-methyl butanoic acid (3.01%) were the major compounds (Garruti et al., 2003).

EFFECTS OF CROPPING SYSTEM, IRRIGATION, MANAGEMENT AND SPACING ON CASHEW APPLE QUALITY

The quality of post-harvested cashew apples has been influenced by the production systems. The integrated fruit production (IFP) and conventional cropping production (CP) systems in dwarf cashew orchard did not influence the firmness of pulp, total soluble solids, and color of

apples. For the titratable acidity and vitamin C content, the IFP system was significantly higher than the CP ones. For the pH variable, a significant difference was observed between the averages of the systems, the value of pH obtained from CP was superior to that of IFP (Andrade et al., 2008).

The influence of spacing on physical-chemical characteristics of peduncles of irrigated early dwarf of cashew (*A. occidentale* L. var. *nanum*) was evaluated. In the Experimental Station of Vale do Curu, located in the county of Paraipaba, Ceará, Brazil, four treatments were evaluated: one traditional (6.0 × 8.0 m) and three densely spaced (4.0 × 3.0, 6.0 × 3.0, and 8.0 × 3.0 m) to which trimming and paring were applied. There were no significant ($p > 0.05$) differences among treatments for the characteristics, such as total soluble solids (TSS), total titratable acidity (TTA), TSS/TTA ratio, vitamin C, and tannin (polymeric, dimeric and oligomeric) contents (Damasceno and Bezerra, 2002).

CASHEW TOXICITY

The study of *in vivo* toxicity of mixture of "cashew apple juice and milk" on mice to confirm or refute the idea that the cashew apple juice consumed with milk would be fatal showed that there was no toxicity of apple "juice-milk" mixture. Instead, the richness of the mixture positively affects erythropoiesis in the studied mice. For the authors, the toxicity of the mixture is not proven on mice; it is permissible to conclude that it is not fatal also for human. So, the idea that the cashew apple juice consumed with milk would be fatal is refuted (Adou et al., 2013). On the other hand, the toxicological assessment of locally produced cashew wine on albino rats (*Rattus norvegicus*) at 10, 7.5, and 5% alcohol content administered to them orally at 1ml/160g body weight using canular for eighteen days was done. Results revealed that at 7% alcohol content and above it caused the distortion of the liver architecture of animals, indicator of toxicity (Awe et al., 2013). Dare et al. (2011) investigated the effects of aqueous extract of *A. occidentale* leaf on pregnancy outcome of Wistar rats. The results showed that the extract of *A. occidentale* should not be taken by pregnant women, even if they suffer of diabetes (Dare et al., 2011). Indeed, for example, intravenous administration of the hexane extract of the bark of (cashew) in normal, healthy dogs produced a significant lowering of the blood glucose levels probably due to the presence of stigmast-4-en-3-ol and stigmast-4-en-3-one (Alexander-Lindo et al., 2004).

EFFECTS OF POSTHARVEST PROCESS ON PHYSICO-CHEMICAL CHARACTERISTICS AND OTHER QUALITY ATTRIBUTES OF CASHEW PSEUDO-FRUITS

Cashew apple is subjected to several processes after the

harvest which influences its physico-chemical characteristics and the quality attributes, because of its highly perishable nature.

The effects of postharvest calcium applications for storage on physico-chemical characteristics, other quality attributes and calcium concentration in tissue of the pseudo-fruits of early dwarf cashew (*A. occidentale* L.), stored under refrigeration at 4°C and modified atmosphere showed that the total soluble solids (Brix) and soluble sugars decreased during storage (Figueiredo et al., 2007). According to the authors, this could be attributed to the consumption of sugars through respiratory metabolism. Cashew apples, irrespective of the dose of calcium used showed significant variations in the acidity according to the time, with trend of reduction. The pseudo-fruits, regardless of the chloride calcium concentration, showed a tendency of decrease in the total vitamin C and an increase in pH during storage. The anthocyanin content showed no variation in treated cashew pseudo-fruits. Low reductions in less polymerized poly-phenolic fraction were observed during the storage period.

The doses of calcium and time of storage had significant effect on compounds, phenol polymeric, oligomeric, and dimeric; also the effect of storage time on cashew apple calcium concentrations was significant. Authors found significant interaction between time of storage and treatment with calcium for total calcium, soluble calcium and insoluble calcium. Cashew apple subjected to the treatment of calcium, showed an increase in the values of total calcium until 15 days of storage, and decreased slightly until the end of the experiment. The treatment of cashew apple from mature dwarf of cashew tree with gamma radiation with doses of 0, 0.5, and 1.0 kGy followed by a storage for nine days at 4°C showed that the levels of vitamin C decreased during storage and according to the applied doses of radiation, while the firmness of the fruits was influenced by both the radiation doses and storage time, increasing during storage and decreasing as the radiation doses increased (Souza et al., 2009).

The combined methods (reduction of water activity, mild heat treatment, pH reduction, addition of ascorbic acid, 1000 ppm sodium benzoate, 600 and 900 ppm of SO₂ addition) were capable of assuring the microbiological stability and sensorial acceptance of the cashew apples during storage at room temperature for 120 days (Mesquita et al., 2003).

The osmotic dehydration of cashew apples showed that the higher the concentration of the osmotic syrup (50, 60, and 70°Brix), induced higher water losses and solute gain during the same period of dehydration. The osmotic dehydration is a relevant factor in the processing of cashew because it removes the water by osmotic pressure and presents the advantage in the characteristics of color, taste and texture, and it also decreased the enzymatic darkness of the fruits during the

dehydration process (Marques et al., 2007). The combined methods (bleaching, osmotic process, heat treatment, and storage) affect the major physical and chemical characteristics of cashew apple (Souza Filho et al., 1999). Changes were pH decrease, soluble solids and reducing sugars increase. Ascorbic acid loss was 23.3% after bleaching, 31.7% after one day osmosis, 35.5% after five days osmosis, 69.0% after heat treatment and 87.3% after 60 days storage at ambient temperature (~ 28°C). The reducing sugars, at the end of the period of storage, represented 83.1% of soluble solids value in the product.

The effects of different osmotic pretreatments on cashew apples, drying kinetics, and product quality were investigated. It was found that drying rates of pretreated fruits decreased owing to the presence of infused solutes. The osmotic pretreatment of the samples showed greater losses of vitamin C and lower levels of water activity (Moreira et al., 2009).

The cashew apples treated at high temperatures (100 to 180°C) showed that generating temperature profiles of heat processes would help to preserve the ascorbic acid content of cashew apples as well as the control of the color development during high-temperature processes (Lima et al., 2010).

Effect of processing on the nutritional quality of cashew juice: Clarification followed by membrane and enzymatic process

Membrane separation processes have been studied as alternatives to heat processes due to their characteristics being conducted at low temperatures, allowing the preservation of heat sensitive compounds, such as vitamins.

Comparing the processing performed in three steps: enzymatic treatment of the pulp, microfiltration for obtaining the clarified juice, and concentration of clarified juice by reverse osmosis showed that tannins were retained by microfiltration membrane and it was not verified in clarified and concentrated juice (Cianci et al., 2005). According to the authors, it is possible to obtain a clarified and concentrated cashew juice with high vitamin C content without using thermal processes, but microfiltration and the inverse osmosis. The sterile filtration and chemical preservation was efficient in decreasing astringency, microbial count, and in retaining nutritional quality of the juice, since soluble solids, total sugar content, and vitamin C are suitable for preservation of cashew apple juice up to three months under refrigeration (Talasila et al., 2011). The cross-flow microfiltration on mineral membranes for the production of clarified cashew apple juice showed that membranes produced a completely clarified juice in which the ascorbic acid content was very close to that of the fresh juice. In contrast, the phenols present at a great quantity in the

raw cashew apple juice were almost completely eliminated during the process (Abreu et al., 2005). But the clarification of cashew apple juice using enzymatic treatment combined with microfiltration decreased the Vitamin C content whereas soluble solids, pH, and acidity were unaffected by the process (Campos et al., 2002).

Clarification of cashew apple juice using clarifying agents

The clarification of cashew apple juice by removing phenols and tannins is an important step in cashew apple processing, because these compounds are responsible for its astringency. Many clarifying agents recommended, such as sago (a refined commercial preparation of starch from cassava (*Manihot esculenta*), starch, gelatin, and poly vinyl pyrolidone (PVP) were used (Jayalekshmy and John, 2004). The clarifying agent, sago at a concentration of 2 g/L, decreased the tannins by 42.85% with visual clarity of 94%. The same clarifying agent with the same concentration along with sterile filtration decreased the tannins by 41.75% with improved visual clarity of 96% (Talasila et al., 2011). 'Sago', a natural commercial starch preparation, is an efficient clarifying agent. Tannin content of the juice clarified with starch was significantly higher than that of sago, PVP and gelatin (Jayalekshmy and John, 2004). The effects of dose of cassava and rice starch, incubation time at 30°C on clarity of cashew apple juice were investigated by Dédéhou et al. (2015b). Cassava starch at 6.2 ml/l for 300 minutes decreased tannins content at 34.2% with visual clarity of 93.75%, while rice starch at 10 ml/l for 193 minutes decreased tannins content at 42.14% with visual clarity of 94.8%. The mechanism of separation of tannin from cashew apple juice is different from different clarifiers, and may probably explain the variations in the juice quality observed. For instance, PVP chelates tannins and sediment it at the bottom. However, starch owing to its great affinity for tannins, removes it from the juice through the technique of flocculation. The efficiency with which different types of starch remove tannins from the juice may vary depending on the size and arrangement of amylose and amylopectin chains. Furthermore, the organic compounds available in the natural starch grains may interfere negatively with the flocculation technique. Sago has further advantages of being substantially cheaper especially in comparison to the traditional use of PVP, which is both costly and to be imported (Jayalekshmy and John, 2004). In addition, clarification with PVP reduced both the chemical and sensorial quality of cashew apple juice (Gyedu-Akoto, 2011).

The clarification methods of cashew apple juices using tannase or gelatin revealed that juices treated with tannase showed a decrease in total tannin (46%), hydrolysable tannin (88%), proanthocyanidins(2%) and turbidity (88%) compared with 39, 50, 32, and 94% for

those treated with gelatin, respectively. Therefore, treatment with tannase when compared with gelatin application was more efficient at reducing hydrolysable tannins, but less efficient at reducing proanthocyanidin levels in the juices. No visual differences were observed for the juices clarified by the two methods (Couri et al., 2003).

The effects of clarification with gelatin, PVP or the adsorbent resin XAD-16, singly or in combination, on the composition and the quality of the juice showed that treatment with gelatin alone (at a concentration of 2.7 to 3.0 g/L at 20°C) resulted in good clarification, and eliminated approximately 94% of tannins. The 2 resins gave the poorest elimination rates of tannins (24% for PVP and 4.3% for XAD-16). Treatment with gelatin followed by adsorbent resins gave a clear, stable and pleasant taste juice with no astringency. Tannin and protein contents were reduced by approximately 99%. These clarification treatments resulted in losses of nutrients, especially ascorbic acid (Quoc et al., 1999).

Effect of thermal treatment on cashew apple juice

The effect of thermal treatment on clarified cashew apple juice at temperatures from 88 to 121°C showed that increasing processing time increased the absorbance at 420 nm. Increasing temperature also showed the rise of browning rate measured at 420 nm. The results obtained for total sugars and the reduction of sugars did not show any definite tendency at any temperature used, and the steady concentration of sugars during thermal treatment showed that sugars did not react with amino acids and therefore did not affect browning. Increasing processing time and temperature had a significant effect on decomposition of ascorbic acid. The correlation of the change in absorbance at 420 nm with loss of ascorbic acid showed an inverse relationship, indicating that ascorbic acid may be the main factor that causes browning in clarified cashew apple juice (Damasceno et al., 2008). During the concentration of cashew apple juice in an industrial plant, 71 volatile aroma compounds were identified (Table 3); of these, 47 were odor active. Alcohols were preferentially recovered in the cashew water phase, notably heptanol: *trans*-3-hexen-1-ol and 3-methyl-1-butanol, accounting for 42% of the total chromatogram area and imparting green grass and fruity aroma in the water phase. Esters represented 21% of the total chromatogram area, especially ethyl 2-hydroxyhexanoate, ethyl *trans*-2-butenate, and ethyl 2-methylbutanoate, and were responsible for the fruity/cashew-like aroma in the water phase. On the other hand, 3-methylbutanoic and 2-methylbutanoic acids were volatile acids that demonstrated the greatest odor impact in the GC effluents of the water phase (Sampaio et al., 2011). According to the authors, further concentration of esters recovered in the water phase, either by partial

Table 3. The volatile compounds identified in the aqueous phase of the cashew juice by chromatography of the different areas expressed by percentage (Sampaio et al., 2011).

Linear retention indices (LRI) (DB-Wax)	Compound	% Area (FID)
-	Ester	20.99
<1000	Ethylpropanoate b	0.12
<1000	Ethyl 2-methylpropanoateb	0.16
1010	Methyl 2-methylbutanoateb	Tr
1014	Methyl 3-methylbutanoatea	0.15
1032	Ethylbutanoatea	0.17
1048	Ethyl 2-methylbutanoatea	0.97
1067	Ethyl 3-methylbutanoatea	3.31
1115	Methyl <i>trans</i> -2-butenateb	0.03
1127	Isoamylacetatea	0.05
1166	Ethyl <i>trans</i> -2-butenatea	0.04
1182	Ethyl 3-methylpentanoateb	0.04
1203	Methyl 2-ethylacrylatec	Tr
1237	Ethylhexanoatea	0.69
1239	Ethyl <i>trans</i> -2-methyl-2-butenatea	-
1298	Ethyl <i>trans</i> -3-hexenoateb	0.05
1338	Ethyl <i>trans</i> -2-hexenoateb	0.09
1407	Ethyl 3-hydroxy-3-methylbutanoatec	0.24
1429	Ethyl octanoateb	Tr
1486	Methyl 2-hydroxy-3-methylpentanoateb	0.03
1522	Methyl 2-hydroxy-4-methylpentanoatec	0.34
1547	Methyl 2-hydroxy-4-methylpentanoatec	13.97
1596	Ethyl 2-hydroxyhexanoateb	0.12
1662	Ethyldecanoateb	Tr
1696	Ethyl 3-hydroxyhexanoateb	0.04
1800	2-Phenylethyl acetateb	0.08
1936	3-Phenylpropyl acetatec	Tr
2134	Ethylcinnamateb	0.30
-	Alcohol	42.06
1038	2-Methyl-3-buten-2-olb	0.29
1096	2-Methyl-1-propanola	1.62
1119	3-Pentanolb	0.05
1159	1-Butanola	0.36
1173	1-Penten-3-olb	0.98
1178	3-Buten-1-olb	0.72
1226	3-Methyl-1-butanola	24.03
1262	1-Pentanola	0.95
1314	<i>Trans</i> -2-Penten-1-olb	0.10
1318	4-Methyl-1-pentanolb	0.29
1323	Cyclopentanolc	0.91
1330	3-Methyl-1-pentanolb	0.56
1359	Hexanolb	4.11
1366	<i>Trans</i> -3-Hexen-1-olb	0.94
1388	<i>cis</i> -3-Hexen-1-ola	4.52
1409	<i>Trans</i> -2-Hexen-1-olb	0.36
1460	Heptanolb	0.14
1502	2-Ethyl-1-hexanolb	0.10
1566	1-Octanola	0.39
1907	Phenylethylalcoholb	0.45
2049	3-Phenyl propanolb	0.19

Table 3. Contd.

-	Aldehyde	1.94
1074	Hexanala	0.11
1141	2-Methyl-4-pentenal ^c	0.12
1209	<i>Trans</i> -2-Hexenal ^b	1.54
1451	Furaldehyde ^b	0.17
-	Ketone/lactone	12.88
<1000	2,3-Butanedione ^b	3.30
1055	2,3-Pentanedione ^b	0.07
1282	3-Hydroxy-2-butanone ^a	7.08
1628	Acetophenone ^b	0.38
1721	γ -Hexalactone ^b	Tr
2025	γ -Nonalactone ^b	Tr
-	Acids	12.62
1445	Aceticacid ^b	Tr
1566	2-Methylpropanoic acid ^b	Tr
1667	3-Methylbutanoic acid ^b	11.63
1667	2-Methylbutanoic acid ^b	-
1846	Hexanoicacida	0.34
1873	Benzylicacid ^b	0.03
2058	Octanoicacid ^b	0.27
2176	Nonanoicacid ^b	0.08
2271	Decanoicacid ^b	0.27
2484	Undecanoicacid	Tr
2560	Phenylaceticacid ^b	Tr
-	Hydrocarbon	0.11
-	Unidentified compounds	5.09
1379	NI	1.05
1414	NI	Tr
1513	NI	2.85
1951	NI	Tr
2437	NI	1.19

NI: Compound not identified; tr: detected in trace amounts (<0.01%). ^aCompound positively identified (pure standard). ^bCompound identified by MS and linear retention index. ^cCompound tentatively identified.

distillation or by alternative technologies, such as per-evaporation, could generate a higher-quality natural cashew apple essence. Besides, among the 19 carotenoids identified in the unheated simulated cashew apple juices, with all-*trans*- β -cryptoxanthin and all-*trans*- β -carotene as the major ones, heating caused the loss of five xanthophylls, whereas two new *cis* isomers and five epoxides or furanoid-derivatives were formed and the levels of all *cis* isomers increased but the amounts of total carotenoids lost were not compensated by those formed. These facts indicated that isomerization and oxidation of both colored and non-colored compounds were the main reactions occurring during heating of carotenoids in aqueous-based and juice systems (Zepka and Mercadante, 2009).

STORAGE STABILITY OF CASHEW APPLE JUICE

The test of the efficiency of chemical preservatives in combination (sodium benzoate and sodium metabisulphite at 0.1 g/L each, sodium benzoate and citric acid at 0.1 g/L each and sodium metabisulphite and potassium metabisulphite at 0.05 g/L each) prolonged shelf life of cashew apple juice up to 20 days. Vitamin C and total sugars of the preserved samples were found to be almost stable. Sensory attributes also revealed good overall acceptability of the juice (Talasila et al., 2012). The shelf life of juice treated with citric acid and benzoic acid at 0.1 g/L each and stored at 4°C was prolonged up to 90 days (Talasila et al., 2011).

Cashew apple juice first hydrolyzed and then micro-

filtered in a 0.3- μ m pore size tubular membrane and stored at lower temperature was still appropriate for consumption after 2-month shelf life, as a vitamin C source and without any haze (Campos et al., 2002). Ultrasound treatment has a potential to be used as an alternative non-thermal technique for traditional thermal pasteurization process for maintaining the quality of beverages prepared from fruit and vegetable juices (Gao and Rupasinghe, 2012).

Storage temperature (frozen, refrigeration or room temperature) differently affect physico-chemical stability of cashew apple juices. For example, the storage of cashew apple juices kept at room temperature for 24 h, refrigerated for seven days or frozen for 120 days showed that the ascorbic acid content in fresh cashew apple juice was 147.29 ± 0.41 mg/ 100 ml and decreased to 6.57% when kept under room temperature. For the juices stored when refrigerated and frozen, reduction rates of ascorbic acid were 1.16%/day and 0.05%/day, respectively. However, the cashew apple juices stored at 4°C reduced polyphenol oxidase (PPO) activity and hydrolyzed cinnamic acid. 5-Hydroxymethylfurfural content in cashew apple juices increased after injury and storage at higher temperatures, indicating non-enzymatic browning. For microbiological stability, in the juices kept at room temperature, an increase in the counts of mesophile bacteria and yeasts and moulds was observed. In the juices stored when refrigerated for seven days, mesophile bacteria counts decreased and yeast and mould counts increased. In the frozen juices, the yeasts and moulds counts remained lower than the initial counts, while mesophile bacteria counts showed decreased variation up to the thirteenth day, and then remained stable (less than one logarithmic cycle) above the initial count (Lavinias et al., 2006).

THE USE OF CASHEW PRODUCTS FOR HUMAN

Fortifying the nutritional quality of some tropical foods

Cashew apple juice is generally used in fortifying some tropical fruits when mixed with other tropical fruits low in vitamin C (for example, pineapple, mango), raised the nutritional quality (Akinwale, 2000).

A ready-to-drink cashew apple juice sweetened with honey in substitution to saccharose showed in the stability studies, that the product maintained good acceptability until the end of the storage period, with regards to the attributes of color, flavor, overall acceptability and the purchase intention. The product maintained satisfactory microbiological quality, in agreement with the current Brazilian legislation. The physicochemical changes did not characterize the lack of stability of the product, except for vitamin C content, which showed an accentuated decrease at the end of

storage (Silva et al., 2008).

Mucamalt (malt drink belongs to the category of brewed drinks that are considered non-alcoholic beverage drink, fortified with vitamin C and other minerals that are of benefit to human health) can be produced with vitamin C obtained from cashew apple fruits (Abdulraheem et al., 2013). Cashew apple juice was also used in the formulation of coconut water/cashew apple juice blends containing 100 ppm of added caffeine. The formulation with 12.5% cashew apple juice and 87.5% coconut water reached the highest hedonic scores. All formulations showed good microbiological quality. The vitamin C content showed a great difference among the formulations which vary according to the proportion of cashew apple juice used (Carvalho et al., 2006).

Cashew apple residues from fruit juice industry as dehydrated fruit powders can be used for wheat flour substitution for cookies formulations. pH, fiber, and protein contents were significantly affected by fruit powder substitution levels during the biscuit-type cookies process. However, cashew apple waste from fruit juice industry can be useful to prepare cookies with good acceptability among consumers. Therefore, the fruit powder addition in biscuit-type cookies formulation seems to be better suited for cookie process and enrichment, since it is possible to use them as partial ingredients for wheat flour substitution as well as functional ingredients in formulated foods (Uchoa et al., 2009).

Other uses

The utilization of cashew apple juice for bioethanol production with optimized fermentation parameters (substrate concentration 10%, pH 6.0, temperature 32.5°C and inoculum level of 8% (v/v) on 24 h which gives maximum yield of ethanol 7.62%, respectively) using immobilized yeast cell technology by *Saccharomyces cerevisiae* was established (Neelakandan and Usharani, 2009). *Acinetobacter calcoaceticus* was able to grow and to produce bio-surfactant on a defined culture medium and on cashew apple juice, reducing the surface tension of both media (Rocha et al., 2006).

Cashew apple juice revealed to be efficient as dairy product for *Lactobacillus casei* growth. The fermented juice with *L.casei* is a good and healthy alternative functional food containing probiotics. *L. casei* was able to overcome the natural microbiota of cashew apple juice dismissing thermal treatment, and thus, reducing the production costs. The fermentation optimal conditions were: initial pH 6.4, fermentation temperature of 30°C, inoculums size of 7.48 Log CFU/ml and 16 h of fermentation (Pereira et al., 2011). However, cashew apple juice is a good source of reducing sugars and can be used as substrate for the production of dextransucrase by *Leuconostoc citreum* B-742 for the

synthesis of oligosaccharides using the crude enzyme (Rabelo et al., 2009), with *Leuconostoc mesenteroides* (Honorato et al., 2007b; Kuila et al., 2011). Cashew apple juice can be used to grow *L. mesenteroides* to produce dextran, mannitol, and oligosaccharides (Honorato et al., 2007b).

Cashew apple juice containing *in vitro* synthesized oligosaccharides can be used as raw material or as ready-drink beverage or as food ingredient in other products by enzymatic process with addition of dextranase enzyme (Honorato et al., 2010) or for *L. casei* B-442 culture and lactic acid production (Silveira et al., 2012). Also, it is feasible to produce surfactin from cashew apple juice by *Bacillus subtilis* LAMI005 (Giro et al., 2009) and to extract sugar (Kuila et al., 2011).

Based on the level of sugar, crude protein, trace metal constituents, and its ability to support the growth of yeasts, the use of cashew apple juice as feed-stock for single cell protein and for wine production was suggested (Silveira et al., 2012).

CONCLUSION

Cashew (*A. occidentale* L.) apple is a good source of antioxidant compounds, reducing sugars, minerals, and some amino acids. Several parameters, such as genetic and climatic variations as well as ecological zones and ripening stage can significantly affect the chemical composition of cashew apples. The quality of post-harvested cashew apples has been influenced by the production systems. Cashew apple is subjected to several processes after the harvest which influences its physico-chemical characteristics and quality attributes. There are many traditional and industrial ways of removing the astringency of cashew apple juice, while clarifying the juice. It is important to encourage the valorization of cashew apples in developing countries by the improvement of the process of cashew apples available in these countries in order to contribute to the nutritional needs of the populations.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of whey storage on physicochemical properties, microstructure and texture profile of ricotta cheese

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The aim of this study is to evaluate the effect of whey storage period (0, 24, 48 and 72 h) on the physicochemical parameters, color, texture and microstructure of fresh ricotta during storage. Sweet whey and acid whey were evaluated based on titratable acidity, pH, fat, cryoscopy, and density, while ricotta was based on yield, fat, protein, ash, acidity, pH, moisture, total solids, color, texture, and microstructure. This was done with analysis of variance in a completely randomized design using Tukey test at 5% probability. Whey pH values increased with storage time. Ricotta made with stored whey had average yield of 5.33%, with decreased fat content and pH, and increased acidity. There were subtle differences in color and texture of ricotta during storage; its hardness and gumminess decreased, resulting in microstructure compression. It is concluded that the production of ricotta with whey stored for up to 72 h makes the product appropriate for consumption.

Key words: Byproduct, fat, fresh cheese, ricotta, whey cheese, organoleptic properties.

INTRODUCTION

Significant milk production and consumers' acceptance of dairy products have increased the production of various

types of cheeses. This has generated significant amounts of liquid waste, from 85 to 95% of the total milk volume,

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called whey (Dragone et al., 2009). In many dairy industries, this byproduct is considered waste and disposed without prior treatment, thus representing the main source of pollution in this sector (Prazeres et al., 2012). The high organic content of whey causes serious environmental impacts due to the high biochemical oxygen and chemical oxygen demand (Magalhães et al., 2011).

However, the biological treatment of whey has high costs and is therefore economically unviable for most dairy industries (Dragone et al., 2009). There is a recurring concern about the applicability of whey in milk products of added value as an effective alternative to overcome environmental issues (Silva et al., 2012). Due to the high moisture content and organic compounds, whey is very perishable, so it requires quick use or application of conservation measures such as refrigeration and/or addition of preservatives (Almeida et al., 2001). Its maximum time of 72 h is recommended for the collection and industrial processing of whey, and maximum temperature of 10°C is recommended for the transport of refrigerated milk whey in isothermal tanks (Brazil, 2013).

The preparation of ricotta is a rational way to use this byproduct, as it has high nutritional value and is considered one of the most cost-effective products that use whey in its composition (Prudencio et al., 2014). Whey produced by rennet coagulation of milk casein is called sweet whey, while that obtained by lactic acid coagulation is called acid whey (De La Fuente et al., 2002). Cheeses produced by acid coagulation with heat treatment emerged as a tool for recovery of milk proteins and/or sweet whey, which have high nutritional value; examples are ricotta (Italy), anari (Cyprus) and manouri (Greece) (Fox et al., 2000). Other varieties are cottage and cream cheese (USA), white cheese (Latin America), karish (Egypt), and cokelek (Turkey). These cheeses must be consumed fresh, within 15 or 30 days (Guneser and Yuceer, 2011).

According to Brazilian law, fresh ricotta is the product obtained from cheese whey albumin with 20% of milk added. It must have cylindrical shape, weigh up to 300 to 1000 g, and unformed or unclear rough crust; it must be soft consistently, not pasty and friable; its texture should be closed with some mechanical holes, and should have white or creamy white color, with typical odor and flavor (Brazil, 2010). However, there is no technical regulation in Brazil to identify the quality of ricotta. This impairs standardization of technological procedures among the different dairy industries and control of physical and chemical, rheological, sensory and microbiological parameters during storage (Carrizo et al., 2011). As cooling helps to maintain the physical and chemical characteristics of whey and also improves the quality of the final product in the dairy industry, the aim of this study is to evaluate the behavior of the physical, chemical, and texture profile parameters of fresh ricotta made with whey

stored under refrigeration.

