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CONTENTS OF THE ISSUE

- i. Copyright Notice
- ii. Editorial Board Members
- iii. Chief Author and Dean
- iv. Contents of the Issue

1. Biotechnical and Systematic Preparation of Artificial Cells (DNA Crown Cells). **1-10**
2. Effects of Yeast and Oxygen on Quality Attributes of Wine Produced From Ethiopian Beetroot. **11-15**
3. Effects of Controlled Atmosphere Storage and Temperature on Quality Attributes of Mango. **17-25**
4. An Overview on the Production of Microbial Copper Nanoparticles by Bacteria, Fungi and Algae. **27-33**

- v. Fellows
- vi. Auxiliary Memberships
- vii. Process of Submission of Research Paper
- viii. Preferred Author Guidelines
- ix. Index



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Biotechnical and Systematic Preparation of Artificial Cells (DNA Crown Cells)

By Shoshi Inooka

Abstract- The first cell biology studies were conducted half a century ago and it has long been known that cells consist of a membrane made of lipid-polymer complexes comprising proteins and carbohydrates complexed with lipids. Many cell biology studies have been based on this understanding of the structure of the cell membrane, yet there have been no reports of a cell membrane associated with DNA.

I have demonstrated that cyto-cells and cyto-particles covered with DNA are formed when cultured cells are mixed with sphingosine-DNA (Sph-DNA). Recently, I have been studying the preparation of artificial cells and have demonstrated the formation of cells (named DNA crown cells) which are surrounded by a membrane comprising lipid-DNA. Moreover, I have synthesized DNA crown cells using a known lipid (monolaurin).

Keywords: *artificial cells, biotechnology, biomedical engineering, DNA crown cells, sphingosine-DNA.*

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Biotechnical and Systematic Preparation of Artificial Cells (DNA Crown Cells)

Shoshi Inooka

Abstract- The first cell biology studies were conducted half a century ago and it has long been known that cells consist of a membrane made of lipid-polymer complexes comprising proteins and carbohydrates complexed with lipids. Many cell biology studies have been based on this understanding of the structure of the cell membrane, yet there have been no reports of a cell membrane associated with DNA.

I have demonstrated that cyto-cells and cyto-particles covered with DNA are formed when cultured cells are mixed with sphingosine-DNA (Sph-DNA). Recently, I have been studying the preparation of artificial cells and have demonstrated the formation of cells (named DNA crown cells) which are surrounded by a membrane comprising lipid-DNA. Moreover, I have synthesized DNA crown cells using a known lipid (monolaurin).

Herein I report three methods for preparing DNA crown cells.

DNA crown cells be prepared by manipulating sphingosine-DNA fibers formed by mixing sphingosine and DNA.

This paper describes these three methods: two successful biotechnical procedures and one systematic procedure.

Keywords: *artificial cells, biotechnology, biomedical engineering, DNA crown cells, sphingosine-DNA.*

I. INTRODUCTION

There has been significant progress in the generation of artificial cells since the first studies in the 1960s (Zhang, Ruder, and Leduc, 2008, Lin, Hansen, Marques and Kiyoshi, 2013, Uruma, Stano, Ueda and Luisi, 2009), yet no artificial cells that can replicate autonomously have been reported to date. Recent work on artificial cells has focused on cell division or replication (Noireaux, Maeda and Libhaber, 2011). I have studied approaches for generating fully operational (self-replicating) artificial cells (Inooka, 2012) and have established a method (Inooka, 2016, Ref. 1) by which artificial cells can be cultivated and produce protein in egg white by combining adenosine with sphingosine (Sph) and DNA. I have studied the mechanism underlying the formation of artificial cells and have demonstrated that artificial cells are generated from Sph-DNA aggregates formed using components in egg white (Inooka, 2016, Ref. 4).

The surface of artificial cells generated from Sph-DNA consists of DNA and these cells are called DNA crown cells. Experiments (Inooka, 2016, Ref. 4)

suggest that DNA crown cells may be proto-cells of artificial cells generated within egg white. I previously reported that cyto-cells and cyto-particles are generated when cultured cells are lysed with Sph-DNA (Inooka, 2000). These cells are also DNA crown cells because their surface consists of DNA. DNA crown cells contain large loops or circles of DNA, similar to the general structure of plasmids. All prepared DNA crown cells can replicate, making it very important to clarify whether the loop structure of DNA is associated with self-replication and thus potentially uncover a new phenomenon applicable to various fields of the life sciences.

Here, I describe three methods for the preparation of DNA crown cells.