MATERIALS AND METHODS

The whey used in this study was obtained from a dairy industry located in Rio Verde - Goiás, that is, the whey is derived from the manufacture of mozzarella cheese. The whey was stored under refrigeration in a cold chamber at temperature of $5 \pm 1^\circ\text{C}$ during 0 (control), 24, 48, and 72 h (treatments 1, 2, 3 and 4, respectively). At the end of the storage period, the whey was isothermally transported to the Instituto Federal Goiano - Campus Rio Verde, Goiás, Brazil, for aseptic processing of ricotta cheese, according to Prudencio et al. (2014).

Whey acidity was previously standardized at 8°D for all treatments by addition of sodium bicarbonate (Behmer, 1999). Whey was heated slowly at 90°C under constant stirring. Lactic acid (0.1% v/v diluted in water 1:10) was added for its coagulate. After 10 min of rest, the supernatant mass was collected and placed in cylindrical molds with holes. It was turned for 1 h and refrigerated at 10°C for 24 h to obtain syneresis. Cheeses were sectioned into four parts, vacuum sealed and kept under refrigeration until they were analyzed. The tests were replicated during storage on 1, 8, 15, and 22 days. Yield was evaluated by the ratio between final mass of ricotta and initial mass of whey (kg), expressed as percentage, according to Zeng et al. (2007).

Sweet whey and acid whey were evaluated for acidity by acid-alkaline titration method No. 920.124 (AOAC, 2005; Brazil, 2006); pH by pHmeter PG 2000/Gehaka® (Brazil, 2006); fat by the Gerber method No. 2000.18 (AOAC, 2005; Pearson, 1976); cryoscopy by electronic microprocessor cryoscope model M90 BR/Laktron® (Brazil, 2006) and density at 15°C with Quevenne lacto-density meter (1.015 to 1.040 g.cm⁻³) (Brazil, 2006).

Ricotta cheeses were analyzed during storage for physicochemical parameters of acidity and pH (AOAC, 2005; Brazil, 2006), and instrumental color and texture. On the eighth day of storage, dry extract composition was evaluated by gravimetric method No. 990.19 (AOAC, 2005; Brazil, 2006), ash by incineration in muffle method No. 930.30 (AOAC, 2005) fat by method No. 2000.18 (AOAC, 2005; Pearson, 1976) and crude protein by micro-Kjeldahl method No. 939.02 (AOAC 2005; Brazil, 2006).

For color and texture analysis of ricotta cheese, cubic samples of 2 cm edge were used. Color was determined with a Colorflex EZ/HunterLab® colorimeter adjusted to daylight illumination D65 and 10° angle through the CIELab system (CIE, 1996). According to the HSV color model, colorimetric space is defined by rectangular coordinates: L*, a* and b* corresponding to brightness from black (0) to white (100), chromaticity green (-)/red (+) and blue (-)/yellow (+), respectively; and cylindrical coordinates: chroma color saturation (C*) and Hue angle (°h) calculated by formulas $(a^{*2}+b^{*2})^{1/2}$ and $\arctang(b^*/a^*)$ (ABNT, 1992).

The texture profile analysis (TPA) was evaluated by texturometer with load cell of 25 kg (CT3/Brookfield®) consisting of double compression test, test speed of 1.0 mm/s, compression distance of 10.0 mm, equivalent to 50% compression, contact force of 3.0 g and acrylic cylindrical probe (TA4/1000), operated by the CT Texture Pro V1.1 Build 7 software (Brookfield Eng. Labs, Inc.). Primary parameter data of hardness, cohesiveness, elasticity index, and secondary of gumminess and chewiness were collected (Buriti et al., 2005).

Microstructure was assessed at the eighth day of storage by scanning electron microscopy (SEM; JSM-6610/Jeol®), with prior lyophilization (Enterprise II/Terroni®), fat extraction in Soxhlet, No. 1122 (IUPAC, 1979) and plating in gold.

All tests were conducted in triplicate and data were analyzed by statistical software and analysis of variance. They were allocated in a completely randomized design by Tukey test at 5% probability as

Table 1. Mean titratable acidity (%), pH, fat (%), cryoscopy ($^{\circ}$ H) and density values (g /mL) of sweet whey and acid whey obtained from the processing of ricotta produced with refrigerated whey stored for zero, 24, 48 and 72 hours.

Parameter	Type of whey	Whey storage (h)			
		0	24	48	72
Acidity	SW	0.09±0.00 ^a	0.10±0.02 ^a	0.09±0.00 ^a	0.09±0.00 ^a
	AW	0.13±0.02 ^b	0.14±0.01 ^{ab}	0.14±0.00 ^a	0.15±0.01 ^a
pH	SW	6.18±0.06 ^c	6.59±0.04 ^b	6.60±0.04 ^b	6.68±0.07 ^a
	AW	5.13±0.10 ^b	5.28±0.18 ^{ab}	5.35±0.06 ^a	5.42±0.21 ^a
Fat	SW	0.57±0.17 ^a	0.58±0.07 ^a	0.63±0.05 ^a	0.61±0.05 ^a
	AW	0.12±0.24 ^a	0.02±0.04 ^a	0.00±0.00 ^a	0.00±0.00 ^a
Cryoscopy	SW	-0.520±0.003 ^{ab}	-0.514±0.005 ^b	-0.518±0.002 ^{ab}	-0.527±0.014 ^a
	AW	-0.582±0.022 ^a	-0.567±0.021 ^a	-0.578±0.018 ^a	-0.582±0.027 ^a
Density	SW	1.02±0.00 ^a	1.02±0.00 ^a	1.02±0.00 ^a	1.02±0.00 ^a
	AW	1.02±0.00 ^a	1.02±0.00 ^a	1.02±0.00 ^a	1.02±0.00 ^a

Means followed by same letter in line do not differ significantly by the Tukey test at 5% probability. SW, Sweet whey; AW, acid whey.

Table 2. Average fat, protein and ash values of ricotta produced with whey stored for zero, 24, 48 and 72 h.

Storage (h)	Parameters (%)			
	Yield	Fat	Protein	Ash
0	5.39 ^a	10.95±1.05 ^a	12.47±0.99 ^a	1.54±0.24 ^a
24	5.21 ^a	10.34±1.80 ^a	11.84±0.90 ^a	1.47±0.11 ^a
48	5.23 ^a	10.08±0.83 ^a	12.42±1.28 ^a	1.49±0.18 ^a
72	5.51 ^a	8.44±1.09 ^b	12.28±1.42 ^a	1.36±0.18 ^a

Means followed by same letter in line do not differ significantly by the Tukey test at 5% probability.

0, 24, 48 and 72 treatments of refrigerated whey. Titratable acidity, pH, fat, cryoscopy and density of sweet whey, acid whey and yield on the day of ricotta cheese processing were evaluated. Ash, fat, protein and SEM of ricotta cheese were evaluated on the eighth day. The other parameters, acidity, pH, moisture, total solids, color and texture were evaluated during storage days of 1, 8, 15, and 22.

RESULTS AND DISCUSSION

Titratable acidity, fat, cryoscopic point and density of sweet whey (0.09%, 0.60%, -0.520 $^{\circ}$ H and 1.02 g/ml, respectively) did not differ with increasing refrigeration period, and unlike pH, increased from 6.18 to 6.68 (Table 1). The physicochemical parameters of sweet whey are consistent with those established by Brazilian law: acidity from 0.08 to 0.14% and pH from 6.0 to 6.8 (Brazil, 2013). In goat milk whey, pH 6.38 was found (Pizzillo et al., 2005). Sweet whey fat contents are related to the cheese manufacturing technology, which was obtained in accordance with the mixing force and milk fat content. Prato cheese whey has levels close to 0.4% (Pinto et al., 2011). For goat cheese whey, the content is higher (1.69%) (Pizzillo et al., 2005) while for fresh cheese

whey, the content is lower (0.20%) (Ordóñez et al., 2005), with variations of up to 0.36% (Pescuma et al., 2010).

For the cryoscopic index, which evaluates the freezing point, values close to zero indicate addition of water; value of -0.520 $^{\circ}$ H for sweet whey confirms suitability for processing, given the proximity to cryoscopy allowed for milk, from -0.530 to -0.550 $^{\circ}$ H (Brazil, 2011). In the acid whey, fat content (0.04%), cryoscopy (-0.577 $^{\circ}$ H) and density (1.02 g/ml) remained stable (Table 1). Acidity increased from 0.13 to 0.15%. There was an increase in pH, from 5.13 to 5.42; which remained lower than the limit of 6.0 (Brazil, 2013). There is an increase in acidity and a reduction in fat content from sweet whey to whey acid. This behavior is due to double clotting process used in ricotta production, in which the addition of organic acid increases acidity and part of the fat is inserted into the protein matrix and remains in the clot (Ordóñez et al., 2005).

The yield of ricotta cheese was not affected by whey storage period, with average value of 5.33% (Table 2). In literature, variable yields have been reported: 3.17% for ricotta with bovine whey (Hawkins et al., 2009), 7.9% for ricotta with goat and bovine milk whey (Borba et al.,

Table 3. Mean acidity (%), pH, moisture (%) and total solids (%) values of ricotta made with whey stored for zero, 24, 48, and 72 h at 1, 8, 15, and 22 days of storage.

Parameter	Storage (h)	Storage (days)			
		1	8	15	22
Acidity	0	0.19±0.05 ^{cB}	0.41±0.08 ^{bB}	0.48±0.13 ^{abC}	0.53±0.06 ^{aC}
	24	0.43±0.04 ^{bA}	0.47±0.08 ^{bAB}	0.48±0.08 ^{bC}	0.64±0.03 ^{aB}
	48	0.45±0.05 ^{cA}	0.45±0.06 ^{cB}	0.61±0.08 ^{bB}	0.70±0.08 ^{aB}
	72	0.46±0.06 ^{cA}	0.53±0.10 ^{cA}	0.77±0.13 ^{bA}	0.90±0.10 ^{aA}
pH	0	5.98±0.28 ^{aA}	5.88±0.26 ^{abA}	5.78±0.03 ^{bcA}	5.69±0.04 ^{cA}
	24	5.92±0.11 ^{aAB}	5.84±0.07 ^{abA}	5.74±0.04 ^{bcAB}	5.64±0.06 ^{cAB}
	48	5.85±0.12 ^{aAB}	5.75±0.08 ^{abA}	5.68±0.04 ^{bAB}	5.60±0.17 ^{bAB}
	72	5.81±0.13 ^{ab}	5.73±0.04 ^{abA}	5.60±0.02 ^{bcB}	5.52±0.08 ^{cB}
Moisture	0	74.60±0.76 ^{abB}	74.99±0.96 ^{abA}	75.39±0.73 ^{aA}	74.01±0.36 ^{bB}
	24	73.88±1.57 ^{bB}	74.67±0.75 ^{abA}	74.94±0.77 ^{aAB}	74.55±1.03 ^{abB}
	48	74.54±0.79 ^{abB}	72.62±0.81 ^{cB}	75.17±0.71 ^{aA}	74.16±1.41 ^{bB}
	72	75.63±0.85 ^{aA}	73.63±1.70 ^{bB}	74.02±1.43 ^{bB}	75.75±1.21 ^{aA}
Total Solids	0	25.40±0.75 ^{abA}	25.01±0.96 ^{abB}	24.61±0.73 ^{bB}	25.99±0.36 ^{aA}
	24	26.12±1.57 ^{aA}	25.33±0.75 ^{abB}	25.06±0.77 ^{bAB}	25.45±1.03 ^{abA}
	48	25.46±0.79 ^{bcA}	27.37±0.82 ^{aA}	24.83±0.71 ^{cB}	25.84±1.41 ^{bA}
	72	24.37±0.85 ^{bB}	26.37±1.70 ^{aA}	25.98±1.43 ^{aA}	24.25±1.21 ^{bB}

Means followed by same lowercase letter in line and uppercase letter in column do not differ significantly from each other according to the Tukey test at 5% probability.

2014), and 16.88% with the addition of 5% milk (Hawkins et al., 2009). The addition of milk whey and calcium salts is favorable for ricotta yield because casein clots strengthen the protein network by improving the rheological properties of the cheese mass (Smithers, 2008). The high whey acidity accelerates clotting and negatively influences the texture, making the cheese mass softer, thus reducing yield. But, slow acidification is related to a slight increase in gel hardness (Lucey, 2004). Thus, the lack of significant differences in yield is also due to acidity correction of whey prior to processing and to the standardized protocol.

Lipids are involved with color, flavor, other sensory characteristics, yield, firmness and texture of cheese (Vargas et al., 2008). According to Brazilian ordinance No. 146/1996, cheeses are classified as extra fat or double cream with over 60% dry basis (d.b.); fat between 45 and 59.9% d.b.; semi-fat between 25 and 44.9% d.b.; low-fat between 10 and 24.9% d.b. and skim with less than 10% d.b. (Brazil, 1996).

The fat content of ricotta ranged from 8.44 to 10.95% wet basis (w.b.), corresponding to 32.15 to 43.90% d.b.. This is classified as semi-fat cheese (Table 2), which is due to the use of whole milk whey with high fat content in the processing (0.60%). The fat content of ricotta (d.b.) increased to 47.29% when 5% milk was added (Hawkins et al., 2009) and decreased to 20.31% when goat milk whey is used in the production of ricotta (Pizzillo et al.,

2005). The fat content of ricotta cheese decreased when whey stored for 72 h was used. This does not affect the whey composition, being statistically equal. It may possibly be due to the weakening of the protein network with longer whey storage periods. Thus, fat globules inserted into the protein matrix were released into the exudation water; another possible factor is lipolysis caused by bacterial activity. The protein content of ricotta cheese remained unchanged with the use of whey stored for a longer period, with average value of 12.25%, in accordance with the whey composition (Table 2). Other authors have found, for ricotta with goat milk whey, values of 6.55% (Pizzillo et al., 2005), 10.3% with bovine and goat milk whey (Borba et al., 2014), and 14.92% with bovine milk whey (Prudencio et al., 2013).

The ash content did not change with treatments, with an average of 1.47% (Table 2). Ash content of 0.93% was reported for ricotta with goat milk whey (Pizzillo et al., 2005), 2.22% for ricotta with bovine and goat milk whey (Borba et al., 2014) and 2.46% when ricotta was made with whey protein concentrate (El Sheikh et al., 2011). For processed ricotta, titratable acidity in lactic acid increased both during storage, especially after the second week, when whey was stored for extended periods (Table 3). Increased acidity is due to the synthesis of metabolite by natural microflora bacteria such as *Lactobacillus* species, even in refrigerated environment, a phenomenon known as post-acidification

(Dermiki et al., 2008). Different results are found in literature. Di Pierro et al. (2011) obtained similar acidity results, from 0.26 to 0.34%, after 30 days of storage. Borba et al. (2014) found no difference up to 14 days; it remained at 0.3%. This was attributed to the absence of starter culture in the manufacture of cheese that resulted in whey.

Thus, the natural microflora of milk and whey and cheese manufacturing technology with the addition of starter cultures strongly influence the final acidity of ricotta. In this study, whey was obtained from Prato cheese manufactured with the addition of mesophilic culture of *Lactococcus lactis*, *Lactococcus* subsp. *Cremonis* and thermophilic culture of *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Lactobacillus delbrueckii* subsp. *Helveticus*.

The pH of ricotta cheeses remained relatively stable, suffering slight decrease during whey storage (Table 3). On the first day, the pH was close to the isoelectric point of whey proteins, in accordance with El-Sheikh et al. (2011), who reported pH of 5.91 and Hauschild et al. (2014), who reported pH of 5.87. Such behavior was consistent with the study of Di Pierro et al. (2011) on ricotta. It attributed this slight pH decrease to the formation of acidic amino acids and free fatty acids, resulting from proteolysis and lipolysis, respectively, and in general, to the occurrence of some buffering effect.

The total solids content of ricotta ranged from 24.25 to 27.37%, corresponding to moisture variation from 72.63 to 75.75% (Table 3). Accordingly, with moisture content above 55%, ricotta is classified as very high moisture cheese according to Brazilian ordinance 146/1996/MAPA (Brazil, 1996). A random behavior of increases and decreases in the moisture content of ricotta cheese during storage were observed. These variations can be attributed to heterogeneous syneresis in the vacuum packaging without moisture absorption (Hauschild et al., 2014). The recoverable total solids content in cheeses is quite variable according to the origin of whey, milk proportion and characteristics of manufacturing technologies that influence yield (Hawkins et al., 2009). Borba et al. (2014) found content of 24.05% for ricotta manufactured only with bovine whey and when milk is added, this content increased to 25.91%. Higher levels are found in ricotta prepared with goat milk whey (30.68%) (Pizzillo et al., 2005), or whey previously treated with calcium concentration or precipitation, which removes hydrophilic lipoproteins that reduce yield (Prudencio et al., 2014).

Variation in the physicochemical composition of ricotta during storage is linked to oxidation and degradation reactions and microbial activity. In summary, these changes become more pronounced after the first week of manufacture, with loss of nutritional and sensory quality. It shows that ricotta is prepared without salt, and is a fresh product for immediate consumption and storage of approximately seven days under refrigeration (Carminati

et al., 2002). Color is extremely important in food products due to its direct influence on appearance and is one of the parameters related to consumers' acceptance (Ramos et al., 2013). There was a decrease of brightness in ricotta prepared with whey stored for 24 h and after the second week of storage (Table 4). Low L^* values are assigned to darkening as a result of oxidation, enzymatic and microbiological degradation, and are undesirable for causing consumers' rejection (Dattatreya and Rankin, 2006). L^* values of 93.36 are found for ricotta containing goat milk (Borba et al., 2014). This is due to the presence of smaller fat globules and conversion of β -carotene to vitamin A (Park et al., 2007).

In this study, a^* coordinate showed decreased values compared to cheeses manufactured with whey on day zero and whey refrigerated for 72 h, and increased values during storage (Table 4). These values tend to be negative in relation to green color due to the presence of riboflavin, vitamin B_2 (Mestdagh et al., 2011), as observed by Borba et al. (2014) (-2.70 values). However, positive values oriented to red in the present study are the result of the addition of natural dye urucum to prato cheese that remained in sweet whey. The b^* coordinate values increased when cheese was manufactured with whey from 0 to 24 h, followed by a slight decline, but did not suffer variations throughout the storage period in these treatments (Table 4).

Possibly, the predominance of the red color over the green color of riboflavin was more pronounced on the first day of whey storage, resulting in the formation of secondary yellow color, and/or transfer of carotenoids from whey to cheese (Sheehan et al., 2009). For the other storage times, compounds of brown color are likely to have prevailed, which are derived from the Maillard reaction, non-enzymatic browning by carbohydrate and protein reactions resulting from the high temperatures used in ricotta processing (Dattatreya and Rankin, 2006). Prudencio et al. (2014) found lower tendency to yellow color on ricotta with whey from fresh cheese, with average b^* value of 15.42. Chroma is the degree of color saturation and low values indicate low intensity and are associated with lower purity and formation of mixed colors (Kubo et al., 2013). c^* followed the same behavior of b^* coordinate, which shows that saturation was more influenced by the tendency to yellow in the color of ricotta.

The color of ricotta measured by the Hue angle decreased during storage. In relation to the whey storage time, shade increased for whey of 24 h, decreased for whey of 48 h, and increased again for whey at 72 h. Kubo et al. (2013) demonstrated the effect of the addition of dye on total color variation, which occurs due to the solubility of the dye in the oil phase of protein-fat cheese matrix. Hue value close to 71° corresponds to the first quadrant of the HSV three-dimensional diagram, between 0° (red) and 90° (yellow). The use of prato cheese whey strongly influenced the color coordinates, especially the

Table 4. Mean values of the color coordinates of ricotta prepared with whey stored for 0, 24, 48 and 72 h at 1, 8, 15 and 22 days of storage.

Coordinate	Storage (h)	Storage (days)			
		1	8	15	22
L*	0	81.56±11.24 ^{bd}	88.03±0.78 ^{ab}	87.07±0.61 ^{ac}	87.51±0.66 ^{aA}
	24	87.87±1.04 ^{bcA}	90.82±1.10 ^{aA}	88.51±1.88 ^{bb}	87.06±0.78 ^{cA}
	48	86.00±0.81 ^{cb}	87.27±0.55 ^{bb}	91.11±1.22 ^{aA}	86.61±0.52 ^{bcA}
	72	83.25±2.32 ^{cC}	88.47±1.04 ^{ab}	85.62±1.06 ^{bd}	83.85±1.20 ^{cb}
a*	0	5.62±0.57 ^{dC}	6.14±0.35 ^{cb}	6.46±0.26 ^{bb}	6.58±0.28 ^{aA}
	24	6.16±0.38 ^{ba}	6.07±0.28 ^{cbC}	6.41±0.32 ^{ab}	6.18±0.56 ^{bc}
	48	6.11±0.32 ^{dAB}	6.29±0.38 ^{cA}	7.48±0.32 ^{aA}	6.45±0.33 ^{bb}
	72	6.04±0.29 ^{ab}	6.04±0.23 ^{ac}	5.98±0.26 ^{ac}	5.07±0.34 ^{bd}
b*	0	16.71±2.41 ^{ac}	17.29±0.48 ^{ab}	17.22±0.41 ^{ac}	17.22±0.42 ^{ab}
	24	19.02±0.46 ^{aA}	18.96±0.47 ^{aA}	19.22±0.54 ^{ab}	18.74±0.47 ^{aA}
	48	17.94±0.46 ^{cb}	18.28±0.40 ^{cbA}	19.60±0.73 ^{ba}	21.19±8.25 ^{aA}
	72	18.79±0.61 ^{aAB}	18.74±0.30 ^{aA}	18.83±0.36 ^{ab}	16.52±0.66 ^{bb}
Chroma	0	17.64±2.43 ^{ac}	18.35±0.48 ^{ab}	18.39±0.42 ^{ac}	18.44±0.47 ^{ab}
	24	19.99±0.50 ^{aA}	19.91±0.51 ^{aA}	20.26±0.57 ^{ab}	19.75±0.45 ^{aA}
	48	18.95±0.50 ^{cb}	19.34±0.46 ^{cAB}	20.67±0.77 ^{ba}	22.47±8.14 ^{aA}
	72	19.74±0.64 ^{aAB}	19.70±0.32 ^{aA}	19.76±0.38 ^{ab}	17.28±0.71 ^{bc}
Hue (°)	0	71.26±1.88 ^{ab}	70.45±1.09 ^{bc}	69.44±0.79 ^{cd}	69.09±0.55 ^{cd}
	24	72.06±0.95 ^{abA}	72.25±0.58 ^{aA}	71.56±0.75 ^{cb}	71.75±1.67 ^{bcB}
	48	71.18±0.74 ^{ab}	71.01±0.90 ^{abB}	70.56±0.53 ^{bc}	71.05±2.10 ^{ac}
	72	72.17±0.60 ^{ba}	72.14±0.60 ^{ba}	72.38±0.64 ^{ba}	72.95±0.74 ^{aA}

Means followed by same lowercase letter in line and uppercase letter in column do not differ significantly from each other according to the Tukey test at 5% probability.

tendency to be red (+a*) and yellow (+b*). The resulting yellowish coloration in the ricotta cheese can be detrimental to consumers' visual acceptance. Thus, this source of whey should be used with caution, with a previous study on sensory acceptance. According to the instrumental texture profile analysis (TPA) of ricotta during storage of 22 days (Table 5), hardness attribute was relatively the same. There were with average values of 1.02, 1.20, 1.68 and 1.01 N, respectively, for control ricotta cheese and for those manufactured with whey of 24, 48 and 72 h. These results were similar to those obtained by Borba et al. (2014), who reported average hardness of 1.94 N on the first 14 days of storage.

Hardness increased when using whey refrigerated for 48 h, and then, hardness values decreased (Table 5). Lipolysis and proteolysis reactions may have affected the stability of the protein matrix and emulsifying agents such as lipoproteins, thus contributing to the increase in hardness (Pereira et al., 2002). Denaturation or a new association among protein may occur during processing, since some whey proteins are sensitive to fluid shear (Almécija et al., 2007), which can interfere with the

homogeneity of the protein network and result in reduction in hardness (Buffa et al., 2001). This attribute suffered great variation due to lack of salts in the processing of ricotta cheeses, which act as strengtheners of the protein network (Tunick et al., 2012). The cheese texture closely depends on the microstructure and chemical composition, mainly concerning fat and salt (Wendin et al., 2000) and total solids contents, pH and maturation time (Bowland and Foegeding, 2001).

Hardness is the amount of force required for compression (N); cohesiveness is the ratio between force and time for the areas of two compressions (dimensionless); elasticity is a recovery measure after the first compression (dimensionless); gumminess is the product of cohesiveness by hardness (N) and chewiness is the product of gumminess by elasticity (N) (Tunick et al., 2012). The average cohesiveness and elasticity values of ricotta did not differ significantly ($p>0.05$) throughout the whey storage. Borba et al. (2014) also observed cohesiveness and elasticity values of 0.47 to 0.74, respectively, during storage. These results show that there were no changes in the deformability of ricotta

Table 5. Average texture attribute values of ricotta prepared with whey stored for 0, 24, 48 and 72 h at 1, 8, 15 and 22 days of storage.

Attribute	Storage (h)	Storage (days)			
		1	8	15	22
Hardness (N)	0	0.86±0.16 ^{aC}	1.02±0.26 ^{aB}	1.06±0.51 ^{aB}	1.15±0.20 ^{aB}
	24	1.26±0.29 ^{aAB}	1.16±0.20 ^{aB}	1.11±0.30 ^{aB}	1.28±0.19 ^{aB}
	48	1.49±0.38 ^{bA}	1.62±0.48 ^{abA}	1.73±0.34 ^{abA}	1.88±0.40 ^{aA}
	72	0.99±0.30 ^{aBC}	1.05±0.15 ^{aB}	1.02±0.28 ^{aB}	0.99±0.25 ^{aB}
Cohesiveness	0	0.33±0.04 ^{abA}	0.32±0.04 ^{bA}	0.43±0.20 ^{aA}	0.33±0.04 ^{abA}
	24	0.36±0.05 ^{aA}	0.35±0.04 ^{aA}	0.41±0.14 ^{aA}	0.36±0.06 ^{aA}
	48	0.36±0.06 ^{aA}	0.35±0.05 ^{aA}	0.38±0.05 ^{aA}	0.39±0.02 ^{aA}
	72	0.34±0.07 ^{aA}	0.37±0.06 ^{aA}	0.45±0.15 ^{aA}	0.34±0.11 ^{aA}
Elasticity	0	0.96±0.47 ^{aA}	0.85±0.30 ^{abA}	0.76±0.18 ^{bA}	0.82±0.02 ^{bA}
	24	0.85±0.24 ^{aA}	0.79±0.02 ^{aA}	0.84±0.28 ^{aA}	0.89±0.23 ^{aA}
	48	0.82±0.02 ^{aA}	0.93±0.29 ^{aA}	0.92±0.30 ^{aA}	0.83±0.02 ^{aA}
	72	0.77±0.03 ^{aA}	0.87±0.16 ^{aA}	0.80±0.02 ^{aA}	0.90±0.26 ^{aA}
Gumminess (N)	0	0.29±0.08 ^{aC}	0.34±0.11 ^{aB}	0.38±0.19 ^{aB}	0.38±0.08 ^{aB}
	24	0.45±0.11 ^{aAB}	0.40±0.05 ^{aB}	0.43±0.08 ^{aB}	0.47±0.12 ^{aB}
	48	0.55±0.20 ^{bA}	0.59±0.24 ^{abA}	0.65±0.17 ^{abA}	0.73±0.15 ^{aA}
	72	0.32±0.08 ^{aBC}	0.39±0.08 ^{aB}	0.43±0.10 ^{aB}	0.33±0.12 ^{aB}
Chewiness (N)	0	0.34±0.19 ^{aA}	0.32±1±0.04 ^{aA}	0.29±0.13 ^{aA}	0.31±0.06 ^{aA}
	24	0.38±0.13 ^{aA}	0.31±0.05 ^{aA}	0.35±0.13 ^{aA}	0.42±0.20 ^{aA}
	48	0.40±0.16 ^{aA}	0.45±0.18 ^{aA}	0.43±0.21 ^{aA}	0.37±0.13 ^{aA}
	72	0.25±0.07 ^{aA}	0.33±0.06 ^{aA}	0.34±0.08 ^{aA}	0.31±0.18 ^{aA}

Means followed by same lowercase letter in line and uppercase letter in column do not differ significantly from each other according to the Tukey test at 5% probability.

due to changes in the chemical structure of components (Ferrandini et al., 2011).

In relation to gumminess, no significant difference ($p>0.05$) during storage was observed; it was only among treatments. This secondary attribute increased when whey refrigerated for up to 48 h was used, then, the gumminess values decreased, showing similar behavior with primary attribute of hardness. Literature shows values greater than 0.90 N when goat and cow milk whey is used (Borba et al., 2014). Although chewiness attribute is secondary, derivative of hardness, it remained stable. Ciabotti et al. (2009) reported higher chewiness value in ricotta made with mozzarella cheese whey (2.15 N). According to the texture profile analysis, ricotta cheese is defined as a viscoelastic food (Fox et al., 2000) with very soft consistency, not pasty and friable (Brazil, 1996, 2010), compressible and not too cohesive, with brittle characteristics (Tunick et al., 2012), stable texture profile during storage, required for marketing and sensory acceptability.

In scanning electron micrographs, rounded dark areas correspond to the position of fat globules, and the bright

area to the protein matrix (Tunick et al., 2012). Images increase by 100x suggests a slight compression and disorder of the protein matrix when ricotta was made with whey stored for a longer period, with losses in granular and rough appearance (Figure 1). Structural changes are observed when there are changes in connections to the whey protein network according to the different manufacturing technologies (Yorgun et al., 2008). During compaction of the microstructure of ricotta with whey stored for 48 h (Figure 1C), the molecular rearrangement of proteins was likely to occur, with strengthened links, as evidenced by increased hardness and gumminess (Table 5). With whey stored for 72 h (Figure 1D), the protein matrix of ricotta loses the spongy characteristic and becomes more compressed. The decrease in interstitial spaces may be the cause and/or effect of the reduced fat content of this treatment (Table 2). There is decrease in hardness and gumminess, suggesting the occurrence of protein bonds is more weakened by proteases, and/or irreversible denaturation.

The structure and texture of acidic coagulation cheeses is closely related to heat and acid levels used during

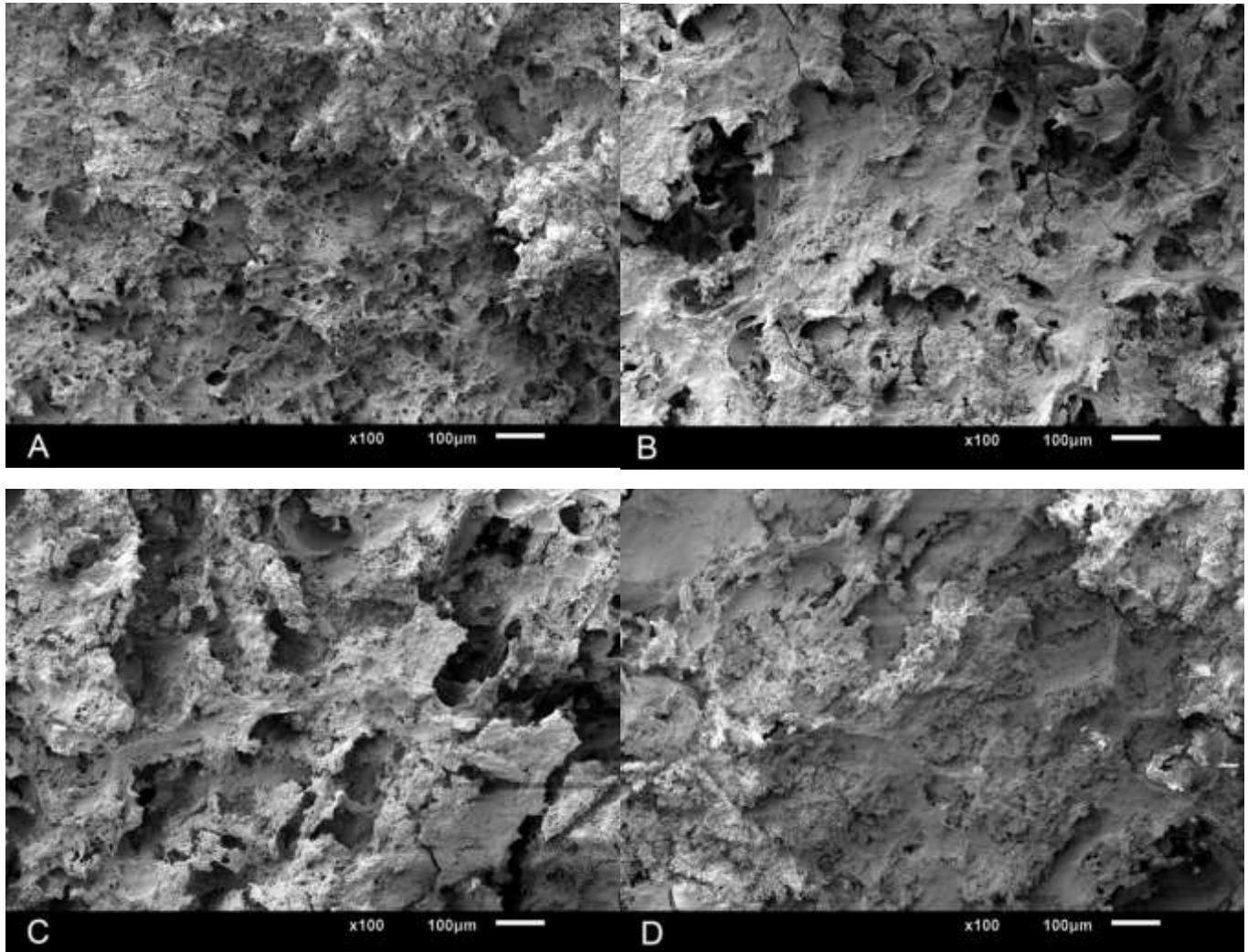


Figure 1. Scanning electronic micrographs at 100x of ricotta made with whey stored for: (A) 0 h - control; (B) 24 h; (C) 48 h and (D) 72 h.

processing as a result of the structural formation of gel and three-dimensional changes of whey protein (Guinee et al., 1993). Lower pH levels, near the isoelectric point of whey proteins, are possibly responsible for the formation of the molten matrix (Tunick et al., 2012). Possibly, immunoglobulins were responsible for this structural change because the isoelectric point (IP) ranges from 5.5 to 8.3 and because they are very sensitive to heat. They interact with β -lactoglobulin (IP 5.2) and bovine whey albumin (IP 4.7 to 4.9) via disulfide bonds (Morr and Há, 1993). Temperature above 70°C causes irreversible denaturation and polymerization of β -lactoglobulin and greater susceptibility to the action of proteases. α -lactalbumin (IP from 4.2 to 5.1) has high denaturation reversibility, around 40% after heating at 95°C due to connections with Ca^{2+} and Zn^{2+} ion (Morr and Há, 1993). Similar microstructure was observed in ricotta made with whey from fresh cheese under similar heat and

acidification conditions applied to the process (Prudencio et al., 2014). In different whey concentration technologies, the authors reported compaction on the protein network.

Conclusion

The yield, protein and ash contents of ricotta cheeses were constant; however, at longer whey storage period, the fat content decreased, while acidity increased inversely proportional to pH. Coloration tends to yellow due to the manufacture of ricotta with whey from prato cheese. In texture profile analysis of ricotta cheeses during storage, there was a balance among rheological forces that make up the structure. Hardness and gumminess of the ricotta decreased with the use of refrigerated whey after 48 h. There was a subtle microstructural difference with the protein network

compaction of ricotta made with whey stored for longer periods. The manufacture of ricotta cheese with whey stored for longer periods within three days under controlled refrigerated environment is a viable alternative for dairy industries, enabling better logistic use of this byproduct.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Influence of vermicompost humic acid on chlorophyll content and acclimatization in banana clone, Enano Guantanamero

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Vermicompost humic acids (VHA) promote plants' growth because they have similar effects with auxins. The aim of this research was to evaluate the effect of VHA in some physiological indicators in the micropropagation and acclimatization phase of banana clone Enano Guantanamero. Six concentrations were used (0, 10, 20, 30, 40 and 50 mg L⁻¹) to evaluate *in vitro* the number of leaves, total chlorophyll content and chlorophyll a-b; also in the acclimatization phase, the plant height, stem diameter and number of leaves at three different times (transplant, 25 days and at the end of this phase-50 days) were determined. Root length and roots dry weight were evaluated at the end of the acclimatization. VHA applied improved total chlorophyll, chlorophyll a-b at concentrations of 20 and 50 mg L⁻¹, but not the number of leaves. In the acclimatization stage, plants height at the end of this period (50 days) were higher with 10 mg L⁻¹ (T1) VHA, the number of leaves increased at 40 mg L⁻¹ (T4) and the stem diameter was higher at both concentrations. In the evaluation of roots length, there were no significant differences, but the number of leaves was higher at 10 and 40 mg L⁻¹ (T1 and T4) and it decreased at 50 mg L⁻¹ (T5); roots dry weight increased at 40 mg L⁻¹ (T4). VHA promoted chlorophyll content under *in vitro* conditions, it reduced the period of acclimatization of banana clone Enano Guantanamero, led to a better growth of plants, and it saved time and resources.

Key words: Acclimatization, humic acid, *in vitro* crop, *Musa* sp.

INTRODUCTION

The banana (*Musa* sp.) constitutes the fourth most important staple food commodity of the world, after rice,

wheat and maize (Hossain, 2014), so it is considered a basic product, exportation, source of employment and income in many developing countries (FAO, 2015). In Cuba from 2013 to 2014, according to FAO (2015), 508,164.00 tons of bananas were produced with a yield of 92,090.00 kg ha⁻¹; this crop together with potatoes and rice are priority food for Cuban population (Juárez, 2013). *In vitro* multiplication of banana crop is an excellent alternative because it allows a fast and massive clonal multiplication, for this reason a number of countries in the world like Israel, France, Australia, many African countries and Cuba are using this technique (Muhammad et al., 2004). In this technique growth regulators are the most expensive medium ingredients although they are required in very small concentrations (Prakash et al., 2002), for this reason, in recent years, there has been a trend to substitute growth regulators traditionally used in plant biotechnology to produce plants via organogenesis, for biological substances, chemical natural derivatives innocuous to the environment, or mixtures of them. This substitution, as well as being an alternative to the use of growth regulators, contributes to the reduction of production costs (Héctor et al., 2007).

In Cuba, several bioactive products are used for this partial or total substitution of growth regulators, such as Pectimorf, Biobras-16, FitoMAS-E, with the aim to improve growth indicators of *in vitro* cultured plants, and also in field conditions (Terry-Alfonso et al., 2014). During the last decade, the use of humic substances (HS) as growth indicator has increased as they have favorable effects on plant growth, especially in the proliferation of roots (Canellas et al., 2002; Trevisan et al., 2010).

The HS, considered as natural polymers, represent up to 90% of organic matter present in soils. They are used as bio-stimulators for plants growth and development because they have effect on the chemical, physicochemical and biological properties of soils (Muscolo et al., 2007). Due to their high availability, agriculture-derived vermicomposts (agro-materials) are excellent raw materials for obtaining HS (Calderín et al., 2012).

The VHA are used in the present research, due to their stimulatory effects known as "like-auxin" analogous to plant hormones presents in plants (Muscolo et al., 2007), so the aim of this work was to evaluate the effect of different VHA concentrations on physiological indicators during micropropagation and acclimatization phase of the banana clone Enano Guantanamero.

MATERIALS AND METHODS

This research was done from June 2012 to September 2013 at the

Havana Bio-factory and the Chemistry Laboratory of the Agrarian University of Havana (UNAH), Cuba.

Plant materials for the determination of physiological indicators (number of leaves, photosynthetic pigments, plant height, stem diameter) were explants of banana (*Musa sp.*) clone Enano Guantanamero (viand type).

Chemical-physical characterization of vermicompost humic acids

As a starting substrate, cattle manure vermicompost with three months of maturation was used. For vermicompost process, the cattle used was fed mainly with grasses, and the manure was processed with African red worm. This vermicompost was used for the humic substances extraction according to International humic substances society (2008) with NaOH (0.1 mol L⁻¹) in a proportion of 1:10 (mg of vermicompost: mL dissolution) and shaking in a MR-1 Mini rocker shaker (BOECO, Germany), for 8 h. Humic acids (HA) were precipitated with HCl (0.01 mol L⁻¹) and then centrifuged and finally they were purified with HCl: HF: H₂O in a proportion of 1:1:98 (mL).

The VHA were structurally modified with acetic anhydride and thionyl chloride according to the techniques described by Andjelkovic et al. (2006), for obtaining the derivatives: acetylated AH (AH-Ac) and AH methylated (AH-Met). For ammonia derivative, 30 mL of NH₄OH (7.5%) and 500 mg of HA were used in a reflow equipment and magnetic stirrer at 45°C for 1 h. The product obtained (AH-NH₃) was washed previously, centrifuged and dried at 50°C.

The percentage of carbon in the HAs and their derivatives were determined by Tyurin method (Nikitin, 1972). The E4/E6 rate was obtained by the relation between absorbance values at 465 and 665 nm in a RayLight Spectrophotometer UV2100 [ultraviolet-visible spectroscopy (UV-vis)]. The inclination parameters were obtained with the following formulation:

$$\text{Inclination} = \frac{E4}{E6} = -6.435 \log \left(\frac{E4}{E6} \right)$$

Where, E4 is value of curve inclination at 465 nm and E6 is value of curve inclination at 665 nm.

The total acidity, the acidity of -COOH groups and -OH groups produced were also calculated, using the procedure described by Canellas et al. (2008a)

The chemical-physical characterization of the main groups presents in the HAs and their derivatives were realized with diffuse reflectance fourier-transformed infrared (DRIFT) (Baes and Bloom, 1989) and chemically by ultraviolet-visible spectroscopy (UV-VIS) (Canellas et al., 2000). Spectral DRIFT was registered in a PE Spectrum-one, obtaining the individual spectra in the frequency range of 700 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹.

The spectral UV-vis was obtained with a 10 mg of VHA dissolved in 100 mL of distilled water, with a wavelength from 200 to 800 nm.

In vitro parameters

Number of leaves

The number of leaves was determined by counting and it was

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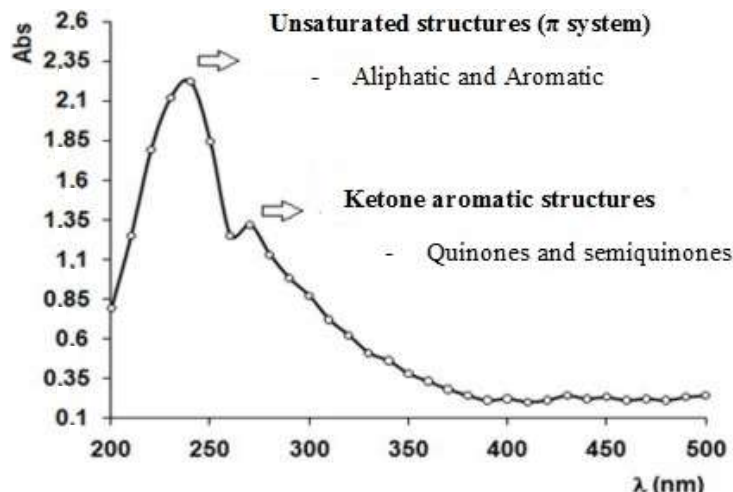


Figure 1. UV-vis spectrum of VHA used in experiments.

expressed as a statistical average (standard error) for each concentration used.

Determination of photosynthetic pigments content

In vitro plants came from four subcultures (multiplication phase) micropropagated in Murashige and Skoog (MS) (1962) medium at Havana Biofabric, under controlled conditions. Different concentrations of VHA were used in the MS medium (0, 10, 20, 30, 40 and 50 mg L⁻¹).

The photosynthetic pigments were determined according to the method of Lichtenthaler (1987), using 1.5 g of macerated green leaves with 25 mL of 45% acetone. The mixture was incubated for 1 h at 40°C in a thermostat bath (DC-3006), and then pure acetone was added to stop the reaction. The mixture was vacuum-filtered and saturated NaCl and NH₄OH (0.02 mol L⁻¹) were added; then it was shaken and separated in a funnel with petroleum ether (50-60°C). In the ether phase (upper phase) were the chlorophylls. The absorbance was determined in a spectrophotometer (RayLihght UV-2100) at wavelengths of λ645 and 663 nm. The chlorophylls content was calculated using the following equations:

1. Total chlorophylls = 8.02 (Abs. λ663 nm) + 20.21 (Abs. λ645 nm)
2. Chlorophyll a = 12.7 (Abs. λ663 nm) - 2.69 (Abs. λ645 nm)
3. Chlorophyll b = 22.9 (Abs. λ645 nm) - 4.68 (Abs. λ663 nm)

Acclimatization

After four subcultures *in vitro* plants were removed from the medium, they were washed carefully with common water and transplanted in trays with 70 holes, filled with a mix of organic matter, earthworm humus and *Trichoderma harzianum* in a proportion of 50:40:10 (g). The plants were grown in a greenhouse for 50 days. Evaluation was done thrice, first one during transplantation just before VHA application, the second one after 25 days (second application of VHA) and the last one at 50th day (culmination of the acclimatization phase). The VHA was sprayed to wet all the aerial parts of the plant. Different concentrations of VHA were used (0, 10, 20, 30, 40 and 50 mg L⁻¹).

The parameters evaluated were: plant height (cm), stem diameter (mm) and number of leaves. At the end of the acclimatization stage, root length (cm) and roots dry weight were also evaluated. Samples

were dried at 105°C for 1 h in a drier (Venticell Medcenter). The biomass was determined in analytical balance (Sartorius, E=± 0.0001 g) and the process was repeated until a constant biomass was obtained. The results were expressed in milligrams (mg).

Statistical analysis

The experimental design was randomized with six levels of VHA (0, 10, 20, 30, 40 and 50 mg L⁻¹) and 20 plants for each concentration. All experiments were performed in triplicate (60 plants in total for each concentration). The statistical analyses consisted of a simple ANOVA (analysis of variance). Tukey's multiple-comparison test was performed when a studied factor showed significance. For these tests, the statistical program STATGRAPH (v. 5.1 plus) was used.

RESULTS

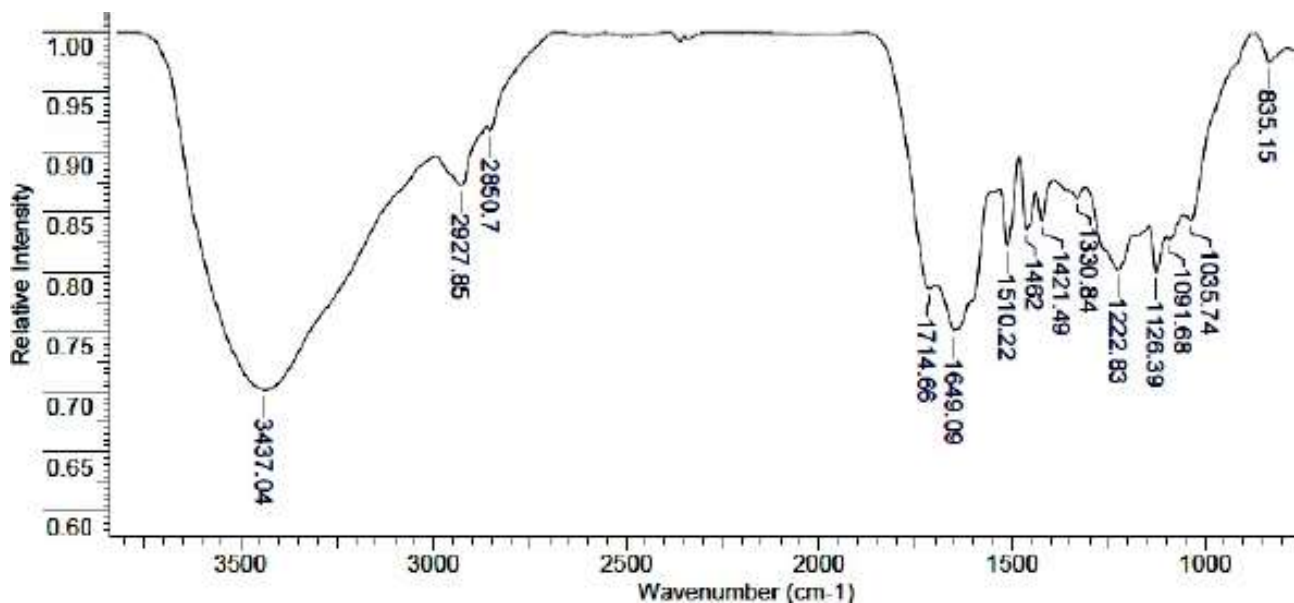
Chemical-physical characterization of vermicompost humic acids

Figure 1 shows the UV-vis spectrum of the VHA obtained. A decrease of absorption with increasing wavelength was observed. An intense peak at shorter wavelengths (200 - 250 nm) was observed in the spectrum. This can be assigned to structures with unsaturated bonds, which are responsible for the high light absorption. Important conjugated instaurations mainly with aromatic nature can be visualized as an intense peak, indicating a high presence of these structures. The UV-vis spectra information allows one to infer in a preliminary way that this VHA has an elevated degree of condensation and aromaticity, as its source of origin is a vermicomposting process.

A shorter peak close to 280 nm was also visualized in the UV-vis spectra because of the presence of quinone structures which are characteristic of these systems (HA). The elemental composition and some chemical

Table 1. Elemental composition, functional groups content, atomic relations and E_4/E_6 of the VHA used in experiments.

C (%)	H (%)	O (%)	N (%)	S (%)	H/C	O/C	C/N	Carboxyl (mol kg ⁻¹)	Phenol (mol kg ⁻¹)	Total acidity (mol kg ⁻¹)	E4/E6
56.7	4.84	34.6	3.07	0.72	0.08	0.61	18.4	9.24	2.03	11.27	4.22

**Figure 2.** DRIFT spectrum of VHA used in experiments. Bands assignments were according to Piccolo (2002).

properties of VHA are shown in Table 1. The E_4/E_6 parameter (relation between extinction coefficients at 465 and 665 nm) was also calculated. For this VHA the value was 4.22 which is similar to the value established for humic substances that came from composted source, which must be below the value of 6.

In Figure 2a wide band of 3437.04 cm^{-1} was observed, associated with inter and intramolecular hydrogen. The extend NH-amide also contributed to this peak.

The bands 2927.85 and 2850.7 cm^{-1} are associated with the wide CH aliphatic symmetric and asymmetric, in which the band 1714.66 cm^{-1} can be associated with the wide C=O from aldehydes and ketones. The band 1649.09 cm^{-1} is also because the wide C=O but from quinones and amides. Characteristics bands of C=C and C=N that are narrow, with aromatics and amides structures, and CN deformation were present at 1510.22 and 1462 cm^{-1} . The band at 1421.49 cm^{-1} was a deformation CH_2 zone corresponding to OH of carbonyl groups and the wide C=O of phenols. Also, the bands 1222.83 and 1126.39 cm^{-1} can be attributed to C=O, OH deformation of carboxylic acids and to a CO of phenols and esters. The bands observed at 1091.68 and 1035.74 cm^{-1} can be assigned to CO of esters groups and the rest of the polysaccharides.

In vitro parameters

Number of leaves

The number of leaves was evaluated in explants of banana clone: Enano Guantanamero (Figure 3). There were no significant differences between the 0 mg L^{-1} and the other concentrations used, except for 20 mg L^{-1} that was lower than the control and the concentration of 50 mg L^{-1} .

Determination of photosynthetic pigments content

The total chlorophyll, chlorophyll a and b are shown in Table 2. The study showed that VHA at 20 and 50 mg L^{-1} induced the higher concentrations for photosynthetic pigments.

Acclimatization

The physiological indicators at this phase were determined thrice according to the application of the VHA. The plant height during transplantation was higher in the control and in 10 mg L^{-1} of VHA. This result is the

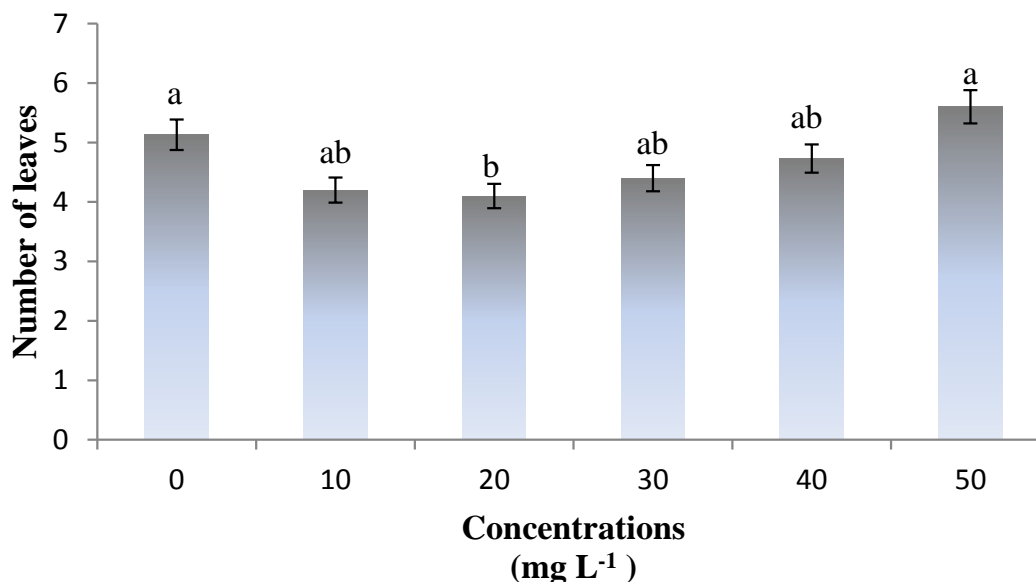


Figure 3. Number of leaves *in vitro* with VHA in MS medium of banana clone: Enano Guantanamero). Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$). Bars represent averages \pm standard error (SE) from three replicate experiments.

Table 2. Photosynthetic pigments content in explants of banana clone Enano Guantanamero with VHA in the MS medium.

Concentrations of VHA	Total chlorophyll (mg g ⁻¹ FW)	Chlorophyll a (mg g ⁻¹ FW)	Chlorophyll b (mg g ⁻¹ FW)
Control	0.206 ^d	0.083 ^c	0.123 ^c
T1 (10 mg L ⁻¹ VHA)	0.202 ^e	0.0422 ^e	0.160 ^d
T2 (20 mg L ⁻¹ VHA)	0.54 ^a	0.162 ^b	0.378 ^a
T3 (30 mg L ⁻¹ VHA)	0.290 ^c	0.060 ^d	0.109 ^c
T4 (40 mg L ⁻¹ VHA)	0.198 ^f	0.038 ^f	0.127 ^c
T5 (50 mg L ⁻¹ VHA)	0.544 ^a	0.203 ^a	0.341 ^b

Different letters indicate significant differences ($p \leq 0.05$).

remaining effect from the *in vitro* phase. After VHA was applied for the second time (25 days) plant height increased by 200% (from 7.7 to 16.64 cm) with 10 mg L⁻¹. Finally, at the end of the acclimatization period, this parameter increased by 346% (from 7.7 to 26.66 cm) (Figure 4).

For plant height at the acclimatization phase is possible to see that with 10 mg L⁻¹ of VHA plants growth more than with the others concentrations used.

The stem diameter was also determined (Figure 5). At the moment of the transplant not significant differences were found for the stem diameter between 0, 10 and 50 mg L⁻¹, but at the end of the acclimatization the diameter increased in 328 and 354% with 10 and 40 mg L⁻¹ respectively.