II. METHODS SUMMARIZED AND RESULTS

a) *Method to prepare DNA crown cells using animal cell materials*

i. *Materials*

Sph (Sigma, USA); DNA (extracted from quail blood lymphocytes); murine fibro-sarcoma cell line L929/LM (L-M cells)

ii. *Procedure*

Step 1 Preparation of assembled sphingosine-DNA (Sph-DNA) and particles

- 1) Sph (10 mM, 30 μ l) was mixed with DNA (50 μ g/ml, 100 μ l), added to distilled water (300 μ l), then examined under a phase contrast microscope and a fluorescence microscope.
- 2) Sph-DNA assemblies were formed, as shown in Fig. 1A. Russert light was observed from the assembly (Fig. 1C). Russert light was not observed in the sample which not contained Sph (Fig. 1B)
- 3) To prepare Sph-DNA particles, Sph (30 μ l) and DNA (100 μ l) were added to 300 μ l distilled water. After mixing, the solution was heated and boiled for several minutes, then concentrated to approximately 100 μ l. To detect DNA, ethidium bromide solution was added and the sample was observed under a fluorescence microscope. Sph-DNA particles were formed and fluorescent particles and aggregates were observed (Fig. 1D). Sph-DNA fibrous assemblies were prepared from a mixture of Sph and DNA and particles were prepared by heating the assembly. Sph-DNA fibers or particles were used as basic materials for the preparation of DNA crown cells.

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Step 2 Cell lysis with Sph-DNA aggregates and particles (Fig. 2)

- 1) L-M cells were cultured in Dulbecco's modified Eagles medium containing 10% fetal calf serum. L-M cells were seeded ($1-2 \times 10^4$ /well) in 96-well plates (FALCON) and confluence cultures ($6-7 \times 10^4$ /well) were used for the experiments.
- 2) Sph-DNA particles (0.3–0.05 ml) were added to the confluent cultures of L-M cells. Cell growth was examined under a phase contrast microscope.
- 3) Sph-DNA aggregates (arrow) were observed on the L-M cells, as shown in Fig. 2A.
- 4) L-M cells surrounding the Sph-DNA aggregates were lysed (Fig. 2B; arrow).
- 5) 24 hours after the addition, L-M cells surrounding the Sph-DNA aggregates formed plaques with the cell lysis (Fig. 2C; arrow).
- 6) 24 hours after the addition, cell particles (arrow) were observed among the lysed L-M cells (Fig. 2D).

Step 3 Formation of DNA crown cyto-cells and DNA crown cyto-particles

- 1) L-M cells were cultured as described in Step 2.
- 2) Sph-DNA particles (0.3 ml) were added to confluent cultures of L-M cells ($6-7 \times 10^4$ /well) and incubated for 3, and 24 hours. The cells were trypsinized, and then the cell pellets were fixed to a glass slide and stained with Giemsa's stain solution.
- 3) Sph-DNA particles were stained with ethidium bromide. The suspension of trypsinized L-M cells (10^5 /well) was incubated with labeled Sph-DNA particles (0.2 ml/well) for 5–60 min in a 96-well plate.

A drop of cells on the slide was observed under a fluorescence microscope.

Sph-DNA particles are cytotoxic and thus can kill neighboring cells, resulting in the generation of DNA crown cyto-cells and DNA crown cyto-particles.

Step 4 Structural integrity of DNA crown cyto-cells

Light microscopic observation showed oval-shaped cyto-cells (Fig.3A). Fluorescence microscopic observation of L-M cells treated with ethidium bromide-labeled Sph-DNA particles (Fig.3B~G) showed fluorescence on the surface of cyto-cells, indicating that Sph-DNA particles were adsorbed on the surface of cells (Fig. 3B), shrunken cells (Fig. 3C), rod-shaped cells (Fig.3D and E), and cocci or rod-shaped cells (Fig.3F). The cocci-shaped cells were $<3.0 \mu\text{m}$ in size (Fig.3G).

Step 5 Structural integrity of DNA crown cyto-particles

Light microscopic observation of L-M cells after 3 hours of Sph-DNA treatment (Fig. 4H~J) showed numerous small particles within the cyto-plasma (arrow) (Fig.4H), and individual or aggregates of particles (Fig.4I and J) were also observed in the separated cyto-plasma, showing the formation of DNA crown cyto-particles.

Figures 4K and 4L show fluorescence microscopic images of DNA crown cyto-particles. Fluorescent particles were incorporated into the cyto-plasma (Fig.4K) and cyto-particles (arrow) and fluorescent aggregates were also observed (Fig.4L), showing that aggregates of cyto-particles were released from cyto-plasma.