The number of leaves was counted for the different concentrations used as is possible (Figure 6).

The number of leaves at the end of the acclimatization with 40 mg L⁻¹ increased in 151%. Plants without VHA

showed a decrease in this important physiological parameter.

Roots length and dry weight are shown in Figure 7.

The roots length did not show any significant differences between the concentrations of VHA used, in the other hand the dry weight of this organ was higher and significant differences were found for 40 mg L⁻¹ of VHA, being this concentration higher to the control in 205%.

DISCUSSION

Vermicompost is a very useful growth medium for most crops because of high content of many available nutrients and plant growth promoters (Arancon et al., 2004). According to Ricci et al. (1995) vermicompost provides P, Ca, Mg, and S like inorganic fertilizers. Additionally, Arancon et al. (2004) showed that vermicompost

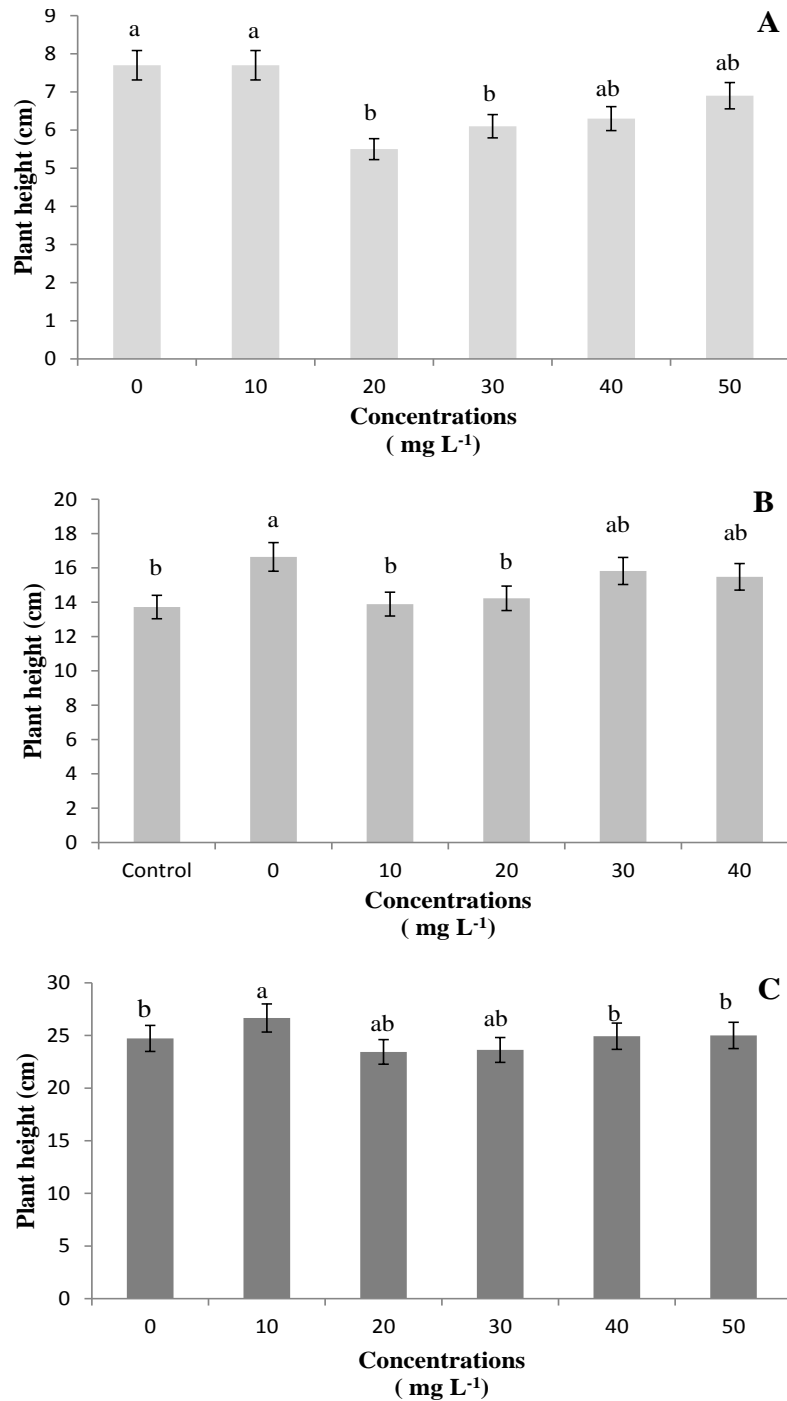


Figure 4. Plant height (cm) at acclimatization phase of banana clones Enano Guantanamero. **A.** During transplantation before VHA application. **B.** After 25 days of transplantation, second application of VHA. **C.** End of the acclimatization process (50 days). Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$). Bars represent averages \pm standard error (SE) from three replicate experiments.

enriched with HS could promote plant growth and nutrient uptake, but it is necessary that this product has good qualities, like the quantities of the diverse elements and

relative percentage of the phenolic and carboxylic acids (Table 1) that are similar to the range reported by the International Humic Substances Society (Ritchie and

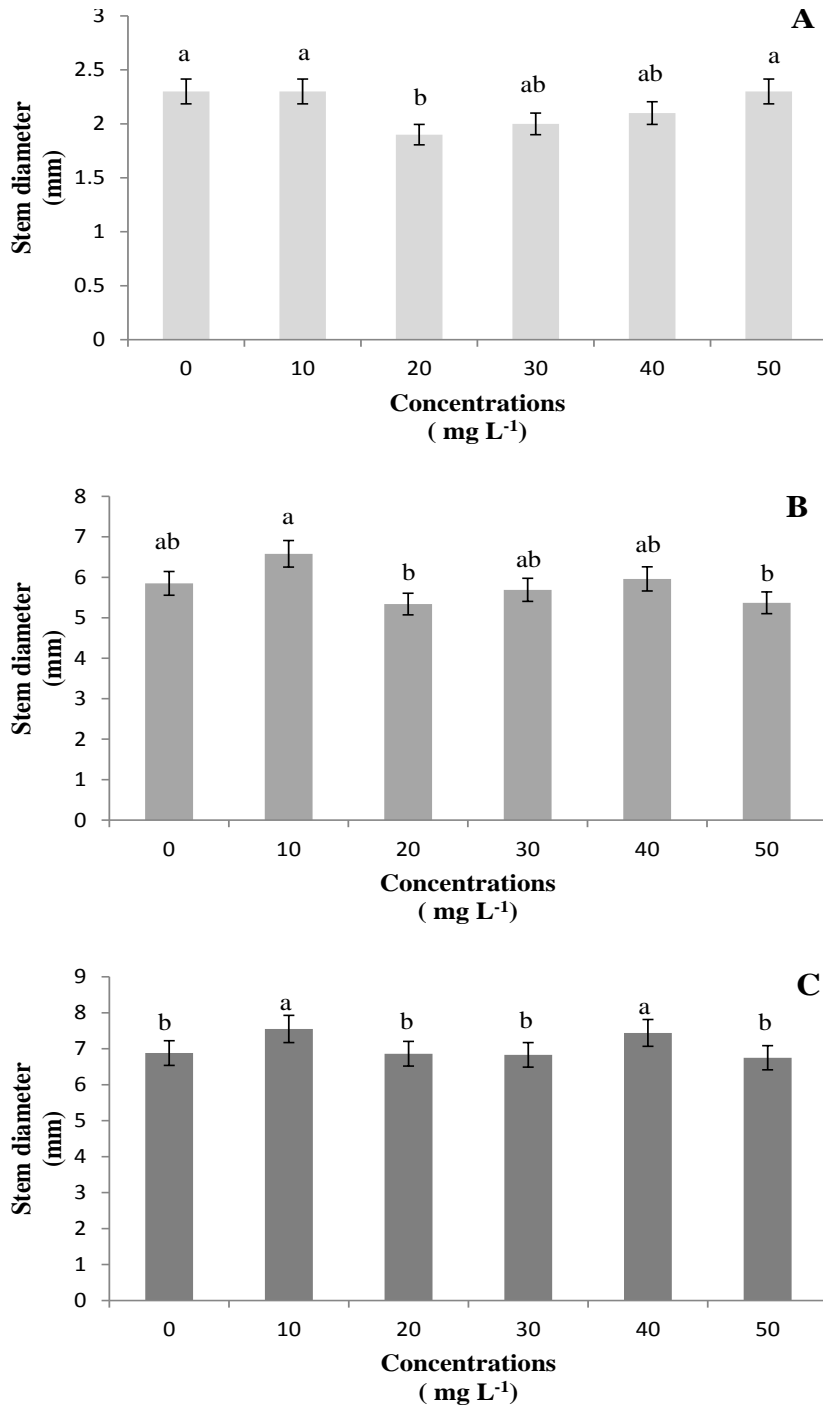


Figure 5. Stem diameter (mm) at acclimatization phase of banana clone Enano Guantanamero. **A.** During transplantation before VHA application. **B.** After 25 days of transplantation, second application of VHA. **C.** End of the acclimatization process (50 days). Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$). Bars represent averages \pm standard error (SE) from three replicate experiments.

Purdue,2003). Also, the rate E4/E6 was 4.22, indicating that the VHA obtained had a great humidification and maturity degree, properties derived from its high degree

of condensation and aromaticity. This result is similar to that obtained by Campitelli and Ceppi (2008) and Fukushima et al. (2009). The VHA from soils with little air

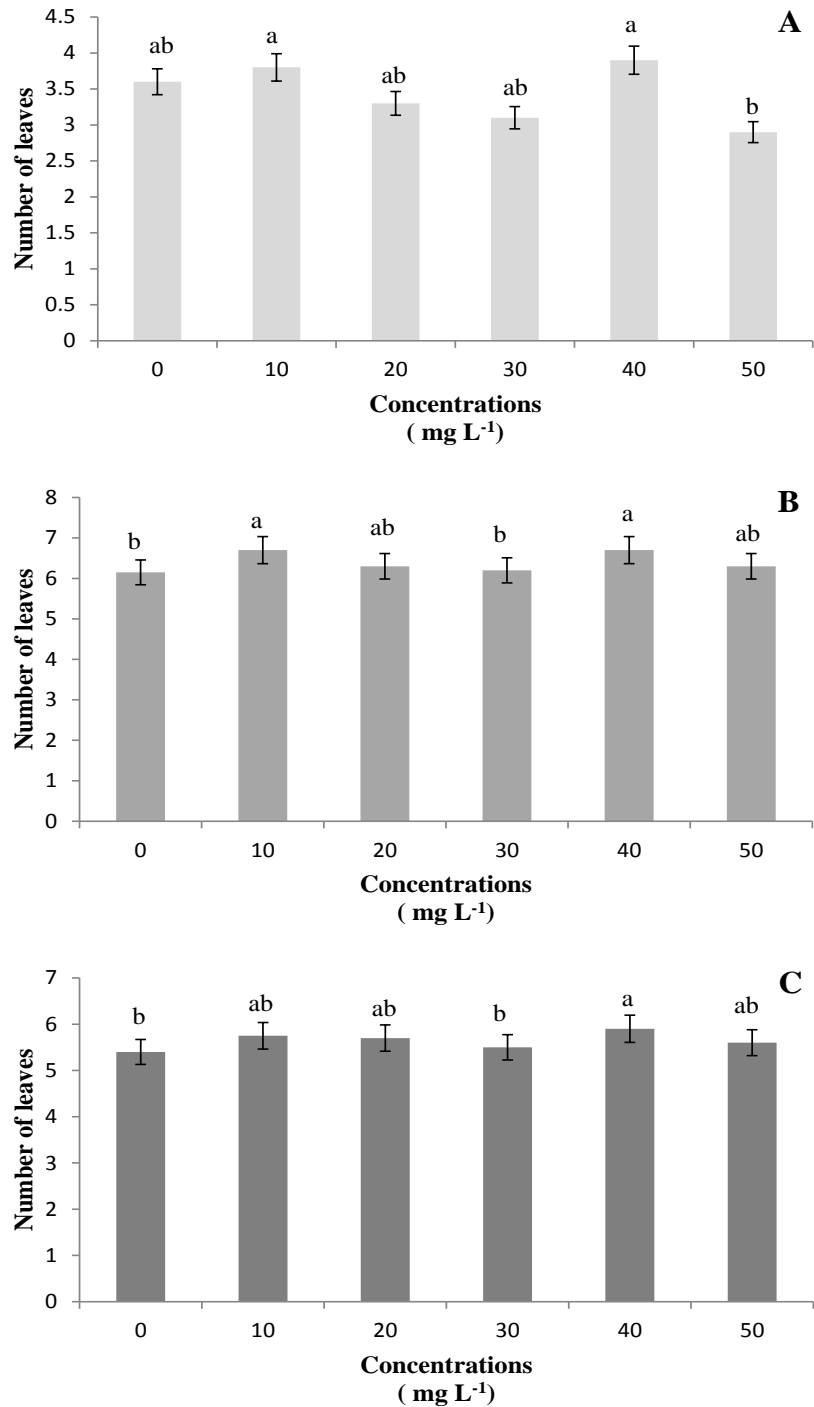


Figure 6. Numbers of leaves at acclimatization phase of banana clone Enano Guantanamero. A: During transplantation before VHA application; B: After 25 days of transplantation, second application of VHA; C: End of the acclimatization process (50 days). Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$. Bars represent averages \pm standard error (SE) from three replicate experiments.

has a higher content of condensed structures due to the elevated rate E4/E6, high fluorescence intensity and more free radicals (Canellas et al., 2008b).

To make the allocations to the main absorption peaks related to the reactions involved in the DRIFT, spectra obtained were taken into account in respect to those

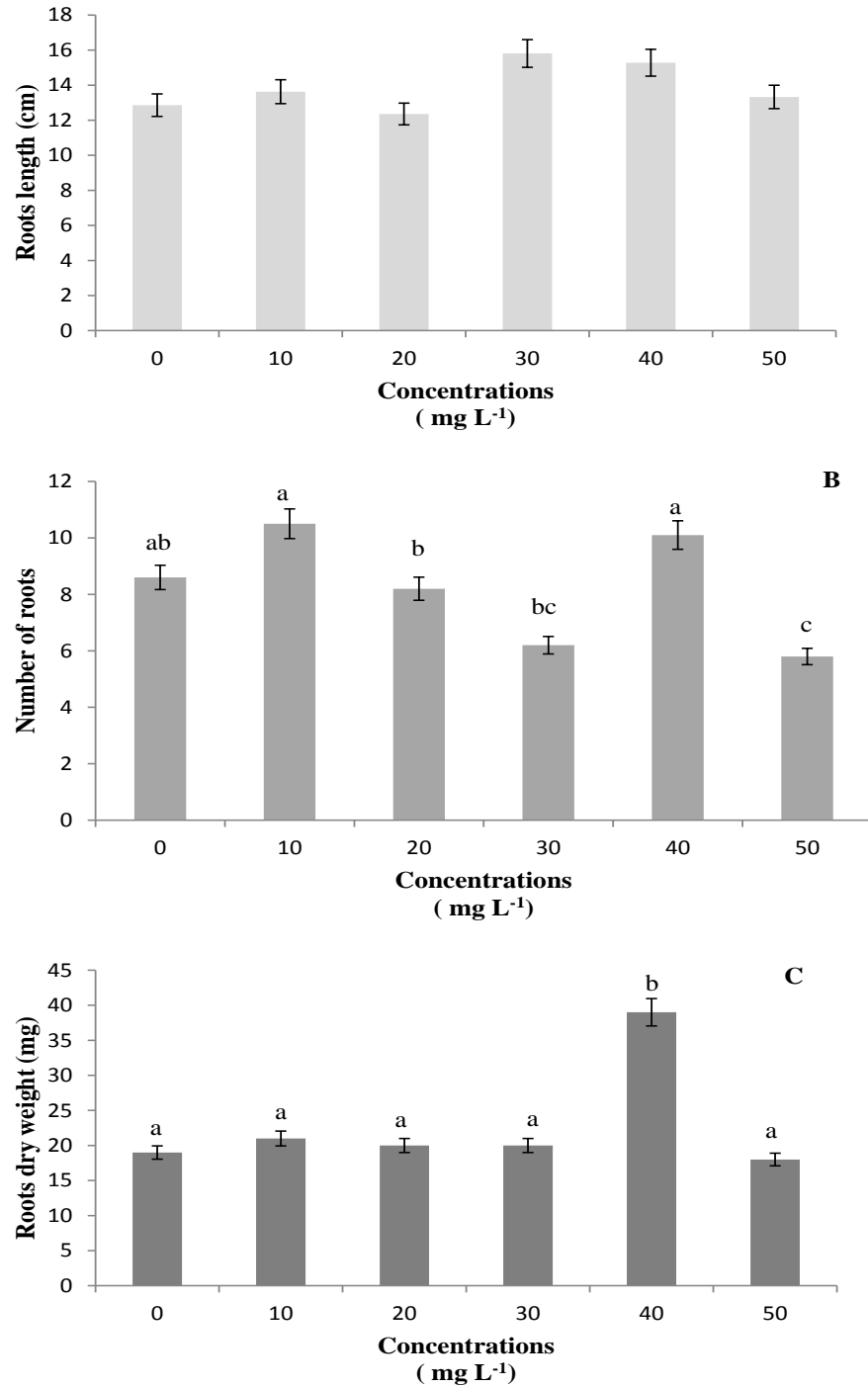


Figure 7. Roots evaluation at acclimatization phase of banana clone Enano Guantanamero. **A.** Root length. **B.** Number of roots. **C.** Roots dry weight. Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$). Bars represent averages \pm standard error (SE) from three replicate experiments.

reported in the specialized literature and research presented by Chen et al. (2009), Dobbss et al. (2009), Spaccini and Piccolo (2009).

All the parameters analyzed in the VHA used in this research demonstrated the great qualities of the product

used, this studies had a great importance in understanding their modes of action and practical use (Muscolo et al., 2013).

In vitro, some parameters were determined to evaluate the influence of VHA in the explant of banana clone

Enano Guantanamero. VHA had no influence over the number of leaves in this crop. Many authors report the increases in plant growth including number of leaves when VHA is used such as da Silva et al. (2015) in *Cattleya warneri* (Orchidaceae), Atiyeh et al. (2002) in tomato (*Solanum lycopersicum* L.) and some species from Cucurbitaceae family, Moghadam et al. (2012) in *Lilium Asiatic* hybrid var. Navona, and some others.

The evaluation of total chlorophyll and chlorophylls a and b showed an increase in the photosynthetic pigments due to VHA influence, but not in all concentrations used.

According to Nardi et al. (2002), the principal effect of HS in the photosynthetic process is not clear yet, but the application of these substances induces an increase in chlorophyll. This does not mean a direct relation with photosynthesis and finally with plant production. It seems that the effects of HS on plants may be selective and variable, depending on their concentration and pH of medium (Nardi et al., 2002), as seen in this research where the effect on photosynthetic pigments depended on VHA concentration. Elevated concentration of HS (100, 200 and 400 ppm) decreased chlorophyll content (Cooper et al., 1998) but in this work, low and intermediate concentration induced reduction in chlorophyll content.

It has been reported that the effect of humified materials on the stimulation of pigment contents depends on the physiological state of plants and the concentrations of the material. In a research on *Phaseolus vulgaris* L., with foliar application of humates extracted from vermicompost, it was found that chlorophyll content could be stimulated or inhibited (Portuondo et al., 2009); and in this investigation with banana *in vitro*, the chlorophyll content was stimulated by different concentrations of VHA.

The acclimatization phase normally for banana is from 60 to 90 days (Granada, 1990), but with the application of VHA this phase was reduced to 50 days for banana clone Enano Guantanamero with an increase in plant height, stem diameter and number of leaves compared with the control.

At the end of the acclimatization 10 mg L⁻¹ was the VHA concentration recommended for plant height and stem diameter and 40 mg L⁻¹ stimulated number of leaves, stem diameter and roots dry weight. All the parameters evaluated decreased without VHA (0 mg L⁻¹) and with the higher concentration (50 mg L⁻¹). According to Singh and Chauhan (2009) applied vermicompost to French bean (*Phaseolus vulgaris* L.) plants induced an increase in several parameters such as germination, height of plant, number of leaves per plant, length of leaves, width of leaves, and so, due to vermicompost treatments improve physico-chemical properties of soil.

In groundnut (*Arachis hypogaea* L.) vermicompost concentrations were used (0, 100, 150, 200 and 250 mg L⁻¹) to evaluate different parameters where the highest root length, shoot length, total leaf area, number of root

nodules, fresh weight, dry weight, chlorophyll and carotenoids were recorded with application of 200 g of VHA at various stages of its growth (25, 50, 75 and 100 days) according to Mathivanan et al. (2012).

Hernández et al. (2012) with VHA concentration of 34 to 46 mg L⁻¹ in rice (*Oryza sativa* L.) obtained significant increases (between 30 and 70% respects to the control) in roots dry weight. In this research in banana with similar concentration (40 mg L⁻¹) an increase of 205% over the control was obtained, this result is similar to the measured of this parameter *in vitro* where 40 mg L⁻¹ induced the higher result (Moya et al., 2014).

Several researches have been reported improvement in many physiological parameters with humic acid. In a perennial ryegrass (*Lolium perenne* L.), HA concentrations were used (0, 100, 400 and 1000 mg L⁻¹) to evaluate different parameters where only 100 mg L⁻¹ favored height of the plant, nitrogen content, roots length and surface of roots (Maibodi et al., 2015). On the contrary, when Can et al. (2008) used 0, 100, 500 and 1000 mg L⁻¹ of humic acid in gerbera plant (*Gerbera jamesonii* L.), the higher concentration used increased root growth. These researches demonstrate that the influence of humic acid or VHA on plant growth is dependent on the concentration used.

Conclusion

The VHA used in this investigation showed an elevated degree of condensation and aromaticity. In this research the ability of humic substances to improve plant development has been proved. This action was reflected in an enhancement of some physiological indicators *in vitro* like total chlorophyll, chlorophyll a-b, and in the acclimatization phase, such as plant height, stem diameter, number of leaves, number of roots and roots dry weight. Two VHA concentrations (10 and 50 mg L⁻¹) increased *in vitro* indicators, while for the acclimatization stage 40 mg L⁻¹ can be recommended for banana for its positive effects on number of leaves and root development. The use of VHA in the acclimatization period reduced this phase to 50 days, which means saving water, nutrients and earlier delivery of plants to farmers.

Conflicts of interests

The authors have not declared any conflict of interests.

Abbreviations

DRIFT, Diffuse reflectance Fourier-transformed infrared; **HS**, humic substances; **MS**, Murashige and Skoog; **UV-VIS**, ultraviolet-visible spectroscopy; **VHA**, vermicompost

humic acids.

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Full Length Research Paper

Study of genetic determinism of harvest index in durum wheat (*Triticum durum* Desf) under semi-arid conditions

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Out of six varieties of durum wheat (*Triticum durum* Desf.), two local varieties with a low harvest index and four others with high harvest indices and short straw imported from France were studied in a diallel cross. The experiment was done in a complete randomized block design with three replications. It was done at the Research Station of the Institute of Technical Big Cultures of Khemis Miliana, located in the semi-arid high Chelif Valley in Algeria. Before spikes emerged from the grains of the six varieties, we did a complete diallel hybridization between them. This resulted in 30 crosses. The heterosis that appeared in F₂ of the harvest index in some hybrids is because the genotypes vary. Nefer variety located close to the parabola shows there is no transgression in character. The effects of additive are confirmed by the Hayman analysis in the harvest index. The most promising breeding stocks that can be used to improve the harvest index of durum varieties are Guem Goum Erkham, Hedba3 and Excalibur.

Key words: Genetic determinism, *Triticum durum* Desf, harvest index.

INTRODUCTION

Before 1925, improvement of harvest index was considered to be due to increase in grain yield. This index is the ratio of the final grain yield to that of aboveground biomass produced at maturity (Bensalem et al., 1991; Guerif and Seguin, 2001). Barrier et al. (1987) considered that the evolution of grain yield was mainly due to the

improvement of the harvest index. However, the role of harvest index in improving yields and generally biomass production remains debatable among some experts in the field.

Jordacijevic R (2009) and Deghais (1993) showed that the use of harvest index for the selection of productive

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varieties is not unanimously accepted among researchers. For Bouzerzour et al. (1998), this parameter indicates the degree of conversion of a part of the aboveground biomass into grain. The weaker the grain quality, the more the harvest index shows an average expression of character. Grain quality, weight and thickness influence harvest index (Anandakumar et al., 2015). Studeto et al. (1986) found 17% harvest index in wheat. Bouzerzour et al. (1998) found 15.71% harvest index in Hedba3 variety.

Under normal growing conditions, harvest index reaches around 50%, and it goes down to about 35% in arid conditions (Richard et al., 1997). Reducing the size of durum wheat stem contributes to reduced sensibility and leads to increased harvest index (Bamoun, 1997). For Sasukuma et al. (1978), the main objective is to select plants that meet the desired criteria (short straw, immune to the end of cycle diseases, biomass production and high index of harvest). The genetic information of local varieties should be exploited to create new varieties with desirable attributes (Ray et al., 2013). In North Africa, Couscous and traditional bread are also made from local varieties of durum wheat (Nefzaoui et al., 2014). This work aims to study the genetic determinism of the harvest index of durum wheat using a diallel cross.

MATERIALS AND METHODS

Genetic stocks

The experiment was carried out in semi-arid conditions with six varieties of durum wheat (*Triticum durum* Desf.) of various origins, including two local cultivars. The two local varieties (Guem Goum Erksam and Hedba 3) take a very long time to grow, have very tall straw, low harvest index, high thousand grain weight and adapt to semi-arid conditions. The durum wheats of North Africa are quite adaptable and contain large genetic diversity (Sourour and Slim-Amara, 2008). The morphological characteristics of the different varieties of Algerian durum wheat can be used in breeding programs with other introduced varieties (Arora et al., 2014).

The other four cultivars: Ardente, Acalou, Nefer and Excalibur have a short growing season, have very short straw, a high number of grains per spike, high yield and a high index of harvest in favorable conditions (Anonymous, 2004). They are susceptible to spring frosts and drought conditions at the end of the growing period. These lines were imported from France and all contain dwarfism gene from Akamodji variety of Chinese origin (D'Amato, 1989).

A complete diallel cross between the six varieties was conducted at the experimental station of the Technical Institute of Big Cultures in Khemis Miliiana, Algeria. Thirty F_1 hybrids were obtained. These were planted and harvested in the F_2 generation.

Experimental design

Two completely randomized tests consisting of two replications were conducted. The first test included six parents of the diallel cross and 30 F_1 hybrids, and the second test included six parents and thirty F_2 hybrids. The size of the basic plot for parent varieties was 3 m². The F_1 and F_2 hybrids were planted in rows of one to two

meters long, depending on the number of grains harvested from the hybrid plants. The space between the lines was twenty cm, and between the plants, 10 cm. The gap between replications was one meter, while the space between the element plots was fifty centimeters.

Measurement and counting

The height of the straw (HS) is measured from the tillering plate to the base of the spike. All stems of a plant are measured and the average is calculated.

Biomass Maturity stage (BM) and the Total Biomass (TB) were evaluated by the weight of ten plants harvested at maturity. The plants were harvested at the tillering point with their spikes. The weight of a group of ten plants was per square meter (g/m²). The number of plants in the parents on which the measures were carried out per square meter was 100.

The grain biomass (GB) in grams per square meter was measured by the number of grains per square meter multiplied by the weight of the grain.

The Harvest Index is equal to Biomass Granules (BG) harvested per square meter on total biomass harvested per square meter (BM) or $IR = BG / BT$. For Desimir et al. (2009); Huang and Gao (2000) and Zarkouna (1985), harvest index is the ratio of grain yield and the harvested biomass yield, $IR = RG/RP$ percent.

Statistical methods

Analysis of variance

Variance analysis was done for the parents in a completely randomized block in the two years. While for F_1 and F_2 , variance analysis was carried out separately.