- iii. *Comments on the possible formation and structure of DNA crown cyto-cells and cyto-particles*

DNA crown cells were generated from cultured cells lysed due to the cytotoxicity of Sph-DNA. Free Sph is a chelator and exhibits strong cytotoxicity, and thus the addition of Sph to cultured cells immediately lyses the cells. However, I found that some agents bind Sph so that Sph lyses cells slowly. Hence, Sph-DNA may exhibit unique cytotoxicity and generate DNA crown cells.

- iv. *Comment on DNA crown cyto-cells*

DNA crown cyto-cells of various sizes can be formed by simply adsorbing Sph-DNA particles on the surface of L-M cells (Fig.5Aa and b). The formation of DNA crown cells may be based on changes in osmotic pressure caused by covering the cell surface with Sph-DNA, resulting in the cells shrinking and formation of rod-shaped or cocci-shaped cyto-cells (Fig.5Ac and d). Cyto-cells are composed of Sph-DNA in the outmost layer, followed by a plasma membrane from L-M cells and nucleus from L-M cells.

DNA crown cyto-cells may contain an L-M cell or L-M cell components.

DNA crown cyto-cells have been generated using two kinds of DNA: quail DNA and DNA from L-M cells.

Here, L-M cells were used as cultured cells and quail DNA was used as DNA.

DNA crown cyto-cells can be prepared from cells other than L-M cells, such as monolayer cells, and from DNA other than quail DNA. Numerus DNA crown cells can be prepared with different combinations of cells and DNA.

- v. *Comments on DNA crown cyto-particles*
DNA crown particles can be formed as follows:

Sph-DNA particles are adsorbed onto cell membranes (Fig.5B a and b) and incorporated into the cells (Fig.5B c and d). These cells are then lysed and used to generate DNA crown cyto-particles (Fig.5B e and f). The surface of these particles therefore consists of the cytoplasm from L-M cells and Sph-DNA particles enclosed in the L-M cell membrane. Many kinds of DNA crown cyto-particles can be generated, similar to the case of DNA crown cyto-cells.

Here, I call DNA crown cells 'DNA crown cyto-cells' or 'DNA crown cyto-particles' to distinguish between DNA crown cells prepared from cell materials and these prepared from Sph-DNA, as described in the following section.

b) *Method to prepare DNA crown cells using egg white components*

i. *Materials*

Sph (Sigma, USA); DNA (Escherichia coli strain B, Sigma); adenosine (Sigma); white Leghorn eggs purchased from a market.

ii. *Procedure*

Step 1 Preparation of the components (F-fraction and D-fraction)

Egg whites were injected with 0.5 ml of adenosine (0.1 M) and the eggs were incubated for 5 days at 37°C, after which the egg whites were collected and kept at 4°C until use. Components were prepared from egg white using a protocol similar to the protocol for extracting DNA. One case is described below.

Egg white (300 μ l) was incubated at 65°C for 30 minutes in a microfuge tube, then 400 μ l of F-solution (DNA extraction kit, Rizo Inc. Japan) was added and the tube contents were mixed. Then, equal volumes (400 μ l each) of phenol and chloroform were added. After mixing, the tube was centrifuged for 10 minutes at $6714 \times g$.

The aqueous phase was separated from the organic phase and an equal volume of isopropanol was mixed with the aqueous phase. This mixture was centrifuged for 10 min at $15107 \times g$ and the upper layer was collected and kept at 4°C and used as the F-fraction. Then, 1 ml of 70% ethanol was added to the tube. The precipitated DNA fraction was dissolved in 50 μ l of distilled water. The fraction was divided between 10 tubes (5 μ l aliquots). This sample was used as the D-fraction. The F-fraction was dried and dissolved in 100 μ g/ml of distilled water. Distilled water (50 μ l) was added to each 5 μ l D-fraction aliquot.

Step 2 Preparation of the compound forming by mixing F-fraction and adenosine (F-A solution)

F-fraction (1.0 ml, 100 μ g) was added to 1.0 ml of adenosine solution (0.1 M). After mixing, 4.0 ml of ethanol was added, the precipitate (F-A compound) was dried, and then the dried precipitate was re-dissolved in 1 ml of distilled water to provide F-A solution

Step 3 Aggregation of Sph-DNA with D-fraction and F-A solution

Aggregates of Sph-DNA could be formed using D-fraction or F-A solution. Sph (90 μ l) was added to DNA solution (40 μ l), then D-fraction (50 μ l) was added. Aggregates of Sph-DNA were formed, as shown in Fig. 6a.