Methods for estimating genetic parameters according to Hayman

The results of the Diallel were analyzed by the method of Hayman (1954). This method is very effective in the analysis of polygenic traits. It is based upon the following parameters:

- i) V_0 = Represents the value of parents (values of the diagonal or selfing).
- ii) V_r = Variance of n families (every family includes a parent and all the crosses performed with that parent).
- iii) W_r = is the covariance of the brothers with the recurrent parent.

The drawing of the parabola is realized with the help of a few points obtained by setting the values of W_r from which we deduce V_r . This curve determines the location of the varieties on the graph.

The regression line $W_r = b.V_r + c$ shows the alignment of the points ($W_r.V_r$) in the graph, while regression line $(W_r + V_r) = bt + c$ determines the prevalence of recessive or dominant genes of the tested varieties. The value "c" is that of the parents used in the experiment (Demarly 1972).

RESULTS AND DISCUSSION

Analysis of variance of parents

The analysis of variance of the characteristics of the two

Table 1. Analysis of variance of the harvest index of the parents of two years.

Variable	C.M				C.V (%)
	Genotype (df = 5)	Environment (df = 1)	Genotype*Environment (df = 5)	Effect blocks df = 2	
Harvest index	626.665***	10.454**	147.132***	29.242	3.95

** and ***: Significant at $p < 0.01$ and $p < 0.001$, respectively,

Table 2. Average value of parents annually.

Genotype	Year 1	Year 2	Average of two years
Excalibur (EX)	29.68 ^a	28.82 ^b	29.23 ^a
Ardente (AR)	23.13 ^b	23.76 ^c	23.41 ^b
Acalou (AC)	21.00 ^b	23.26 ^c	22.12 ^b
Nefer (NE)	28.78 ^a	33.77 ^a	31.49 ^a
Guem Goum Erkhama (GE)	12.00 ^d	13.12 ^e	12.45 ^d
Hedba3 (H3)	19.18 ^c	18.12 ^d	18.31 ^c
Average	22.29	23.48	23.96

years (Table 1) shows:

- i) Very highly significant ($p < 0.001$) effects of genotypes.
- ii) Very highly significant ($p < 0.001$) effects of the interaction genotype * environment.
- iii) A highly significant effect ($p < 0.01$) is found in environment.

Study of parents

The ranking of harvest index averages, according to Newman-Keuls test at the 5% threshold, identified four groups:

- * Genotypes Excalibur and Nefer formed group (a).
- * Genotypes Acalou and Ardente formed group (b).
- * Genotype Hedba3 formed group (c).
- * Genotype Guem Goum Erkhama formed group (d).

The average of character is 22.29 in the first year while in the second year the average is 23.48 (Table 2). The average harvest index has increased from 22.29 in the first year to 23.48 in the second year. There is a reduction in harvest index in second year in the Excalibur variety.

The genotypes Acalou, Nefer had their harvest index increasing from the first to the second year, while harvest index of the variety Ardente remained stable.

The harvest index of local varieties remained low compared to the introduced genotypes. Nefer genotype had the highest harvest index in the second year, while Guem Goum Erkhama had the lowest harvest index in

both years of testing.

Analysis of hybrid variance

The analysis of variance hybrid (Table 3) shows a highly significant genotype effect ($p < 0.001$), while the effect blocks shows a highly significant effect (0.01).

Average F₁ and F₂ hybrids

The harvest index of F₁ hybrid records an average of 21.66%. The number of groups formed by the test of Newman Keuls at the 5% threshold is 17. The extreme value of 34.36 is achieved the hybrid AC/GE which forms the "A" group. The hybrid AR/H3 11.64% with a harvest index represents the group L (Table .4).

The average harvest index in F₂ is 40.22%. Classification of averages showed the presence of sixteen homogeneous groups. The highest value is recorded by crossing AR/NE with 61.56% harvest index. This hybrid is the "A" group. Hybrid AC/GE, AC/H3 and AR/H3 form the "L" group with the lowest values (18.18, 18.84 and 18.94 respectively) (Table 4).

DISCUSSION

The harvest index and grain yield of parents, F₁ and F₂ hybrids evolved between the first and second year. Local varieties have the lowest indexes compared to the introduced genotypes. These findings are consistent with those found by Bouzerzour et al. (1998). According to

Table 3. Analysis of variance of the harvest index of F₁ and F₂ hybrids.

Character	CM			Coefficient of variation
	Genotype (df = 29)	Blocks (df= 1)	Residual (df = 30)	
Harvest index F ₁	69.37**	0.07	0.38	2.90
Harvest index F ₂	2302.20***	45.06**	929.69	3.36

** and ***: Significant at p<0.01 and p<0.001, respectively.

Table 4. Ranking of hybrid F₁ and F₂ according to their averages.

Hybrid	Code hybrid	Value F ₁	Value F ₂
Ardente/Excalibur	AR/EX	18.09 ^{hi}	29.26 ^{ijk}
Acalou/Excalibur	AC/EX	25.35 ^d	46.50 ^{de}
Acalou/Ardente	AC/AR	30.60 ^b	49.38 ^{cd}
Nefer/Excalibur	NE/EX	23.01 ^{ef}	57.31 ^{bc}
Nefer/Ardente	NE/AR	31.10 ^b	60.03 ^b
Nefer/Acalou	NE/AC	15.00 ^k	32.21 ^{hij}
GuemGoum Erkham/Excalibur	GE/EX	15.07 ^k	25.66 ^{ijkl}
Guem Goum Erkham/Ardente	GE/AR	18.03 ^{hi}	41.49 ^{defgh}
Guem Goum Erkham/Acalou	GE/AC	25.54 ^d	25.77 ^{ijkl}
Guem Goum Erkham/Nefer	GE/NE	15.97 ^k	32.51 ^{hij}
Hedba3/Excalibur	H3/EX	24.47 ^{de}	42.38 ^{defg}
Hedba3/Ardente	H3/AR	14.78 ^k	39.25 ^{efgh}
Hedba3/Acalou	H3/AC	19.05 ^h	36.79 ^{fghi}
Hedba3/Nefer	H3/NE	22.33 ^f	34.61 ^{ghi}
Hedba3/Guem Goum Erkham	H3/GE	24.47 ^{de}	36.77 ^{fghi}
Excalibur /Ardente	EX/AR	22.18 ^f	40.54 ^{efgh}
Excalibur/Acalou	EX/AC	18.48 ^h	55.75 ^{bc}
Excalibur/Nefer	EX/NE	29.42 ^b	61.16 ^b
Excalibur/GuemGoum Erkham	EX/GE	16.28 ^{jk}	22.78 ^{kl}
Excalibur/Hedba3	EX/H3	19.40 ^{gh}	35.42 ^{ghi}
Ardente/Acalou	AR/AC	26.91 ^c	37.72 ^{fghi}
Ardente/Nefer	AR/NE	17.69 ^{hij}	65.46 ^a
Ardente/Guem Goum Erkham	AR/GE	16.51 ^{ijk}	44.67 ^{def}
Ardente/Hedba3	AR/H3	11.64 ^l	18.94 ^l
Acalou/Nefer	AC/NE	30.73 ^b	56.18 ^{bc}
Acalou/Guem Goum Erkham	AC/GE	34.36 ^a	18.18 ^l
Acalou/Hedba3	AC/H3	24.39 ^{de}	18.84 ^l
Nefer/Guem Goum Erkham	NE/GE	22.36 ^f	49.21 ^{cd}
Nefer/Hedba3	NE/H3	20.60 ^g	54.56 ^{bc}
Guem Goum Erkham/Hedba3	GE/H3	15.86 ^k	37.37 ^{fghi}
Average		21.66	40.22

Houshmand and Vanda (2008), the characteristics that lead to higher grain yield are harvest index and thousand-kernel weight. We found very highly significant correlations between harvest index and grain yield of 0.903 *** and 0.923 *** F₁ and F₂ respectively.

The high harvest index is frequently associated with the

avoidance of water stress (Keim and Kroustad, 1981; Fussel et al., 1991). Le Gouis et al. (2000) showed an increase of harvest index while production of wheat aerial dry matter remained stable. The results show a hybrid vigor level between 3 and 19% compared to the parental average.

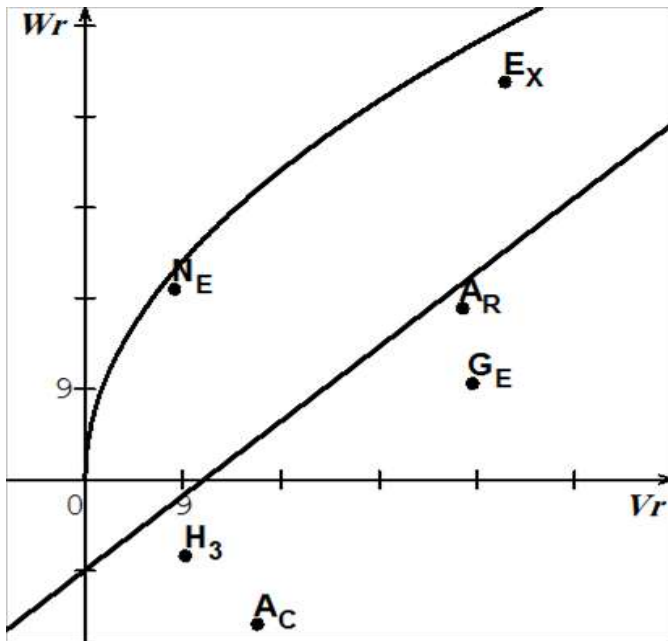


Figure 1. Relation between W_r covariance and V_r variance en F_1 .

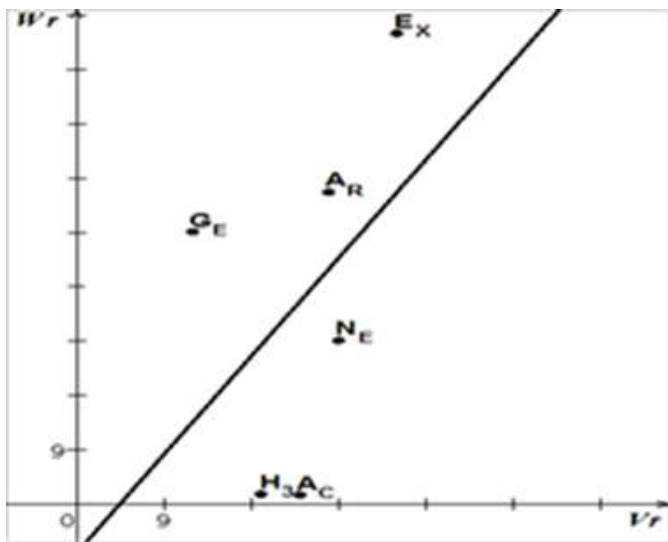


Figure 2. Relation between the sum of covariance and variances value of parent t: $(W_r + V_r) / t$ en F_1 .

Stoddart (2003) finds a heterosis of 17% for the harvest index in wheat. Hanifi-Mekliche and Boukecha (2008) found a positive heterosis of 19.35% for the harvest index in durum wheat. The result of the crossing involving parents from more divergent groups showed a maximum heterosis (Singh et al., 2014). Maria (1984) found a heterosis of wheat harvest index without giving figures. A

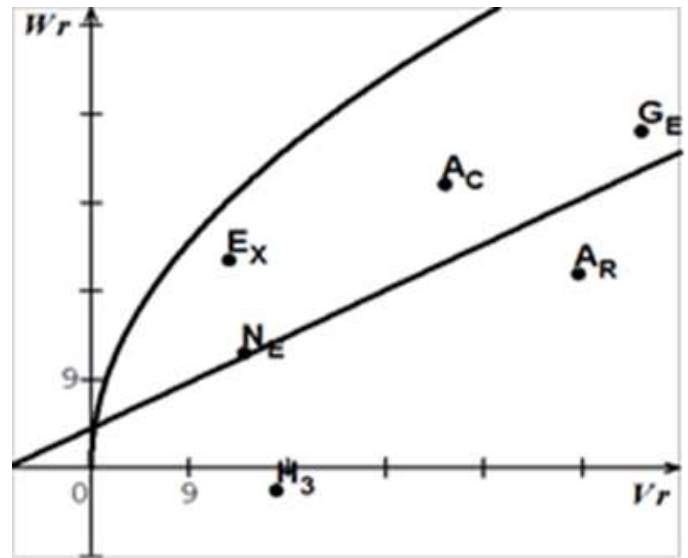


Figure 3. Relation between W_r covariance and V_r variance en F_2 .

heterosis (in the full sense of the word) between 5 and 44% is found in the following F_2 hybrids: AC/AR, GE/AR, H3/GE, GE/AC, AC/GE, NE/GE, AR/AC, and NE/AR.

The cross between local variety and variety introduced makes the hybrids to have elevated harvest index. When the genetic distances are high among parents, they involve a significant heterosis in offspring (Zamaniafard et al., 2015).

Graphic interpretation

The position of the regression lines W_r / V_r of diallel cross allows us to carry out the analysis of genetic potential of parents. The graph (Figure 1) reveals that F_1 crop index is governed by a superdominance. Hedba 3 genotypes Acalou Ardente include more recessive genes for positive action on the expression of character; however, genotypes Guem Goum Erkhame and Excalibur have many recessive and dominant genes. The dominant genes have a negative effect on the expression of character. The location of Nefer variety near the parabola shows equality between W_r and V_r therefore this genotype cannot be transgressive for the character (Figure 2).

The harvest index in F_2 is under the effect of partial dominance (Figure 3). Gebhardt (1990) showed that harvest index was under the influence of dominant genes. Acalou genotypes Guem Goum Erkhame and Ardente have many recessive genes and dominant genes. Their position gives hopes for the possibility of transgression in future generations. In order to reach a transgressive segregation, the presence of genetic divergence between

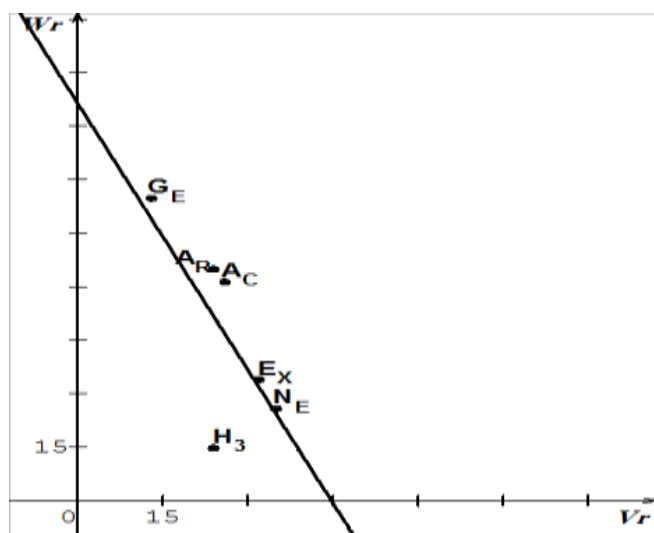


Figure 4. Relation between the sum of covariance and variances value of parent t: $(W_r + V_r) / t$ en F_2 .

parents is paramount (Ahmad et al., 2014). Dominant genes have a positive effect on the expression of character (Figure 4). It is possible to combine within a single genotype favorable dominant genes and recessive genes to obtain interesting hybrids (Berthelem et al., 1974; Abdus et al., 2003). The accumulation of dominant genes and recessive genes with good complementarity leads to better genetic efficiency (Gallais, 1967). In order to benefit from a transgressive segregation genetic divergence between the parents is mandatory (Ahmad et al., 2014). Kumar et al. (2014) have also provided similar results of genetic divergence in wheat.

Confirmation of the stability of heterosis of harvest index found in AC/AR hybrids, GE/AR, H3/GE, GE/AC, AC/GE, NE/GE, AR/AC and NE/AR can be done over several years and in different environments to better understand their behavior over several generations.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Comparative phylogenetic analysis of intergenic spacers and small subunit rRNA gene sequences of two microsporidian isolates from *Antheraea mylitta*

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Two microsporidian isolates extracted from infected tasar silkworms (*Antheraea mylitta*) collected from forest area in Deoghar district, Jharkhand, India were subjected to PCR amplification using intergenic spacer (IGS) region and small subunit rRNA (SSU-rRNA) gene specific primers followed by cloning and sequencing. The IGS and SSU-rRNA gene sequences were analysed to derive the identity of the microsporidian isolates and establish their phylogenetic relationships. The phylogenetic analysis of test isolates included assessment of variation in sequences and length of IGS and SSU-rRNA genes with reference to 16 different microsporidian sequences. The results proved that IGS sequences have more variation than SSU-rRNA gene sequences. Analysis of phylogenetic trees reveal that both test isolates have very close relationship with each other as well as with three *Nosema* reference species viz., *N. philosamia* and *N. antheraea* isolated from *Philosamia cynthia ricini* and *Antheraea pernyi* in China respectively, and *N. disstriae* isolated from *Malacosma disstriae* in Canada. The test microsporidian isolates revealed closer relationship with other *Nosema* reference strains compared to *Nosema* sp. (NIK-1s_mys) from India. The study results indicate that the IGS or/and SSU rRNA sequence based analysis is suitable and valuable to ascertain phylogenetic relationships between various microsporidian strains/species.

Key words: Microsporidia, *Antheraea mylitta*, small subunit rRNA, intergenic spacer, phylogenetic relationship.

INTRODUCTION

Antheraea mylitta Drury (Lepidoptera, Saturniidae) a tropical tasar silkworm found in the states of Jharkhand,

and Andhra Pradesh in India are reared on forest trees, viz. *Terminalia arjuna* Haines, *Terminalia tomentosa*

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Table 1. Details of the microsporidian isolates: Their place of collection, host and morphology.

S/N	Name of microsporidian isolates	Host	Place of collection (forest area/village), district, latitude/longitude/elevation	Spore size (μm)	
				Length	Width
1	MIJ-2pD	<i>Antheraea mylitta</i>	Pindari forest area, District: Deoghar, Jharkhand, India. 24°28'49.88N/86°42'0.00E/249.8	5.10±0.20	3.20±0.01
2	MIJ-4cD	<i>Antheraea mylitta</i>	Chechai forest area, District: Deoghar, Jharkhand, India. 24°28'49.88N/86°42'0.00E/249.8	4.50±0.12	2.56±0.01

MIJ, Microsporidia India Jharkhand.

Haines and *Shorea robusta* Roxb. Tasar silkworm rearing generates substantial rural employment in India. However, rearing of *Antheraea mylitta* often causes infectious diseases like microsporidiosis, virosis, bacteriosis and muscardine. Among these, the most devastating is microsporidiosis caused by the microsporidian *Nosema* sp. causing severe cocoon crop loss (Singh, 2011).

Microsporidia are a diverse group of spore-forming obligate intracellular parasites that include more than 1300 described species under 160 genera (Corradi and Keeling, 2009). They infect a wide range of invertebrates and vertebrates including insects, fishes and mammals (Wittner and Weiss, 1999; Weiss, 2001). They have the smallest genomes among eukaryotic organisms and cause a variety of important medical, agricultural, veterinary, sericulture and ecological impacts (Keeling and Fast, 2002). The ultrastructural and phenotype-based classification systems faced several problems that were largely overcome through ribosomal DNA (r-DNA) sequence analysis (Baker et al., 1995; Hung et al., 1998). Several studies have been attempted to classify microsporidian species and strains through amplification and sequencing variable regions of the genome such as the ribosomal Internal Transcribed Spacer region (ITS), Inter-Genic Spacer region (IGS), Large Sub-Unit (LSU) and Small Sub-Unit rRNA (SSU rRNA) gene (Huang et al., 2008; Santin et al., 2009; Dong et al., 2010; Li et al., 2012). In fungi, the noncoding spacer regions of rDNA, which evolve rapidly, have been utilized in inferring phylogeny among more closely related taxa. The IGS region have been examined in the course of evolutionary and taxonomic studies of fungi (Erland et al., 1994; Molin et al., 1993; Aminnejad et al., 2009). Nowadays, the non-coding spacer regions (ITS/IGS) have been found valuable to study and establish relationships among closely related taxa particularly in fungi and other organisms. The ITS / IGS sequence regions can develop variations within genera that distinguish them at intra species level. The taxonomic value of ITS/IGS region is due to their significant heterogeneity in length and nucleotide sequences. This study targeted the SSU-rRNA gene and IGS region located in between the SSU-rRNA and 5S-rRNA gene cluster of the microsporidia. The IGS region that has the most rapidly evolving sequence

provides significant data considered phylogenetically useful for delineating relationships within species (Hillis et al., 1991).

The aim of our study was to identify and delineate microsporidian isolates based on IGS and SSU rRNA gene sequences. In order to analyze the IGS and SSU rRNA gene sequences, we had amplified IGS and SSU rRNA gene followed by cloning and sequencing. We defined and analyzed inter and intra-individual variations in the IGS and SSU rRNA gene sequences of test microsporidian isolates. The heterogeneity of r-DNA sequence derives the genetic variations of isolates and elucidates phylogenetic relationship among defined microsporidian species/strains.

MATERIALS AND METHODS

Collection, isolation and purification of microsporidian spores

Two strains of microsporidia were extracted from individual infected moths of *A. mylitta* collected from the forests areas of Deoghar district Jharkhand, India by maceration and suspended in 0.85% NaCl followed by filtration through layers of cheese cloth and centrifugation at 3500 r/min for 10 min. The spore pellet obtained was further purified through density gradient ultracentrifugation using Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) described by Undeen (Undeen and Alger, 1971). The details of microsporidian isolates, places of collection, host and size are given in Table 1.

Spore morphology (length and width)

The morphology of purified fresh spores was observed under phase contrast microscope (Carl Zeiss-AXIO, Humburg, Germany) and measurements were recorded according to the method of Undeen (Undeen and Vavra, 1997). Fresh spores were spread in water agar on glass micro-slides and measured using an ocular micrometer under phase contrast microscope. All the measurements are presented in micrometers as mean values of 12 individual observations.

DNA extraction and purification

Genomic DNA was extracted from the sporoplasm using the glass bead method (Undeen and Cockburn, 1989). DNA concentration

Table 2. Detail of SSU-rDNAs sequences submitted in NCBI of two microsporidian isolates.

S/N	Microsporidian isolates ID	Sequences details			
		SSU-rRNA (bp)	Gene bank accession no.	IGS (bp)	Gene bank accession no.
1	MIJ-2pD	1232	KU187949	270	KP151550
2	MIJ-4cD	1232	KU187950	281	KP151552

and quality was determined both by spectrophotometry at 260 and 280 nm and on 0.8% agarose gel, using a known quantity of λ DNA (10 ng/ μ l) as a standard before use in subsequent PCRs. Any possibility of host DNA contamination was checked using insect mitochondrial primers. A working solution of DNA (10 ng/ μ l) was prepared in sterile double distilled water and the concentration and purity was determined as per standard protocol.

PCR amplification of SSU-rRNA gene

The genomic DNA of both microsporidian isolates were amplified using SSU-rRNA gene primers [Forward-5'-CACCAGGTTGATTCTGCCTGAC-3' and Reverse - 5'-GATATAAGTCGTAACATGGTTGC-3'] as previously described (Wang et al., 2006). The amplified products excised from the agarose gel were eluted using the QIA quick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR amplification of IGS region

Genomic DNA from the two microsporidian isolates was amplified with primers (forward primer - 5'-CGTCGTCTATCTAAGATGGTATTATC-3' Reverse primer - 5'-TACAGCACCCAACGTTCCCAAG-3') designed from *Nosema bombycis* SSU-rRNA gene sequences (D85503 and D85504) having product size between 1115 and 1141 bp (26 mer) (Huang et al., 2008). PCR amplification was carried out in 20 μ l, using 10 ng DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 2 mM MgCl₂, and 1 U of Taq Polymerase (MBI Fermentas, USA). The amplification conditions were: 94°C denaturation for 3 min, followed by 35 cycles of 94°C for 60s, 55°C annealing for 2 min, and extension at 72°C for 30 s, with a final extension of 10 min on a thermal cycler (MJ Reaserch). The primers generated expected fragment size of a range between 510 and 515 bp [117 bp-16s SSU rRNA gene, 279 bp-IGS, and 115 bp-5s rRNA gene]. The amplified products were visualized on 1.2% agarose gel and purified by QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Cloning and sequencing of SSU-rRNA gene and IGS region

The purified DNA fragments were ligated into a pJET1.2 cloning vector in the presence of T4 DNA ligase (CloneJET PCR Cloning Kit, Thermo Scientific) at 22°C. The ligated products were transfected into JM101 competent cells and plated on ampicillin agar plates. White colonies were selected, and plasmids were isolated from the cells using the GeneJET plasmid miniprep kit (Fermentas Life Sciences). After isolating plasmid DNA from transformed *Escherichia coli* cells, the plasmids were digested with restriction enzyme (Bgl II) to check whether they contained the desired insert. Three clones from each of the microsporidian

isolates were sequenced using DNA sequencing kit (BDT version 3.1) on a semi-automatic DNA sequencer (ABI Prism 310, Applied Biosystems, Perkin Elmer) with M13 universal primers at Eurofins Genomics India Pvt. Ltd., Bangalore, India. The sequences were cleaned of any vector contamination using a vector screen program, (NCBI, Bethesda, Maryland, USA). The final sequences were deposited in NCBI GeneBank and the details are given in Table 2.

Phylogenetic analysis of SSU-rRNA and IGS-Sequences

The analysis of SSU-rRNA and IGS sequence homology was carried out using BLAST search from NCBI database. In contrast, 16 non-redundant microsporidian sequences from *Nosema/Vairimorpha* species including an out-group *Glugoides intestinalis* for IGS and *Encephalatazoon hellem* for SSU-rRNA were retrieved from the NCBI database. These sequences were aligned with IGS and SSU-rRNA gene sequences of test microsporidian isolates including an Indian *Nosema* reference strain in CLUSTAL W program (Higgins et al., 1994) (Table 3). The molecular phylogenetic trees were constructed from aligned sequences using maximum likelihood using the branch and bound option (with 500 bootstrap replicates) of the MEGA program (Version 6.0) (Tamura et al., 2013). The cloned and sequenced IGS and SSU-rRNA gene sequences of the test isolates and 16 *Nosema* reference strains were checked for sequence similarity using the Sequence Identity Matrix in BioEdit software (Hall, 1999) (Tables 4 and 5).

RESULTS

Morphological characterization

The spore sizes of both the isolates ranged from 4.50 to 5.10 μ m in length and 2.56 to 3.20 μ m in width. Details are given in Table 1.

Molecular characterization

PCR amplification, cloning and sequencing of IGS fragment and SSU-rRNA gene

The PCR amplification for both the microsporidian isolates using targeted IGS primer sets was successfully carried out. The amplified fragment consisted of a partial region of SSU-rRNA gene followed by complete sequences of IGS and 5S rRNA gene with an expected fragment size of approximately 500 bp. Substantial full-length sequences of the IGS cloned gene was obtained

Table 3. Taxon, source, host and Gene Bank Acc. No. of microsporidian species used in the phylogenetic analysis.

Accession number	Organism name	Host	Order	Family
KP151550	MIJ-2pD	<i>Antheraea mylitta</i>	Lepidoptera	Saturniidae
KP151552	MIJ-4cD	<i>Antheraea mylitta</i>	Lepidoptera	Saturniidae
KP177890	NIK-1s_mys	<i>Bombyx mori</i>	Lepidoptera	Bombycidae
FJ767862	<i>Nosema philosamia ricini</i>	<i>Philosamia Cynthia</i>	Lepidoptera	Saturniidae
DQ073396	<i>Nosema antheraeae</i>	<i>Antheraea pernyi</i>	Lepidoptera	Saturniidae
HQ457431	<i>Nosema disstriae</i>	<i>Malacosma disstriae</i>	Lepidoptera	Lasiocampidae
AY960987	<i>Nosema plutellae</i>	<i>Plutella xylostella</i>	Lepidoptera	Plutellidae
HQ457432	<i>Nosema fumiferanae femiferane</i>	<i>Choristoneura</i>	Lepidoptera	Tortricidae
JF443684	<i>Nosema bombycis</i>	<i>Bombyx mori</i>	Lepidoptera	Bombycidae
HQ891818	<i>Vairimorpha</i> sp.	<i>Bombyx mori</i>	Lepidoptera	Bombycidae
AF394525	<i>Glugoides intestinalis</i>	<i>Daphnia magna</i>	Cladocera	Daphniidae
EU338534	Uncultured <i>Nosema arsakia</i>	<i>Eurema blanda</i>	Lepidoptera	Pieridae
FJ969508	<i>Nosema</i> sp. <i>PA armata</i> Baly	<i>Phyllobrotica</i>	Coleoptera	Chrysomelidae
FJ772435	<i>Nosema heliothidis armigera</i>	<i>Helicoverpa</i>	Lepidoptera	Noctuidae
AY747307	<i>Nosema spodopterae</i>	<i>Spodoptera litura</i>	Lepidoptera	Noctuidae
JN882299	<i>Nosema</i> sp. <i>HA atrilinata</i>	<i>Hemerophila</i>	Lepidoptera	Geometridae
JN792450	<i>Endoreticulatus</i> sp.	<i>Bombyx mori</i>	Lepidoptera	Bombycidae
DQ445481	<i>Nosema bombycis</i>	<i>Bombyx mori</i>	Lepidoptera	Bombycidae
L19070	Encephalitozoon hellem	<i>Homo sapience</i>	--	Encephalitozoonidea

for both the isolates. Similarly, SSU-r RNA gene for both microsporidian isolates amplified with an expected fragment size of 1232 bp. Appreciable full-length sequences of the SSU-rRNA cloned gene were obtained and the sequences were successfully submitted to the NCBI-Gen Bank. The individual accession details for the submitted IGS and SSU rRNA gene sequences are given in Table 2.

Length and sequence variation in IGS and SSU-rRNA gene sequences

The IGS and SSU-rRNA gene sequences of 16 different microsporidians species with similar homology downloaded from NCBI were utilized for analysis. The sequence similarity of IGS sequence between test microsporidian isolates was observed to be 86%, while, SSU-rRNA gene sequence similarity was 99%. The average sequence similarity between test isolates and all reference *Nosema* sp. for IGS was 75%, whereas, 97% similarity was found in case of SSU-rRNA gene. Highest (90%) IGS sequence similarity was observed between the test isolates [MIJ-2pD and MIJ-4cD], and a reference strain *Nosema antheraeae*, while, both test isolates had least (34%) similarity with *Vairimorpha* reference species (Table 4). On the other hand, SSU-rRNA gene sequence similarity between test and reference microsporidians strains were very high with both test isolates showing

about 99% similarity with almost all reference *Nosema* strains except NIK-1s_mys. Accordingly, it was clearly observed that test isolates had substantial low similarity for IGS sequences compared to SSU-rRNA gene sequences (Tables 4 to 5). As expected, a very low level of similarity was observed between both IGS and SSU-rRNA gene sequences of test isolates and out-group species: *Glugoides intestinalis* for IGS and *Encephalatozoon hellem* for SSU-rRNA (Tables 4 to 5).

Phylogenetic analysis based on IGS and SSU-rRNA gene sequences

Two dendrograms were constructed based on IGS or SSU-rRNA gene sequences. In both cases, test isolates separated from reference microsporidia species with above 50% bootstrap value (Figures 1 and 2). The analysis of both phylogenetic trees manifested that the test isolates and 16 *Nosema* reference strains including an out-group separated into two major clades. In the IGS phylogenetic tree, the test microsporidian isolates and three *Nosema* reference species [*N. philosamia* (FJ767862.1), *N. antheraeae* (DQ073396.1) and *N. disstriae* (HQ457431)] grouped in a single clade, while in SSU-rRNA gene phylogenetic tree one additional uncultured *Nosema* sp. (EU338534) joined the test isolate group. The other eleven *Nosema* reference species including Indian *Nosema* sp. (NIK-1s_mys)

Table 4. IGS sequences similarity matrix of 18 different microsporidians strain including two experimental isolates.

Sequence	MIJ-2pD (KP153550)	MIJ-4cD (KP153552)	NIK_1s_mys (KP177890)	Nosema sp. (FJ767862)	Nosema antheraeae (DQ073396)	Nosema disstriae (HQ457431)	Nosema plutellae (AY960987)	Nosema fumiferanae (HQ457432)	Nosema bombycis (JF443684)	Vairimorpha sp. (HQ891818)	Glugoides intestinalis (AF394525)	Uncultured Nosema (EU338534)	Nosema sp.PA (FJ969508)	Nosema heliothidis (FJ772435)	Nosema spodopterae (AY747307)	Nosema sp.HA (JN882299)	Endoreticulatus sp. (JN792450)	Nosema bombycis (DQ445481)
<i>MIJ-2pD (KP153550)</i>	ID	0.86	0.64	0.86	0.88	0.81	0.71	0.68	0.66	0.34	0.34	0.65	0.64	0.65	0.62	0.66	0.65	0.65
<i>MIJ-4cD (KP153552)</i>	0.86	ID	0.69	0.90	0.90	0.85	0.75	0.71	0.69	0.37	0.36	0.69	0.69	0.67	0.66	0.68	0.69	0.69
<i>NIK_1s_mys (KP177890)</i>	0.64	0.69	ID	0.69	0.67	0.68	0.69	0.66	0.81	0.38	0.36	0.80	0.78	0.76	0.76	0.78	0.73	0.73
<i>Nosema sp. (FJ767862)</i>	0.86	0.90	0.69	ID	0.90	0.87	0.74	0.70	0.69	0.37	0.37	0.70	0.70	0.66	0.66	0.69	0.69	0.69
<i>Nosema antheraeae (DQ073396)</i>	0.88	0.90	0.67	0.90	ID	0.84	0.73	0.69	0.68	0.38	0.34	0.69	0.67	0.65	0.64	0.68	0.68	0.68
<i>Nosema disstriae (HQ457431)</i>	0.81	0.85	0.68	0.87	0.84	ID	0.73	0.67	0.69	0.39	0.36	0.70	0.69	0.66	0.64	0.69	0.69	0.69
<i>Nosema plutellae (AY960987)</i>	0.71	0.75	0.69	0.74	0.73	0.73	ID	0.87	0.71	0.40	0.35	0.71	0.68	0.67	0.67	0.68	0.66	0.66
<i>Nosema fumiferanae (HQ457432)</i>	0.68	0.71	0.66	0.70	0.69	0.67	0.87	ID	0.67	0.39	0.34	0.67	0.67	0.65	0.65	0.68	0.65	0.65
<i>Nosema bombycis (JF443684)</i>	0.66	0.69	0.81	0.69	0.68	0.69	0.71	0.67	ID	0.39	0.38	0.86	0.78	0.84	0.82	0.84	0.74	0.74
<i>Vairimorpha sp. (HQ891818)</i>	0.34	0.37	0.38	0.37	0.38	0.39	0.40	0.39	0.39	ID	0.31	0.38	0.39	0.39	0.37	0.39	0.37	0.37
<i>Glugoides intestinalis (AF394525)</i>	0.34	0.36	0.36	0.37	0.34	0.36	0.35	0.34	0.38	0.31	ID	0.35	0.35	0.36	0.35	0.40	0.34	0.34
<i>Uncultured Nosema (EU338534)</i>	0.65	0.69	0.80	0.70	0.69	0.70	0.71	0.67	0.86	0.38	0.35	ID	0.78	0.79	0.84	0.82	0.75	0.75
<i>Nosema sp.PA (FJ969508)</i>	0.64	0.69	0.78	0.70	0.67	0.69	0.68	0.67	0.78	0.39	0.35	0.78	ID	0.75	0.76	0.79	0.73	0.73
<i>Nosema heliothidis (FJ772435)</i>	0.65	0.67	0.76	0.66	0.65	0.66	0.67	0.65	0.84	0.39	0.36	0.79	0.75	ID	0.80	0.84	0.74	0.74
<i>Nosema spodopterae (AY747307)</i>	0.62	0.66	0.76	0.66	0.64	0.64	0.67	0.65	0.82	0.37	0.35	0.84	0.76	0.80	ID	0.83	0.77	0.77
<i>Nosema sp.HA (JN882299)</i>	0.66	0.68	0.78	0.69	0.68	0.69	0.68	0.68	0.84	0.39	0.40	0.82	0.79	0.84	0.83	ID	0.75	0.75
<i>Endoreticulatus sp. (JN792450)</i>	0.65	0.69	0.73	0.69	0.68	0.69	0.66	0.65	0.74	0.37	0.34	0.75	0.73	0.74	0.77	0.75	ID	1.00
<i>Nosema bombycis (DQ445481)</i>	0.65	0.69	0.73	0.69	0.68	0.69	0.66	0.65	0.74	0.37	0.34	0.75	0.73	0.74	0.77	0.75	1.00	ID

clustered separately with test isolates in three small sub groups in both cases (Figures 1 and 2). In addition, the reference *Vairimorpha* species separated alone in the same clade with all reference *Nosema* sp. and test isolates in both generated dendrograms. As expected, *G. intestinalis* (AF394525) and *E. hellem* (L19070) used as an out-group in IGS and SSU-rRNA based dendrograms, respectively got separated

from both major clades in the constructed trees (Figures 1 and 2).

DISCUSSION

The present study aimed at characterization of two strains of microsporidia isolated from the tasar silkworm *A. mylitta* based on morphology and

phylogenetic analysis of variations in IGS and SSU-rRNA gene sequences.

Morphological characterization

Morphological studies did not reveal any major variations in spore width and length of the isolates. Since spore size for a given species may vary

Table 5. SSU-rRNA gene sequences similarity matrix of 18 different microsporidians strain including two experimental isolates.

Sequence	MIJ-2pD (KU187949)	<i>Nosema heliothidis</i> (FJ772435)	<i>Nosema</i> sp.SC (FJ767862)	<i>Nosema spodopterae</i> (AY747307)	<i>Nosema bombycis</i> (JF443577)	MIJ-4cD (KU187950)	<i>Nosema</i> sp.PA (FJ969508)	<i>Nosema</i> antheraeae(DQ073396)	<i>Nosema</i> sp.HA(JN882299)	<i>Nosema</i> disstriae(HQ457431)	<i>Nosema</i> bombycis(DQ445481)	<i>Nosema</i> fumiferanae(HQ457432)	<i>Nosema</i> plutellae (AY960987)	<i>Endoreticulatus</i> sp.(AF240355)	Uncultured <i>Nosema</i> (EU338534)	<i>Vairimorpha</i> sp. Sd (HQ891818)	<i>Nosema</i> sp. NIK-1s (AY713309)	<i>Encephalitozoon hellem</i> (L19070)
<i>MIJ-2pD</i> (KU187949)	ID	ID	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.97	0.98	0.82	0.47	0.42
<i>Nosema heliothidis</i> (FJ772435)	0.99	ID	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.97	0.98	0.82	0.47	0.42
<i>Nosema</i> sp.SC (FJ767862)	0.99	0.99	ID	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.97	0.98	0.82	0.47	0.42
<i>Nosema spodopterae</i> (AY747307)	0.99	0.99	0.99	ID	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.97	0.98	0.82	0.47	0.42
<i>Nosema bombycis</i> (JF443577)	0.99	0.99	0.99	0.99	ID	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.97	0.98	0.82	0.47	0.42
<i>MIJ-4cD</i> (KU187950)	0.99	0.99	0.99	0.99	0.99	ID	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.96	0.98	0.82	0.47	0.42
<i>Nosema</i> sp.PA (FJ969508)	0.99	0.99	0.99	0.99	0.99	0.99	ID	0.99	0.99	0.99	0.99	0.99	0.98	0.97	0.98	0.82	0.47	0.42
<i>Nosema antheraeae</i> (DQ073396)	0.99	0.99	0.99	0.99	0.99	0.99	0.99	ID	0.99	0.99	0.99	0.99	0.98	0.96	0.98	0.82	0.46	0.42
<i>Nosema</i> sp.HA(JN882299)	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	ID	0.99	0.99	0.99	0.98	0.96	0.98	0.82	0.47	0.42
<i>Nosema disstriae</i> (HQ457431)	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	ID	0.99	0.99	0.98	0.96	0.98	0.82	0.47	0.42
<i>Nosema bombycis</i> (DQ445481)	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	ID	0.99	0.98	0.97	0.97	0.82	0.47	0.42
<i>Nosema fumiferanae</i> (HQ457432)	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	ID	0.98	0.97	0.97	0.82	0.47	0.42
<i>Nosema plutellae</i> (AY960987)	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	ID	0.96	0.97	0.81	0.47	0.42
<i>Endoreticulatus</i> sp.(AF240355)	0.97	0.97	0.97	0.97	0.97	0.96	0.97	0.96	0.96	0.96	0.97	0.97	0.96	ID	0.95	0.80	0.48	0.40
Uncultured <i>Nosema</i> (EU338534)	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.97	0.97	0.97	0.95	ID	0.81	0.46	0.42
<i>Vairimorpha</i> sp. sd(HQ891818)	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.81	0.80	0.81	ID	0.39	0.43
<i>Nosema</i> sp. NIK-1s (AY713309)	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.46	0.47	0.47	0.47	0.47	0.47	0.48	0.46	0.39	ID	0.53
<i>Encephalitozoon hellem</i> (L19070)	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.40	0.42	0.43	0.53	ID

with respect to the host, the morphological characteristics are not considered for delineating identity of microsporidian isolates (Brooks et al., 1972).

Molecular characterization

Genotyping studies based on the ribosomal RNA

small subunit gene (SSU-rRNA) sequence analysis are a promising tool for organism identification (Zhu et al., 2010; Ku et al., 2007). The ribosomal DNA comprises of highly conserved SSU gene sequences that occur in multiple copies within the eukaryotic genome and has been widely exploited since many years, for deriving phylogeny inference (Liu et al., 2012). The phylogenetic relationships of several micro-

sporidian genera including *Vairimorpha* and *Nosema* were studied based on their SSU-rDNA sequence similarity (Baker et al., 1994; Dong et al., 2010). Three different microsporidia species showed 100% identity of SSU r-RNA gene sequences while their ITS, IGS and 5S sequences varied (Dong et al., 2010). Molecular variations have been established among *Trichosporon* isolates from close geographical locations based

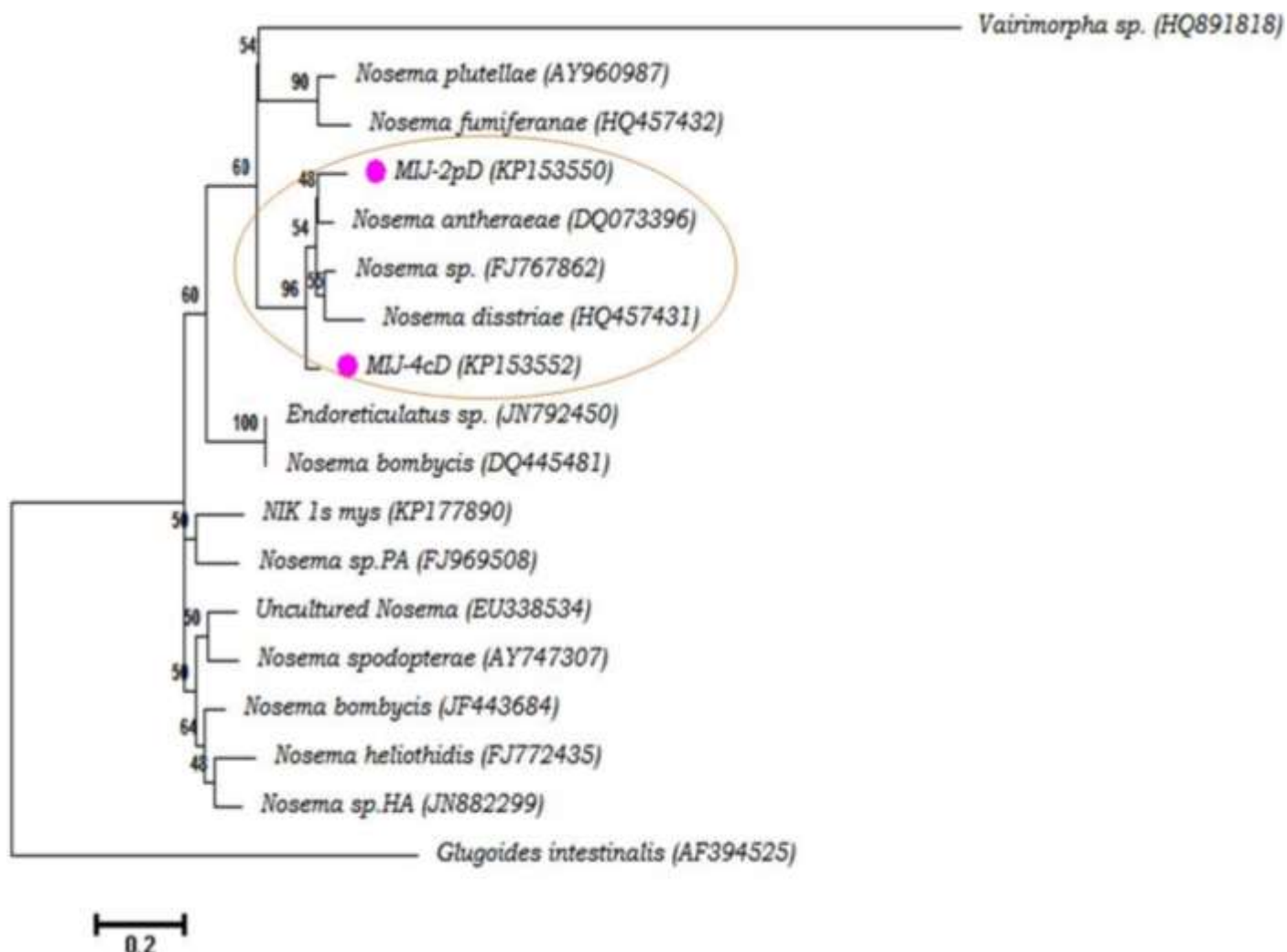


Figure 1. Phylogenetic tree based on IGS sequences. Eighteen different microsporidians IGS sequences were analyzed based on maximum likelihood approach using MEGA 6 (Tamura, 2013) run with 500 bootstrap replication. Number of each node indicates bootstrap value.

based on restriction patterns of IGS (Sugita et al., 2002). Various studies have indicated that, molecular phylogenetic analysis based on IGS region might be a better tool to investigate intra-specific divergence and would provide significant molecular evidence for classification and evolutionary studies of microsporidia (Dong et al., 2010; Sagastume et al., 2010; Liu et al., 2013). A recent report indicated differentiating of *N. ceranae* strains from different geographic origins in Europe based on sequence analysis of highly variable regions of IGS sequence and a part of r-RNA that corresponded to IGS region and their virulence (Dussaubat et al., 2012).

Based on these reports we targeted the IGS as well as SSU-rRNA sequences for phylogenetic analysis of key microsporidia isolates and also made a comparative

study of the said gene sequences with 16 different *Nosema/Vairimorpha* strains to derive a phylogenetic inference for both microsporidian strains isolated from *A. myllita*. The IGS and SSU-rRNA gene sequences of the isolates indicated that they belong to the genus *Nosema*. Comparative sequence similarity analysis of IGS and SSU-rRNA sequence for the test isolates and reference strains revealed that SSU-rRNA gene sequence is highly conserved. Sequence similarity analysis revealed that, the IGS sequence (280 bp) showed 70% similarity between test and reference microsporidian isolates while SSU-rRNA (1240 bp) showed 99% similarity. The dendrograms of both test isolates based on IGS and SSU-rRNA gene sequences revealed close genetic relationship with three *Nosema* species, that is, *Nosema philosamia*, and *Nosema antheraeae* from China and

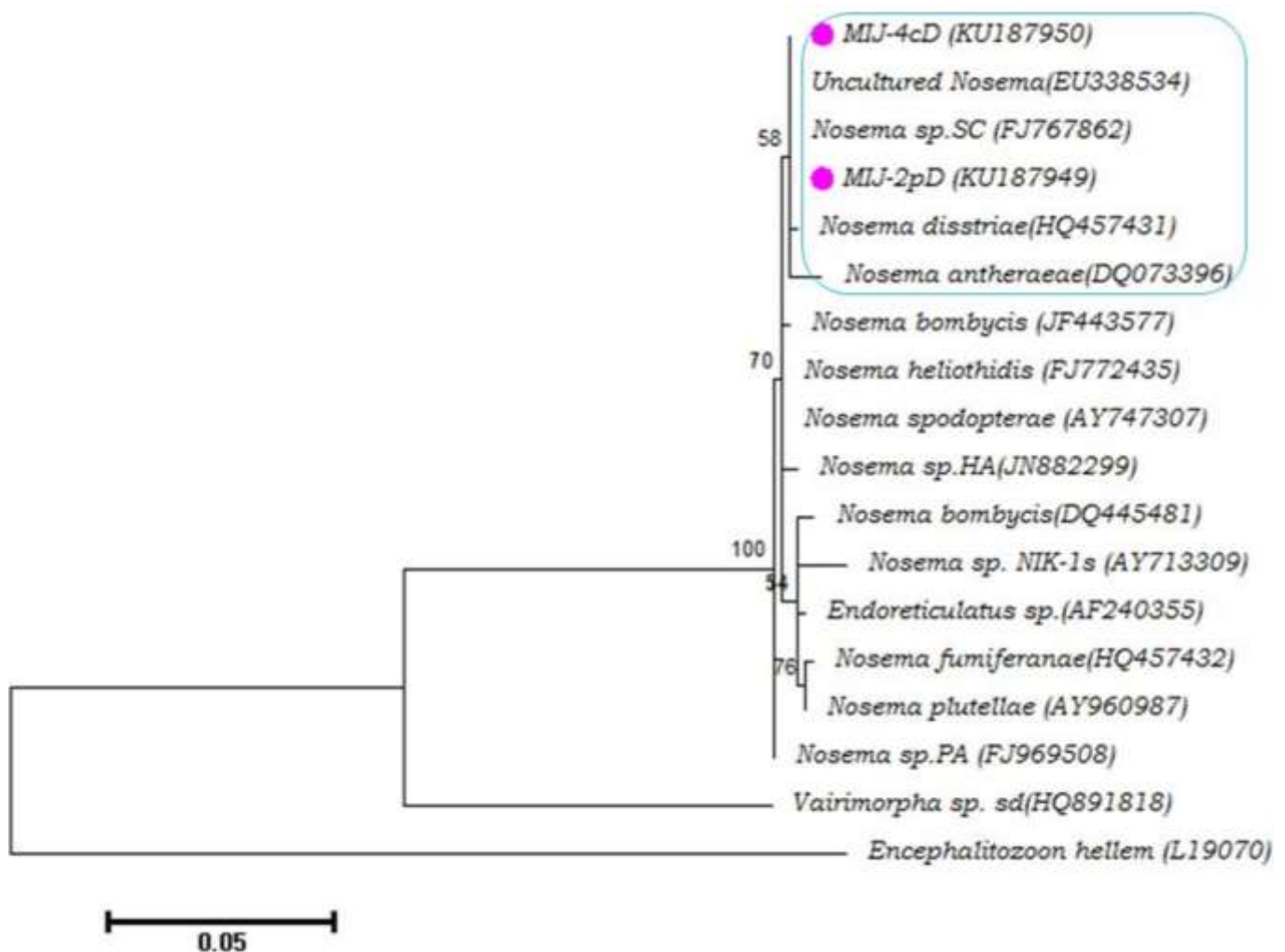


Figure 2. Phylogenetic tree based on SSU-rRNA gene sequences. Eighteen different microsporidians IGS sequences were analyzed based on maximum likelihood approach using MEGA 6 (Tamura, 2013) run with 500 bootstrap replication. Number of each node indicates bootstrap value.

Nosema disstriae from Canada. Since, the test isolates were extracted from *Antheraea mylitta* the genetic similarity of test isolates with *N. philosamia*, and *N. antheraeae* support host specification of the microsporidian strains. The trees generated from the IGS and SSU-rRNA gene sequences of the test microsporidia isolates revealed a closed as well as complex phylogenetic relationship of the test isolates with each other due to evolutionary process. Thus, the sequence based phylogenetic analysis of IGS and SSU-rRNA genes provided additional molecular evidence for the classification and evolutionary study of microsporidian isolates on species as well as genus level.

In recent times, the ITS/IGS genes of ribosome were utilized for identification of parasite species and strains as well as a tool for molecular diagnosis. The SSU rRNA

gene sequence variation used for establishing phylogenetic relationship between different species in the same genus can also be used for the classification of microsporidia at the species level. Consequently, sequence and phylogenetic analysis based on the combination of SSU rRNA gene and IGS of ribosome might provide additional molecular evidence for the classification and study of evolution of microsporidia. Thus, the gives strong evidence of genetic recombination in *N. antheraeae* or *N. philosamia* and a molecular method for defining species in closely related *Nosema* species. Further, it also revealed, the SSU-rRNA and IGS sequence variability among the microsporidian isolates that would be a rich source of information and could serve to differentiate these isolates in order to give us insight into their origins as well as the spread of this

economically important disease of the tasar silkworm in India.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and characterization of methanogenic bacteria from brewery wastewater in Kenya

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The production of biogas from renewable resources is becoming a prominent feature of most developed and developing countries of the world. A study was undertaken to characterize methanogenic microbial community found in brewery waste water. Their performance with regards to methane production was also studied. Thirty-two isolates were obtained using brewer thyglycollate agar medium. Characterization of the isolates was done by culture and biochemical methods. 65% of the isolates were found to be positive with Gram staining reaction, while 35% were negative. The isolates were identified by method of polymerase chain reaction (PCR). From the phylogenetic analysis, thirteen isolates were clustered into genus *Bacillus* sp., isolate 9^{3b} was closely related to *Bacillus subtilis* strain, while isolates 20^{a1}, 17¹ and 7 closely related to *Bacillus methylotrophicus* isolate 10 was grouped together with *Bacillus tequilensis*, isolate 31 was clustered together with *Bacillus licheniformis*, while isolates 13², 25², 15, 26² and 18² were closely related to *Lysinibacillus* sp. and isolate 19¹ was clustered together with *Lactobacillus casei*. The study also shows that three isolates 3², 18¹ and 4 were closely related to *Ralstonia pickettii*, *Providencia rettgeri* and *Myroides odoratimimus*, respectively. The presence of isolates 20^{1a}, 17¹ and 7 with abilities to ferment different sugars, hydrolysis starch, liquefy gelatin, split amino acid tryptophan, produce catalase enzyme and hydrogen sulphide gas suggests their involvement in biogas production. The percentage methane content in the total gas produced at pH 8 varied significantly ($p < 0.001$) for all the temperature ranges. The highest concentration of methane for most isolates was recorded at temperatures of 35 and 37°C for all the pH ranges.

Key words: Biogas, characterization, methanogenic bacteria, pH, temperature, wastewater.