For F-A solution, Sph (90 μ l) was added to DNA solution (40 μ l). After mixing, F-A solution (50 μ l) was added and mixed.

Aggregates of Sph-DNA were formed, as shown in Fig. 6c.

Using D-fraction or F-A solution, two types of aggregates were formed: mucoid type (Fig. 6a) and crystal type (Fig. 6c). Staining the aggregates with ethidium bromide resulted in the observation of Russert light in mucoid type (Fig. 6b) and crystal type (Fig. 6d) aggregates, indicating that these aggregates contain DNA.

Figure 6a shows mucoid type aggregates formed using D-fraction, whereas Fig. 6c shows crystal type aggregates formed using F-A solution.

Crystal type aggregates were also formed using D-fraction and mucoid type aggregates were also formed using F-A solution. Therefore, the forces driving the aggregation of Sph-DNA were the same in D-fraction and F-A solution.

Step 4 Preparation of DNA crown cells

DNA crown cells could be formed using either D-fraction or F-A solution.

Using D-fraction, Sph (90 μ l) was added to the DNA solution (40 μ l) and then D-fraction (50 μ l) was added. After mixing, F-fraction (100 μ l) was added. Using F-A solution, Sph (90 μ l) was added to DNA solution (40 μ l). After mixing, F-A solution (50 μ l) was added and mixed, and then F-fraction (100 μ l) was added, resulting in the preparation of DNA crown cells. A typical cell stained with ethidium bromide is shown in Fig. 7.

I only show Fig. 7 to illustrate DNA crown cells because this paper has a page limitation. A detailed description has been published (Inooka, 2016, Ref. 4). Comments on possible mechanisms of formation and the structure of DNA crown cells.

DNA crown cells are generated from aggregates of Sph-DNA.

Sph-DNA aggregates may be formed as follows:

When Sph is mixed with DNA, thread-like fibers are formed (Fig. 1A).

These fibers may aggregate upon the addition of D-fraction or F-A solution, resulting in the formation of aggregates of Sph-DNA. The addition of F-fraction (which includes lipids) results in the formation of DNA crown cells.

Therefore, the membrane of the cells may consist of Sph, with DNA and Sph in the middle. The cells may be empty or may contain liquid such as distilled water.

D fraction contains adenosine and lipids and F-fraction contains lipids. The components have not been identified and the use of these complex components allows the preparation of various DNA crown cells using DNA rather than Escherichia coli cells.

c) *Method to systematically prepare DNA crown cells using adenosine-monolaurin*

i. Materials

Sph (Sigma, USA); DNA (Escherichia coli strain B, Sigma, USA);

Adenosine (Sigma, USA and Wako, Japan); monolaurin (Tokyo Kasei, Japan)

ii. Procedure

Step 1 Preparation of the compound formed by mixing adenosine and monolaurin (A-M)

A-M was prepared by adding 0.4 ml (0.1 M) monolaurin to 0.4 ml (0.1 M) of adenosine solution. After mixing, 0.15 ml of ethanol was added and the precipitate was collected and dried. The A-M precipitate was dissolved in 1.0 ml distilled water and used.

Step 2 Preparation of aggregates of Sph-DNA with A-M

Sph (90 μ l, 10 mM) was added to 40 μ l of DNA (1.7 μ g/ μ l). After heating the mixture, A-M solution (50 μ l) was added.

To observe aggregates, one drop of ethidium bromide solution was added to one drop of the Sph-DNA-A-M mixture, then a drop of this mixture was placed on a glass slide and observed using phase contrast microscopy and fluorescence microscopy.

Two types of aggregates, mucoid type (Fig. 8a) and crystal type (Fig. 8b and c), were prepared. Fluorescence was observed on the surface of the crystal type (Fig. 8d), suggesting the presence of DNA.

Thus, aggregates of Sph-DNA could be prepared in a simple manner.

Step 3 Synthesis of DNA crown cells:

To synthesize DNA crown cells, Sph (90 μ l, 10 mM) was added to 40 μ l of DNA (1.7 μ g/ μ l). After heating the mixture, A-M solution (50 μ l) was added and monolaurin solution (50 μ l, 0.1 M) was added to the Sph-DNA-A-M mixture. After mixing, the cells were observed as described above.

Cells of various sizes were formed (Fig. 9) and the surfaces of all cells were covered with DNA (Fig. 11), indicating that all cells were DNA crown cells.

A typical cell as observed under a phase contrast microscope is shown in Fig. 10.