INTRODUCTION

Readily available energy for domestic, agricultural and industrial applications defines the utmost attractive features of a developing community (Rabah et al., 2010). Energy is the source of economic growth and thus its

consumption reflects the state of development of a nation. The growing interest in the search for cleaner source of energy globally, has been heightened by the allied harmful environmental, health and social effects of

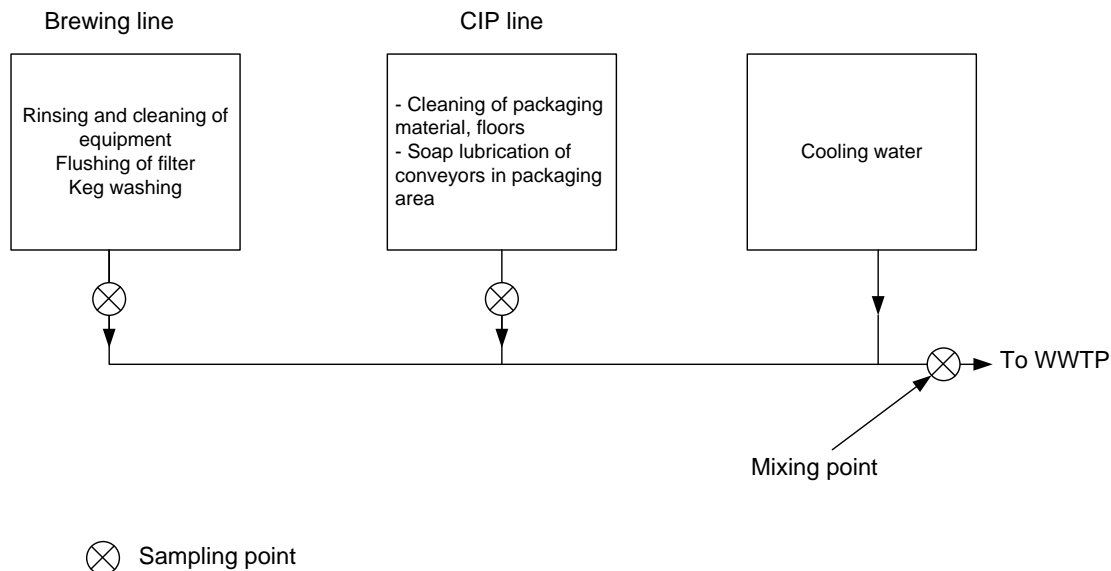


Figure 1. Sampling points.

dependence on fossil fuel (Sárvári Horváth et al., 2016; Sayibu and Ofoso, 2015). Biogas is a promising alternate energy source as the technology of its production may combine the treatment of various organic wastes with the generation of an energy carrier, methane, for the versatile applications with direct reduction in the production costs for processing industries. Most countries in the World have focused interest in the production of biogas from renewable resources. Biogas is produced when bacteria degrade biological materials in the absence of oxygen, in a process known as anaerobic digestion (Weiland, 2010; Horváth et al., 2016). The great varieties of diverse microbes that participate in the microbial food chain gradually degrade the complex molecules essentially to a mixture of CH_4 and CO_2 (Bayer et al., 2004). The environmental and internal factors usually control the actions of the various microbes, involving members of the Eubacteria and Archaea. In addition, the composition of the microbial consortium is determined by numerous factors, including substrate ingredients, temperature, pH, mixing or the biodigester geometry (Cirne et al., 2012). A lot has been done on the general biogas production technology, albeit, the microbial communities involved have not been fully documented (Kröber et al., 2009), indicating that various microorganisms in the analysed fermentation samples of the biogas plants are still unclassified or unknown. This study focused on the isolation and characterization of methanogenic bacteria from brewery wastewater and evaluation of the effect of

temperature and pH on the quantity of methane produced.

MATERIALS AND METHODS

Collection of waste water samples

Samples of brewery waste water from Keroche industries were used as an inoculum. The samples were collected in glass sampling bottles that were pre-treated by washing with 70% ethanol and later rinsed with distilled water and dried overnight in an oven at 105°C , for disinfection and drying of the sampling bottles (APHA, 2005; World Health Organization, 2008). They were stored in a refrigerator at 4°C without further treatment. The sampling points included the Brewing line; Clean in Place (C.I.P) and the Mixing point, as illustrated in Figure 1.

Isolation of waste water bacteria

Brewer thyglycollate media from Oxoid was used for cultivation of the anaerobic bacteria. It consisted of 1.0 g lab-Lemco¹ powder, 2.0 g yeast extract, 5.0 g peptone, 5.0 g glucose, 5.0 g sodium chloride, 1.1 g sodium thioglycollate, 0.002 g methylene blue and 1.0 agar at $\text{pH } 7.2 \pm 0.2$ per litre. One millilitre of each sample was inoculated at the base of each sterilized test tube containing the medium using a sterile syringe and incubated at 37°C in anaerobic jar. Observations for growth were made after every 12 h. Serial dilutions of 12 h old bacteria culture in the ratios of 10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} were transferred to Petri-dishes containing brewer thyglycollate media with modification and spread over the surface with a sterile glass spreading rod. Each dilution series was

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used to inoculate a series of plates with three plates at each dilution level and incubated in an anaerobic jar. Anaerobic jar was evacuated by placing a kindled candle, which quenches immediately the left over oxygen. The jar was incubated for a period of 72 h at 37°C. The colonies that emerged on the plates were sub-cultured repeatedly on fresh plates to obtain pure isolates.

Characterization of the isolates

Morphological and cultural characteristics of pure colonies were used to perform preliminary characterization (Holt et al., 1994). The cell shape and arrangement characteristics were observed under the compound microscope after standard staining of the isolates. Three percent (w/v) KOH test (Gregersen, 1978) was used to determine gram characteristics of isolates. Among the biochemical tests conducted were triple sugar iron, gelatine liquefaction, motility, starch hydrolysis, H₂S production, catalase test and indole production test. Molecular characterization was used to confirm the identity of the isolates. Total bacterial DNA was extracted according to the procedures described by Marmur (1961). Bacterial 16S rRNA genes of the pure isolates were amplified (Plate 2) and used as a template for amplification of 16S rRNA gene. PCR amplification was performed using PeQlab advanced Primus 96 Hamburg thermal cycler (Applied Biosystems), using universal primers pair combination of forward primer 8F forward 5'-AG (A/G) GTTTGATCCTGGCT-3' and 1492R reverse, 5'-CGGCTACCTTGTTACGACTT-3' according to the position in relation to *Escherichia coli* gene sequence (Lane, 1991).

DNA was amplified in a 50 µl mixture containing 0.30 µl of gene script Taq, 2.5 µl (10 pmol/ µl) of 8F forward primer, 2.5 µl (10 pmol/µl) of 1492R reverse primer, 10 µl of template DNA (10 ng/µl), and 6.0 µl of dNTP's mix (1.25 mM), 5.0 µl PCR 10 x buffer with mgcl₂ and 23.7 µl of PCR water. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 32 cycles: Initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, primer annealing at 49°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min (Roux, 1995). Agarose gel 1%, stained with ethidium bromide was used to confirm amplified PCR products. Successfully amplified PCR products were purified by QIAquick purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions (Sambrook et al., 1982). Sequencing was performed by Macrogen through ABI prism big dye terminator. The 16S rRNA gene sequences were viewed and edited by Chromas pro software (www.technelysium.com.au). Aligning of the sequences was achieved using CLASTAL W 1.6 software, and was compared to the public databases through BLAST search program on the National Center for Biotechnology Information (NCBI) Website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic relationship (Figure 2) was performed by the Maximum likelihood method using Mega 5 software (Tamura and Nei, 1993; Tamura et al., 2007).

Identification of methanogenic bacteria

Colonies of methanogenic bacteria were identified on Petri plates using Fluorescent test in which a blue-green fluorescence, characteristic to this metabolic group of bacteria (Dhadse et al., 2012) was observed and was distinct from the white-yellow fluorescence normally observed with non-methanogenic bacteria. To confirm this group, the isolates were sub-cultured in brewer thyglycollate broth media and incubated anaerobically in batch digesters for 7 days in an mrc laboratory equipment water bath Bo-200. Effects of temperatures and pH on the methane quantity were also studied under temperatures of 30, 35, 37 and 40°C and pH variations of 6, 7.2 and 8. The experiments were carried out three times. The gas produced was analysed using Biogas 5000 analyser, with CH₄ and CO₂ accuracy of ±0.5% of measurement reading after

calibration. The cumulative percentage of CH₄ produced was based on optical density (OD). The OD 600 nm for all the isolates were scaled down to OD of 1.0 for comparison. The OD values were determined using Eppend of Bio photometer AG 22331 Hamburg. Each experiment started after a preliminary operation of 5 min in order to minimize the effects of environmental changes and gas phase differences. The cumulative volume of the gas produced during the incubation period was estimated using ideal gas law (Equation 1). The Initial pressure was indicated on the KIF LAB Labotporp vacuum pump made in (France) during air evacuation and the final pressure was as indicated by barometer on the biogas analyzer 5000.

$$V_{gas} = \frac{(P_2 - P_1)V_r T_a}{P_a T_r} \quad (1)$$

Where, V_{gas} is cumulated volume of gas produced (mL); P_1 is the initial pressure in the digester as indicated by the vacuum pump (kPa); P_2 is the final pressure after incubation period (kPa); P_a is ambient pressure; T_a is ambient (Initial) temperature (K); T_r is temperature of the digester (K) and V_r is the capacity of the digester. Methane produced in mL was calculated according to Equation 2:

$$CH_{4(gas)} = \%CH_4 \times V_{gas} \quad (2)$$

Calibration of the equipment

Two calibration setups were performed once every week, the zero' and 'span'. Zero experiments. The zero experiment was the point at which the gas analyzer was calibrated when there was none of the methane gas present (in the open field). Span zero was at the point at which the gas analyser was calibrated when a known quantity of the methane gas was present (using cooking gas from Total Kenya). Zeroing of the gas analyser was undertaken at the start of each week's monitoring.

RESULTS

Most colonies were observed to grow within two to three days of incubation at 37°C. The colony morphology of the isolates obtained from brewery waste water ranged from circular, entire, flat to filamentous (Table 1). They were smooth or entire and the colour ranged from white to cream and bluish. 65% of the isolates were Gram positive, while 35% were Gram negative and they ranged from short to long rods (Plate 1). The biochemical characteristics of these isolates are given in Table 2. BLAST analysis of the partial sequences (Table 3) showed that 81.25% were from the genus *Bacillus* within the Firmicutes in the domain bacteria with similarities between 70 and 100%. Among these were *Bacillus subtilis*, *Bacillus licheniformis*, *Lactobacillus casei* and *Bacillus methylophilus*. Five isolates from the bacillus group belonged to genus *Lysinibacillus* sp. with percentage similarities between 95 and 97. Three isolates had 6.25% each and belonged to the genera *Ralstonia* (isolate 3²), *Providencia* (isolate 11) and *Myroides* (3²) with similarities of 77, 96 and 98%, respectively.

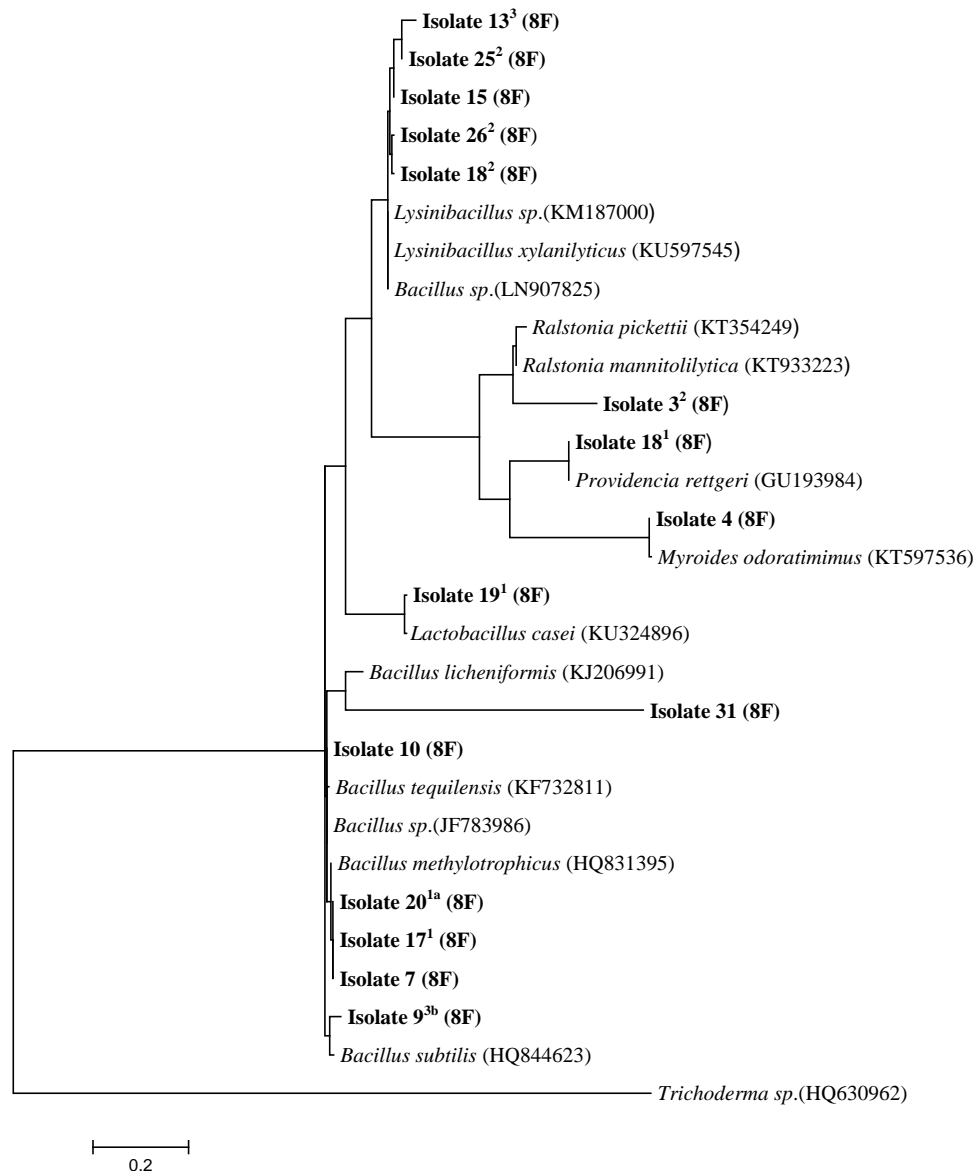


Figure 2. The evolutionary history inferred by using the Maximum Likelihood method based on the Tamura and Nei model (1993) and Tamura et al. (2007). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 nucleotide sequences. Evolutionary analyses were conducted in MEGA6. The gene sequence of *Trichoderma sp.* (HQ630962.1) was used as an out-group.

Thirteen isolates were clustered into genus *Bacillus* sp., in the phylogenetic analysis with isolate 9^{3b} being closely related to *B. subtilis* (HQ844623) strain while isolates 20^{1a}, 17¹ and 7 closely related to *B. methylotrophicus* (HQ831395), isolate 10 was grouped together with *Bacillus tequilensis*, isolate 31 was clustered together with *B. licheniformis* (KJ206991) while isolates 13², 25², 15, 26² and 18² were closely related to *Lysinibacillus sp.* (KM187000) and isolate 19¹ was clustered together with *L. casei* (KU324896). The study also showed that three isolates including 3², 18¹ and 4 closely related to *Ralstonia pickettii* (KT354249), *Providencia rettgeri*

(GU193984) and *Myroides odoratimimus* (KT597536), respectively. Isolates 4, 17¹, 18¹, 18², 19¹, 20^{1a}, 25², 26² and 31 were isolated from brewing line sample while isolates 3², 7, 9^{3b}, 10, 13 and 15¹, were from the mixing point (Figure 1).

Effect of temperature and pH on the quality of methane production

Table 4 shows the means of the quality of methane gas produced by different isolates with variations in

Table 1. Morphological characteristics of bacteria isolates obtained from brewery waste water.

Isolate	Colony characterization				Cell characterization	
	Color	Form	Elevation	Margin	Gram reaction	Arrangement
1	Cream	Oval	Flat	Entire	+	Rods
2	Cream	Oval	Slightly raised	Entire	+	Rods
3 ¹	Bluish/clear	Oval	Slightly raised	Entire	-	Rods
3 ²	Clear/Bluish	Oval	Slightly raised	Entire	-	Rods
4	White	Irregular	Flat	undulated	-	Rods
5	White	Filamentous	Flat	Filiform	-	Rods
6	Cream	Oval	Raised	Entire	+	Rods
7	Cream	Oval	Raised	Entire	+	Rods
8	White	Oval	Flat	Entire	+	Rods
9 ²	Clear/bluish	Oval	Raised	Entire	+	Rods
9 ^{3a}	Clear/Bluish	Oval	Raised	Entire	+	Rods
9 ^{3b}	Clear/Bluish	Oval	Raised	smooth	-	Rods
10	Bluish/clear	Oval	Slightly raised	Entire	+	Rods
11	Bluish/clear	Oval	Raised	Entire	-	Rods
12	Bluish/clear	Oval	Raised	Entire	-	Rods
13 ²	White	Irregular	Flat	Undulated	+	Short rods
13 ³	White	Irregular	Flat	Undulated	+	Rods
14	White	Irregular	Flat	Undulated	-	Rods
15 ¹	Cream	Oval	Raised	Entire	+	Short rods
16	White	Irregular	Flat	Lobate	+	Rods
17 ¹	Clear/Bluish	Oval	Raised	Entire	+	Short rods
17 ²	Clear/bluish	Oval	Raised	Entire	+	Short rods
18 ¹	Cream	Oval	Raised	Entire	-	Rods
18 ²	Cream	Oval	Raised	Entire	+	Rods
19 ¹	Clear/bluish	Oval	Raised	Entire	+	Rods
20 ^{1a}	Cream	Oval	Raised	Entire	+	Rods
25 ²	Cream	Oval	Raised	Entire	-	Rods
26 ¹	Clear/bluish	Oval	Raised	Entire	-	Rods
26 ²	Clear/Bluish	Oval	Raised	Entire	+	Rods
27 ¹	Clear/bluish	Oval	Raised	Entire	+	Rods
28 ²	Clear/bluish	Oval	Raised	Entire	+	Rods
31	Clear/bluish	Irregular	Flat	Undulated	+	Rods

temperature and pH. Generally, the percentage methane content in the total gas produced at pH 8 varied significantly ($p < 0.001$) for all the temperature ranges with reduction in the total volume of gas produced with increase in temperature. Most isolates were observed to float in the digesters at temperature 40°C, with a corresponding least quality of methane and volume of the total gas produced (Figures 3 and 4). The highest concentration of methane for most isolates was recorded at temperatures of 35 and 37°C for all the pH ranges. The methane concentration for isolate 17¹ increased from temperature 30, 35 to 37°C followed by a drop of temperature 40°C. In addition, its best quality was observed at pH 8 for at least 75% of the temperatures studied. Isolate 18² had its best quality at temperatures

35 and 37°C, at pH 7. There was no significant difference ($p < 0.001$) in the quality of methane for isolates 26², 25² and 20^{1a} for pH 6 and 8 at temperatures 35 and 37°C (Table 4).

DISCUSSION

From the detailed BLAST analysis, the genus *Bacillus* were found to be the most prominent indicating a possibility of this group playing an important role in biogas production process as discussed by Horváth et al. (2016), Li et al. (2013) and Kröber et al. (2009). This is also comparable to the results obtained by Rabah using abattoir waste as the inoculum (Rabah et al., 2010) and

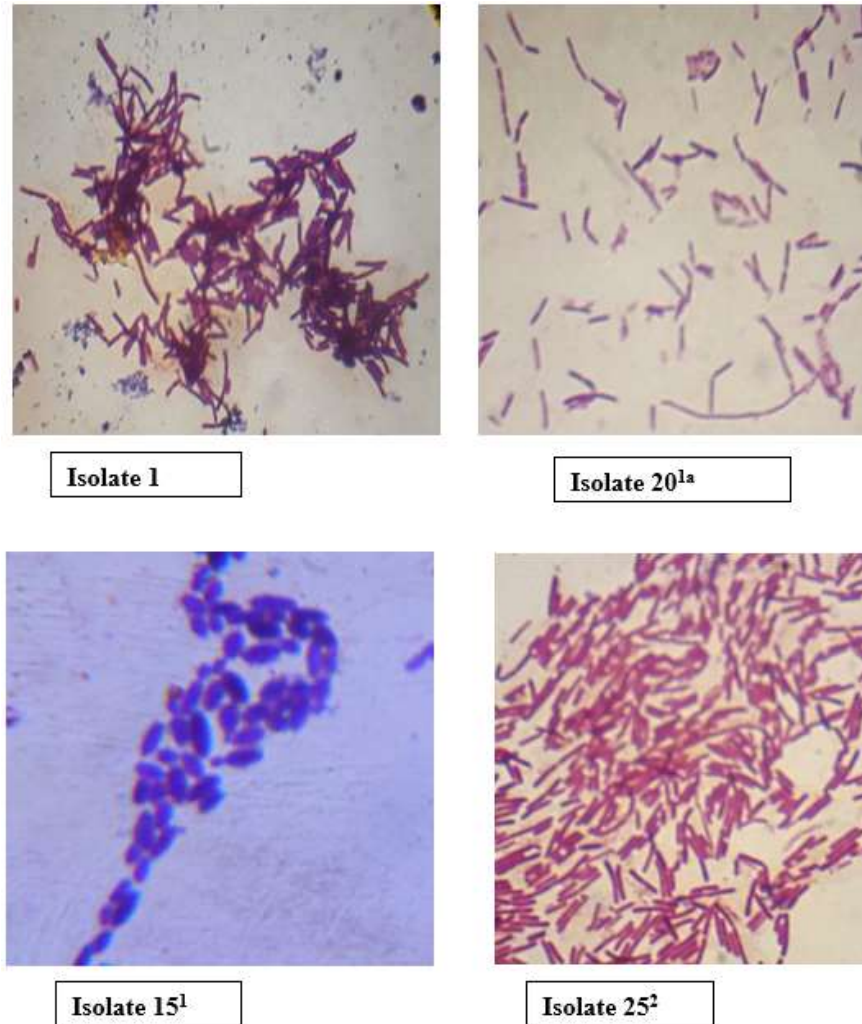


Plate 1. Gram reaction of the selected bacterial isolates. Gram positive rods (1), gram positive rods (20^{1a}), gram positive short rods (15¹), gram negative rods (25²).

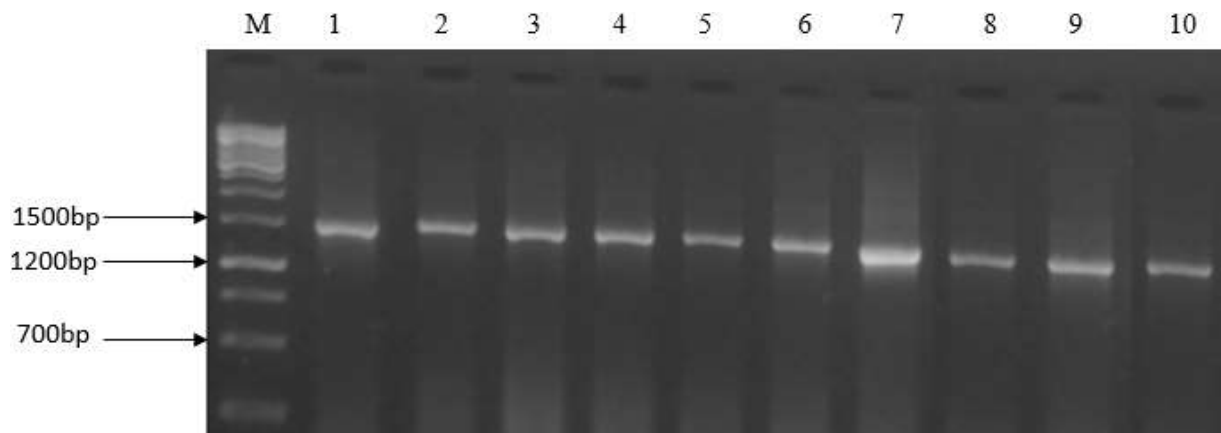


Plate 2. PCR amplified 16S rDNA products from representative isolates among the isolates brewery waste water using universal primers bac 8F and bac 4392R. Lanes 1 (9^{3b*}), 2 (13^{3*}), 3 (18^{1*}), 4 (25^{2*}), 5 (26^{2*}), 6 (15^{1*}), 7 (18^{2*}), 8 (3^{2*}), 9 (20^{1a*}), 10 (17^{1*}) and (M*) M-1500 bp, Molecular marker size. *The figures within the brackets are the isolate numbers.

Table 2. Biochemical characteristics of bacterial isolates obtained from brewery waste water.

Isolate	Starch	Catalase	Indole	Motility	Gelatin	Butt	Slant	Fluorescence	H ₂ S
1	+	+	-	+	-	++	++	+	+
2	+	-	-	+	+	++	++	++	+
3 ¹	+	-	-	+	+	++	++	++	+
3 ²	-	+	-	+	-	++	++	+	-
4	+	+	-	-	-	++	++	+	+
5	-	+	-	-	+	++	++	+	+
6	+	+	-	+	-	+++	++	++	+
7	+	+	-	+	+	++	+++	+	+
8	+	+	-	+	+	++	++	+	+
9 ²	+	+	-	+	+	++	++	+	+
9 ^{3a}	+	+	-	+	+	++	++	+	+
9 ^{3b}	+	+	+	+	+	++	++	+	+
10	+	+	+	+	+	+++	++	-	+
11	+	+	-	+	+	++	++	++	+
12	+	-	-	+	+	++	++	+	+
13 ²	+	-	-	-	+	++	+++	+	-
13 ³	+	-	-	+	-	+++	+++	++	+
14	-	+	-	+	-	++	+++	+	-
15 ¹	-	+	-	-	-	++	++	+	+
16	+	+	-	+	+	++	++	+	+
17 ¹	+	-	-	+	+	+++	+++	+++	+
17 ²	+	+	-	-	+	++	+++	++	+
18 ¹	+	+	-	+	+	++	+	+	-
18 ²	+	-	-	+	+	+	+++	++	+
19 ¹	+	-	-	-	+	+	+++	+	+
20 ^{1a}	+	+	-	+	+	++	++	++	+
25 ²	+	+	-	-	-	+++	+++	+	+
26 ¹	-	-	-	+	-	++	++	++	+
26 ²	-	-	-	+	+	++	++	+++	+
27 ¹	+	+	-	+	+	++	++	++	+
28 ²	+	-	-	+	+	++	+++	+	+
31	+	-	-	+	+	++	++	++	+

Table 3. BLAST analysis results of the isolates from brewery waste water nearest neighbours in the data bank and their percentage relatedness.

Isolate	Next neighbour	Accession number	Similarity (%)
9 ^{3b} -(bac 8F)	<i>Bacillus subtilis</i> strain AIMST 7.Os.2	HQ844623.1	94
	<i>Bacillus licheniformis</i> strain BNR143	KT074465.1	94
	<i>Bacillus tequilensis</i> strain HS10	KP743123.1	94
18 ¹ -(bac 8F)	<i>Bacillus thuringiensis</i> serovar indiana strain HD521	CP010106.1	100
	<i>Bacillus cereus</i> strain S2-8	JF838294.1	100
	<i>Bacillus anthracis</i> strain Ames_BA1004	CP009981.1	100
25 ² -(bac 8F)	<i>Bacillus</i> sp. MSB1-25E	KT030900.1	96
	<i>Lysinibacillus fusiformis</i> strain L13	KU179364.1	96
	<i>Lysinibacillus sphaericus</i> strain C2-37c-8	JX517244.1	96
	<i>Lysinibacillus xylanilyticus</i> strain 11W6RMR3-2	KT728728.1	96

Table 3. Contd.

26²-(bac 8F)	<i>Lysinibacillus boronitolerans</i> strain KnMuC3-2	KF032677.1	97
	<i>Lysinibacillus</i> sp. BFE17K1	<u>KM187000.1</u>	97
15-(bac 8F)	<i>Lysinibacillus</i> sp. DB14515	KP670240.1	97
	<i>Lysinibacillus xylanilyticus</i> strain RD_AZIDI_12	KU597545.1	97
18²-(bac 8F)	<i>Lysinibacillus xylanilyticus</i> strain MA	KT030900.1	95
	<i>Lysinibacillus fusiformis</i> strain L13	KU179364.1	95
20^{1a}-(bac 8F)	<i>Bacillus amyloliquefaciens</i> strain Y1	<u>KJ616752.1</u>	97
	<i>Bacillus methylotrophicus</i> strain Nk5-1	<u>HQ831395.1</u>	97
	<i>Bacillus subtilis</i> strain yxw4	<u>KF278950.1</u>	97
	<i>Bacillus methylotrophicus</i> strain NMTD14	<u>HQ844484.1</u>	97
17¹-(bac 8F)	<i>Lysinibacillus boronitolerans</i> strain KtTA1-2	<u>KF025654.1</u>	97
	<i>Lysinibacillus</i> sp. Je33-2	HF563553.1	97
10-(bac 8F)	<i>Bacillus subtilis</i> strain F111	HQ647257.1	98
	<i>Bacillus tequilensis</i> strain ADIP3	KF732811.2	98
	<i>Geobacillus</i> sp. CRR1-HN-1	JQ695928.1	98
3²-(bac 8F)	<i>Ralstonia mannitolilytica</i> strain 4903	KT933223.1	77
	<i>Ralstonia pickettii</i>	KT354249.1	77
	Uncultured bacterium clone Ap.ba-F-DM-HN-1-46	KT354249.1	77
11-(bac 8F)	<i>Providencia rettgeri</i> strain IITRP2	GU193984.1	96
	Uncultured <i>Providencia</i> sp. clone F2jun.39	GQ417423.1	96
	Uncultured bacterium clone PB16	GU166190.1	96
16-(bac 8F)	<i>Bacillus licheniformis</i> strain RTS	EF644417.1	95
	<i>Bacillus</i> sp. J26	JF783986.1	95
	<i>Bacillus tequilensis</i> strain EB-95	KU258071.1	95
	<i>Bacillus subtilis</i> strain 1201	EU982509.1	95
19¹-(bac 8F)	<i>Lactobacillus casei</i> strain L1	KM350161.1	95
	<i>Lactobacillus casei</i> strain MSJ1	KU324896.1	95
	<i>Lactobacillus casei</i> strain EM2	KM350160.1	95
3²-(bac 8F)	<i>Myroides odoratimimus</i> strain LZ1306-2-5	KT597536.1	98
	<i>Myroides odoratimimus</i> strain YRL08	EU373415.1	98
7-(bac 8F)	<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> strain L09	JN700139.1	98
	<i>Bacillus methylotrophicus</i> strain CR1	KP851947.1	98
	<i>Bacillus subtilis</i> strain EPP2 2	JQ308548.1	98
31-(bac 8F)	<i>Bacillus licheniformis</i> strain R2	KJ206991.1	70
	<i>Bacillus licheniformis</i> strain SMR1	KF600749.1	70
	<i>Bacillus subtilis</i> strain VJJS-01	DQ872516.1	70

in line with that of Onwuliri et al. (2016) in which *Bacillus*, *Yersinia* and *Pseudomonas* species were found to be

responsible for biogas production from cow dung. In the literature, *Bacilli* are described as aerobic or facultative

Table 4. Means for quality of % methane gas produced by different isolates.

pH	Temperature (°C)	15 ^{^1}	17 ^{^1}	18 ^{^1}	18 ^{^2}	20 ^{^1a}	25 ^{^2}	26 ^{^2}	3 ^{^2}	9 ^{^3b}
6.0	30	0.52±0.02 ^{cd}	0.72±0.02 ^d	0.29±0.07 ^a	0.48±0.02 ^c	0.33±0 ^a	0.31±0 ^b	0.41±0.03 ^b	0.29± ^{ab}	0.24±0.02 ^a
6.0	35	0.64±0.05 ^d	0.93±0.03 ^e	0.62±0.07 ^c	0.67±0.07 ^d	0.69±0.06 ^d	0.5±0.03 ^{cd}	0.79±0.03 ^h	0.28±0 ^a	0.74±0.01 ^{bc}
6.0	37	0.38±0.01 ^{abc}	1.12±0.02 ^f	0.49±0.03 ^b	0.42±0.03 ^{bc}	0.58±0 ^c	0.47±0.01 ^c	0.29±0 ^a	0.29±0.01 ^{ab}	0.35±0 ^a
6.0	40	0.33±0.01 ^{ab}	0.34±0.01 ^b	0.23±0 ^a	0.26±0 ^a	0.28±0 ^a	0.3±0 ^b	0.25±0 ^a	0.33±0 ^{ab}	0.29±0.01 ^a
7.2	30	0.32±0.01 ^{ab}	0.21±0 ^a	0.33±0.01 ^a	0.73±0 ^d	0.48±0.02 ^b	0.21±0 ^a	0.44±0.06 ^{bc}	0.38±0.01 ^{bc}	0.43±0.04 ^a
7.2	35	0.24±0.02 ^a	0.33±0.01 ^b	0.43±0.01 ^b	1.12±0.03 ^f	0.48±0.02 ^b	0.21±0.01 ^a	0.47±0.03 ^{bcd}	0.43±0.01 ^c	0.63±0.04 ^b
7.2	37	0.44±0.02 ^{bc}	0.51±0.08 ^c	0.72±0.08 ^d	1.15±0.03 ^f	0.5±0 ^b	0.78±0.01 ^e	0.5±0 ^{cde}	0.6±0 ^d	0.62±0 ^b
7.2	40	0.47±0.01 ^{bc}	0.25±0.02 ^{ab}	0.52±0 ^{bc}	0.75±0.02 ^d	1.3±0 ^f	0.76±0.02 ^e	0.51±0.04 ^{cde}	0.59±0.09 ^d	0.61±0.01 ^b
8.0	30	0.86±0.14 ^e	0.75±0.01 ^d	1.1±0 ^e	0.97±0.08 ^e	0.55±0 ^{bc}	0.55±0 ^d	0.55±0.02 ^{def}	0.52±0.03 ^d	0.83±0.19 ^c
8.0	35	0.45±0.01 ^{bc}	2.3±0 ^h	2.2±0 ^f	0.77±0 ^d	0.77±0 ^e	0.52±0.03 ^d	0.58±0 ^{ef}	0.73±0 ^e	2.3±0 ^d
8.0	37	0.42±0 ^{bc}	2.1±0 ^g	1.08±0.01 ^e	0.42±0 ^{bc}	0.7±0 ^{de}	0.51±0.01 ^{cd}	0.66±0.02 ^g	2.1±0 ^f	0.7±0 ^{bc}
8.0	40	0.37±0.02 ^{ab}	0.71±0.05 ^d	1.1±0 ^e	0.34±0.02 ^{ab}	0.5±0.03 ^b	0.51±0.01 ^{cd}	0.63±0 ^{fg}	0.77±0 ^e	0.77±0 ^{bc}
LSD	-	0.133	0.093	0.104	0.107	0.069	0.045	0.080	0.092	0.189
CV%	-	17.1	6.3	7.8	9.2	6.6	5.5	9.2	7.8	14.4

Means with same letter are not significantly different.

anaerobic; rod shaped, Gram positive, motile, flagellated bacteria, either catalase positive that belongs to the division Firmicutes with varying ecological diversity. They are most commonly found in soil, waste water, milk, dust and plant surfaces.

The pH ranges of brewery wastewater is reported as 6.5 to 8.2 (Janhoappliedm et al., 2009; Caliskan et al., 2014; Zheng et al., 2015). The study considered pH values of 6, 7.2 and 8 in order to investigate the effect of pH on the growth of the bacteria which has a direct impact on the concentration of methane gas produced by the different isolates (Harris et al., 1984). This could be attributed to different adaptations levels by individual isolate. The low pH of 6 and temperature of 30°C, and high pH of 8 and temperature 40°C, may have inhibited the growth of some isolates, resulting in low concentrations of

the methane produced. Production of methane at low pH is essential for digestion to progress from the anaerobic acid phase to the methane production phase. Presence of isolate 25², 26² and 20^{1a} which were acid tolerant is consistent with literature (Ladapo et al., 1997). At pH 7.2, most of the isolates were able to adjust and increase in numbers especially at temperatures between 35 and 37°C. The isolates observed floating in the digesters could indicate a possibility of death for these isolates as they could not adapt easily to the high pH and temperature of 40°C.

CONCLUSIONS AND RECOMMENDATIONS

The study demonstrated that brewery waste water harbour diverse bacteria species with potential biogas production. Biochemical properties of

some isolates like ability to ferment different sugars, hydrolysis of starch, liquefying of gelatin, amino acid tryptophan split, and production of catalase enzyme and hydrogen sulphide gas suggests their involvement in biogas production. Since most isolates adapted easily at temperatures of 35 and 37°C, with the highest quality of methane gas, these conditions could be exploited as optimal working conditions.

More research is required to assess whether the isolates in this study possess unique physiological characteristics to explore their full potential.

Conflict of Interests

The authors have no affiliations with or involvement in any organization or entity with any financial or non-financial interests in the materials

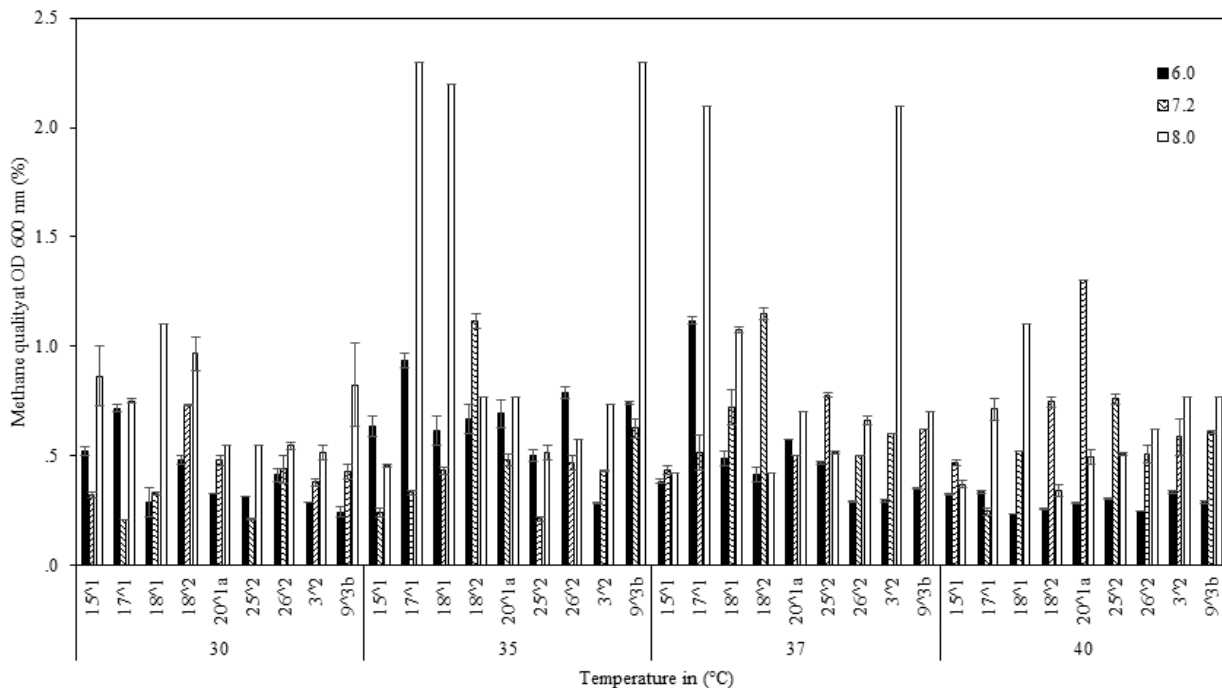


Figure 3. Effect of temperature and pH on the quality of the methane produced at OD 600 nm.

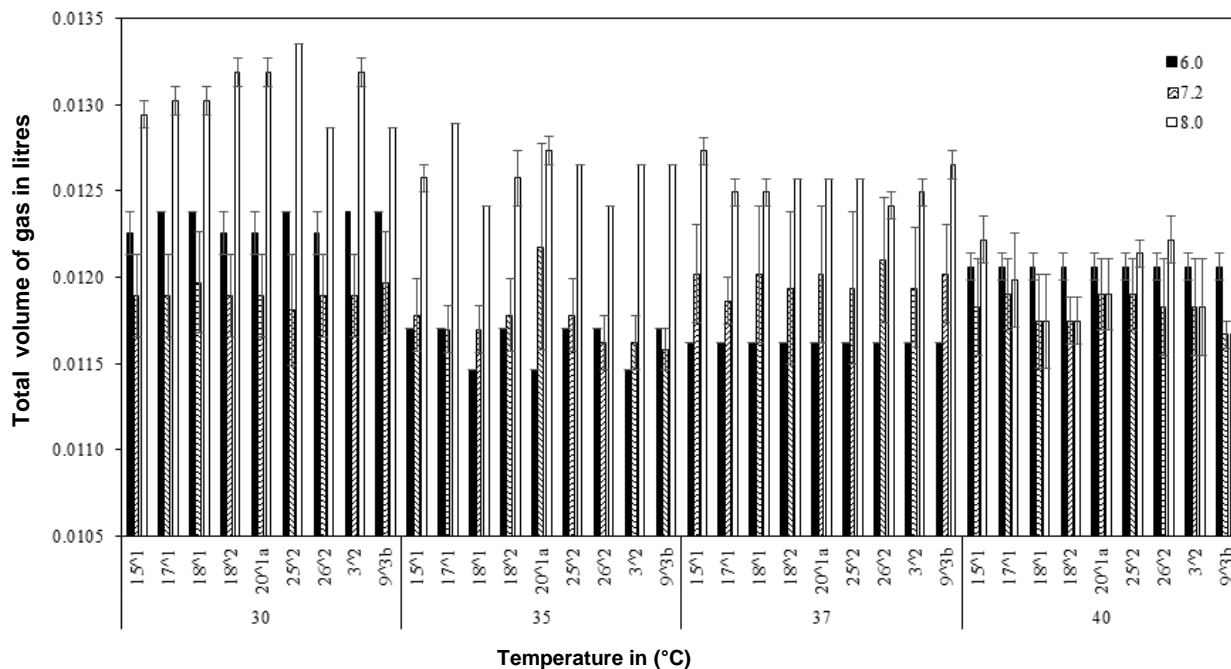


Figure 4. Effect of temperature and pH on the total volume of gas produced.

discussed in this manuscript.

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Full Length Research Paper

Production of alkaline proteases by alkalophilic *Bacillus subtilis* during recycling animal and plant wastes

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The production of extracellular alkaline protease by *Bacillus subtilis* was studied with submerged fermentation. A new strain of *Bacillus* sp. was isolated from alkaline soil, which was able to produce extracellular alkaline protease. The production of alkaline protease involved the use of agricultural or animal wastes at pH 8 and temperatures at 37°C. Results showed that growing *B. subtilis* sub sp. *subtilis* under optimized growth resulted in production of alkaline protease with enzyme activity of 1412.5 U/ml, while with pomegranate peel at a concentration of 3%, the enzyme activity reached 3600 U/ml; further increase in pomegranate concentration did not however, lead to additional enzyme activity. Among various nitrogen sources, yeast extract was found to be the best inducer of alkaline protease. Among metal salts, KNO₃ and NH₄Cl were found to increase protease production. The maximum enzyme production (3600 U/ml) was observed with pomegranate peels of fermentation medium in the presence of yeast extract, potassium nitrate and ammonium chloride.

Key words: Production, alkaline protease, *Bacillus subtilis*, animal wastes, enzyme activity.

INTRODUCTION

Marketable proteases have reached up to 60% of the total industrial enzymes' market, and they represent one of the three largest groups of industrial enzymes known. The proteolytic enzyme has been severally applied in industries including the pharmaceutical, food and detergent industries (Moon and Parulekar, 1991). Proteases of commercial importance are produced from microbial, animal and plant sources (Patel, 1985). Almost all living organisms can produce alkaline protease at 32 to 45°C and pH 8 to 9 (Akcan and Uyar, 2011). Microbial

proteases are produced from high yielding strains, including species of *Bacillus* sp., which is the most important group of bacteria that are involved in the enzyme industry. This bacterium is known to produce proteolytic enzymes quite effectively (Kaur et al., 1998). Generally, alkaline proteases are produced using submerged fermentation due to apparent advantages in enzyme production characterized by defined medium, and process conditions, and advantageous in downstream despite the cost-intensiveness for medium components

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(Prakasham et al., 2006). The researchers of microbial proteases production are always in search of new and cheaper methods to improve the protease production as well as to reduce the market cost of this enzyme (Mukherjee et al., 2008). Recently, efforts have been made to explore new resources to reduce the protease production cost by improving the yield, and using cost-free or low-cost feedstocks or agricultural byproducts as substrate(s) for protease production (Sandhya et al., 2005). Currently, production of the enzyme by using cheap materials has attracted attention of many researchers such as Shaheen et al. (2008) and Boominadhan et al. (2009) who used crushed crab shells, acid wastes (Johnvesly et al., 2002), fish wastes (Ellouz et al., 2001), chicken-feather (Fakhfakh et al., 2009), maize extract with molase (Maal et al., 2009), wastes of rice and dates (Khosravi-Darani et al., 2008), protein by-products from lather industry (Kumar et al., 2008), wheat wastes (Gouda, 2006), that prepared peptone from sardine wastes with wastes of flour industry (Haddar et al., 2010). Researchers are always trying to find the most appropriate alternatives that are also cheaper. In view of this, the aim of the present study was to estimate the feasibility of utilizing easily available substrates in submerged fermentation for the production of alkaline protease by *B. subtilis* by using abundant natural resources and cheaper wastes of growth conditions of bacterium to enhance protease enzyme production and the effect of addition of nitrogen and metal salts sources on enzyme activity.

MATERIALS AND METHODS

Bacterial isolate

Bacillus subtilis isolated from soil during November 2009 at the Eastern Province of Saudi Arabia, were used in this research. The isolate was identified in the Plant Protection Department, Faculty of Science and Agriculture in King Saud University by Biolog Systems by Al-Yahya et al. (2007). Identified isolates were evaluated for their ability to produce alkaline protease by Al-Khalidi (2014). *B. subtilis* sub sp. *subtilis* was the highest active isolate in alkaline protease activity and thus used for the study.

Source of natural wastes

Kitchen wastes as well as agricultural and animal wastes were collected from farms in sterile plastic bags (Sonia Sethi et al., 2012). The following wastes were used, bran, seeds of linen, date, olive, peels of mango, carrot, orange, banana, pomegranate and potatoes were also used. Corn husks, onion peel, egg shells, sheep bones and manure, camel manure and pigeon feathers were taken by replacing casein in the growth media.

Media preparation

After washing wastes, it was left to dry air in an oven at 70°C for 5 days followed by grinding. 0.75 g of each type was kept in a flask with 25.0 ml H₂O and inoculated with 500 µl of the isolate, then incubated with shaking water bath (90 rpm) at 37°C for 5 days (Al-

Khalidi, 2014). The enzyme was extracted and examined for protease production.

Protease assay

Proteolytic activity in the bacterial cultural media was determined by using the spectrophotometric method (Rao and Narasu, 2007). 500 µl of filtrate, representing the enzyme, was added to 100 µl of 1% w/v casein (substrate) soluble in phosphate buffer at pH 8. 50°C for 30 min and the reaction was stopped by the addition of 2.0 ml trichloroacetic acid (10%) and the reaction was left for 30 min at room temperature in the dark. The precipitate was separated by centrifugation at 10000 rpm. 1000 µl from the filtrate was added to 5.0 ml NaOH (0.5 mol/l) and 500 µl folin Ciocalteu Phenol reagent. The resulted color was read using UV-1800 spectrophotometer (model no a1145806148cd). The absorbance was read at 660 nm. One unit of proteolytic enzyme activity was defined as the amount of casein that hydrolyzed during 30 min incubation at 37°C for milliliter of solution of extract. All experiments were conducted in triplicate and the mean at three with standard deviation (SD) was represented.

Effect of different waste concentrations on enzyme production

To test the best kind of waste as substrate of enzyme production, 0.1, 0.5, 1.0, 3.0, 6.0 and 12 g/100 ml H₂O were distributed in flasks contain 250 distilled water and cultured with the bacteria; the flasks were shaken at 90 rpm in a water bath at 37°C for 5 days, the enzyme activity was measured for enzyme production (Al-Khalidi, 2014).

Effect of some salts and nitrogenous sources on the enzyme activity

0.5 g/100 ml H₂O of the following salts (NaCl, ZnCl₂, cobalt chloride, yeast extract, peptone, potassium nitrate and ammonium chloride) were dissolved and poured into flasks containing the waste and inoculated with 500 µl of the bacterium. Flasks were incubated at 37°C for 5 days and then examined for alkaline protease production (Al-Khalidi, 2014).

Produced enzyme in rotein media or in natural wastes

The optimized media of enzyme production (10 g fructose, 5 g KNO₃, 150 g NaCl, 5 g K₂HPO₄, 0.4 g MgSO₄, 0.2 g CaCl₂ and 10 g Tween-80) dissolved in one liter of distilled water, was prepared (Al-Khalidi, 2014). The optimized media and natural waste media and mixing media of both were inoculated with 25.0 µl of bacteria with incubation at 37°C for 5 days. The produced enzyme was separated by centrifugation at 10000 rpm (Al-Khalidi, 2014).

Statistical analysis

The results obtained were analyzed statistically using version of SPSS16 program where transaction averages were compared at the abstract level (0.05) using the least significant difference test (LSD) (Norusis, 1999).

RESULTS

Pomegranate peel proved to be the best media used for enzyme production; as shown in Table 1, the activity

Table 1. Effect of natural wastes on alkaline protease activity.

Plant and animal wastes (3 g/100 ml)	Optical density (OD)	Protease activity (U/ml)
Bran	0.3316	157±5.57
Linen seeds	0.4108	208.5±16.73
Date seeds	0.3380	159±16.04
Olive seeds	0.2172	102±4.58
Mango peel	1.2512	1007.5±35.00
Carrots Peel	0.8630	640±90.04
Orange peel	1.0723	840±48.80
Banana peels	0.4322	227.5±15.21
Pomegranate peel	3.9524	3075±525.00
Potatoes peel	0.6120	400±30.41
Corn husks	0.6745	455±44.23
Onions peel	0.9147	687.5±41.61
Eggshells	0.0832	39±1.50
Pulp peel	0.1666	80±5.00
Shrimp peel	0.4976	287.5±19.53
Fish guts	0.1865	86±5.90
Sheep bones	0.2965	139±4.00
Sheep manure	0.2064	97.5±7.70
Camel manure	0.1680	80±5.29
Pigeon feathers	0.3111	143.5±10.48

Table 2. Effect of pomegranate peel on alkaline protease activity.

Pomegranate peel concentration (g/100 ml)	Optical density (OD)	Protease activity (U/ml)
0.1	0.2948	137.5±2.50
0.5	0.9988	767.5±50.19
1	2.1409	1837.5±121.24
3	4.0000	3600±0.00
6	4.0000	3600±0.00
12	4.0000	3600±0.00

measured was 3075 U/ml, followed by 1007.5 U/ml for mango peel. Orange peel yielded protease enzyme with an activity of 840 U/ml followed by onion peel, carrot peel, corn husks and potato peels with 687.5, 640, 455 and 400 U/ml as enzyme activities, respectively. Shrimp peel yielded enzyme with activity of 287.5 U/ml, followed by banana peel recording 227.5 U/ml. Among all the plant and animal wastes investigated in this study, egg shells produced alkaline protease enzyme with the lowest activity of 39 U/ml (Table 1).

The effect of various concentrations of pomegranate peel on alkaline protease activity is shown in Table 2. The results revealed that the alkaline protease activity progressively increased from 137.5 U/ml at concentration 0.1 g to 3600 U/ml at concentration 3 g of pomegranate peel; further increase in pomegranate peel did not

Table 3. Effect of some salts and nitrogen sources to the best of pomegranate peel concentration on alkaline protease activity.

Some salt and nitrogen sources (0.5 g/100 ml)	Optical density (OD)	Protease activity (U/ml)
NaCl	3.9422	3540±60.00
CoCl ₂	1.8934	1600±5.00
ZnCl ₂	3.7352	3352.5±52.50
Yeast extract	4.0000	3600±0.00
Peptone	3.6877	3312.5±130.00
KNO ₃	4.0000	3600±0.00
NH ₄ Cl	4.0000	3600±0.00

however, alter the activity of the enzyme (Table 2).

Table 3 revealed the effect of some salts and nitrogen sources of pomegranate peel concentration on alkaline protease activity. The result indicated that adding yeast extract with potassium nitrate and ammonium chloride increased enzyme activity to 3600 U/ml. Among all salts and nitrogen sources investigated, the alkaline protease enzyme obtained in the presence of cobalt chloride had the lowest activity (1600 U/ml) (Table 3).

More so, the alkaline protease enzyme activity of 3600 U/ml obtained after growing *B. subtilis* subsp. *subtilis* in media containing pomegranate peels (3 g/100 ml) (Table 4) was significantly higher than that recorded after growing in optimized medium (1412.5 U/ml). The enzyme

Table 4. Comparing of activity alkaline protease in the optimal medium with/without pomegranate peel, and in medium with pomegranate peel only.

Type of medium	Optical density (OD)	Protease activity (U/ml)
Optimal medium	1.6863	1412.5±59.11
Optimal medium with Pomegranate peel	4.0000	3600±0.00
Pomegranate peel medium	2.3164	2010±76.61

activity after mixing pomegranate with the optimized media was, however, 2010 U/ml.

DISCUSSION

Bacillus species are considered to be the most important sources of protease and have been used for enzyme production using submerged fermentation (Gaurav et al., 2015). Several substrates obtained from a kitchen wastes as well as agricultural and animal wastes were collected from farms and tested for protease production (Table 1). Adding natural and cheap nutrients, considered as wastes, to bacterial growing media led to high production of alkaline protease enzymes with increasing activity. Alkaline protease production by *B. subtilis* using submerged fermentation was influenced by physiological and chemical nature of substrates and associated with growth of the microbial strain (Abid et al., 2017). In this study, maximum enzyme production (3075 U/ml) was observed with pomegranate peels; further studies were carried out using it as a substrate. Results proved that pomegranate peels were the best additive.

The principal components of primary cell wall are composed of the cellulose microfibrils and pectins, and they play a significant role in the structuration (Agoda-Tandjawa et al., 2012). It is understood that the mechanical properties of pomegranate peel gels are administered mainly by pectins and/or fibrous material. Also, the presence of calcium and/or sodium ions and the biopolymer concentrations were reported to have an influence on the properties of the mixed cellulose and pectins.

Pomegranate peel at concentration of 3% recorded high enzyme activity reaching 3600 U/ml (Table 2). With increase in concentration, there is no difference in the rate of the enzyme activity production. This steadiness in activity may be due to change of enzyme. Units ranging from free to conjugated, which contains the active sites on the enzyme are totally occupied by the substrate. Results proved that adding yeast extract and potassium nitrate increased enzyme activity (Table 3). Generally, adding nitrogenous compounds stimulate microorganism growth and enzyme production. Some microorganisms use these compounds in enzyme production and not in growth. Growing *B. subtilis* subsp. *subtilis* under optimized medium (10 g fructose, 5 g KNO₃, 150 g NaCl, 5 g K₂HPO₄, 0.4 g MgSO₄, 0.2 g CaCl₂ and 10 g Tween-

80) dissolved in one liter of distilled water, enzyme activity reached 1412.5 U/ml (Table 4), while with pomegranate peel, high enzyme activity reaching 3600 U/ml was recorded. The pomegranate peel contains many ions of metals necessary for the growth of bacteria, and contributes to the stability of the enzyme protease. Kushwaha et al. (2013) pointed out that pomegranate peel powder was evaluated for its minerals value: Sodium (mg/kg) 362.74, potassium (mg/kg) 6679.50, calcium (mg/kg) 728.23, magnesium (mg/kg) 524.80, phosphorus (mg/kg) 57.01, iron (µg/g) 18.33, copper (µg/gm) 4.67, zinc (µg/g) 9.63. The effect of nitrogen sources on protease production is shown in Table 3. Yeast extract increased protease production by *B. subtilis*, while Yang et al. (2000) found that protease production by *B. subtilis* Y-108 was repressed by most of the nitrogen sources. Addition of metal salts sources was investigated. Among these metal salts, KNO₃ and NH₄Cl were found to increase protease production (Table 3). For maximum alkaline protease activity, there is a need for a divalent cation like Ca²⁺, Mg²⁺ and Mn²⁺ or a combination of these cations. These cations were also found to enhance the thermal stability of *Bacillus* alkaline protease (Paliwal et al., 1994). These cations are believed to protect the enzyme against thermal denaturation, and also play a vital role in maintaining the active conformation of the enzyme at high temperatures. In addition, specific Ca²⁺ binding sites influence the protein activity and stability apart from the catalytic site described for proteinase K (Maheshwari and Saraf, 2015).

Conflict of Interests

The authors did not declare any conflict of interests.

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