A layer-like ring or membrane is observed outside. A typical cell as observed under a fluorescence microscope is shown in Fig. 12. Russert light was observed in the outmost layer.

iii. Comments on the possible mechanism underlying structure formation of synthetic DNA crown cells

DNA crown cells can be prepared using the purified reagents sphingosine, DNA, adenosine, and monolaurin.

During DNA crown cell formation, Sph-DNA aggregated with A-M solution and branches of Sph-DNA

fibers were formed. These branches may spontaneously seal, resulting in the formation of cells.

Accordingly, the cell structure may comprise Sph outside, DNA in the middle, and Sph inside, which is the same as cells formed with D-fraction or F-A solution.

Many types of DNA crown cells can be generated using this method.

Artificial cells were generated using Sph-DNA and nucleosides, including uridine (Inooka, 2016, Ref. 3), showing that compounds prepared using combinations of nucleosides and monolaurin may aggregate Sph-DNA. Moreover, compounds prepared using combinations of nucleosides and lipids related to monolaurin can also form aggregates of Sph-DNA. Here, I used Escherichia coli DNA, but DNA crown cells can also be prepared using DNA from other sources.

Thus, various DNA crown cells consisting of different components can be prepared by combining nucleosides, lipids and DNA.

iv. Summary of Comments

Here, three basic methods were described for preparing DNA crown cells.

Interestingly, these cells can self-replicate (Inooka, 2017).

All DNA artificial cells contain Sph-DNA fibers in the membrane of the cells, whereas the contents of the cells can differ.

DNA crown cyto-cells contain two types of DNA and whole L-M cells may be encapsulated within DNA crown cyto-cells.

Therefore, L-M cells can grow. Enzymes in L-M cells may stimulate Sph-DNA in DNA crown cyto-cells, resulting in the multiplication of DNA.

DNA crown cyto-particles are enclosed by a cell membrane. The DNA in DNA crown cyto-particles may stimulate enzymes in the membrane of the cells, resulting in division of the DNA in the artificial cells.

In contrast, DNA crown cells formed using adenosine-lipids may be empty or contain water. It is unclear how these DNA crown cells could self-replicate. Some components to stimulate division of DNA may be contained in egg white. Thus, DNA crown cells (cyto-cells and cyto-particles) prepared from cell materials differ from those prepared using egg white components or A-M compound.

The current method for preparing DNA crown cells is performed easily.

Cells whose membranes consist of DNA-lipid have not previously been reported.

DNA crown cells are artificial cells containing a large loop of DNA, similar in structure to general plasmids. Studies using DNA crown cells may provide new findings in a wide number of fields in the life sciences

III. CONCLUSION

Here, I described two biotechnological methods using cell materials and egg components, and a systematic method using A-M compound to prepare artificial cells (DNA crown cells: cells whose membrane consists of DNA). These DNA crown cells were formed using Sph-DNA. Mixing Sph with DNA resulted in the formation of fibrous sphingosine-DNA.

DNA crown cells prepared using cell materials resulted in Sph-DNA fibers covering the surface of the target cell or being enclosed by the cell membrane, resulting in the formation of DNA crown cells.

DNA crown cells prepared using the components of eggs or A-M compound resulted in fibrous sphingosine-DNA spreading with the components or A-M compound, and these sphingosine-DNA bilayers may spontaneously seal, resulting in the formation of DNA crown cells.

A cell membrane associated with DNA has not previously been reported.

Therefore, studies using DNA crown cells may provide new findings in a wide range of fields in cell biology and the life sciences in general.

IV. ACKNOWLEDGEMENTS

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Figure Legends

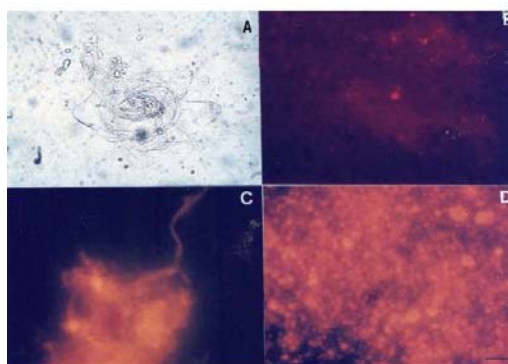


Figure 1: Sphingosine-DNA (Sph-DNA) assembly and particles

A: Sph solution was mixed DNA solution and examined under a phase contrast microscope. Fibrous assembly was observed. Sph solution was mixed phosphate -buffered saline without DNA (Fig1B). Sph solution was mixed with DNA solution (Fig1C). A drop of echidium-bromide solution was added, and they were examined under a fluorescence microscope. B: Russert was not observed. C: Russert were observed on fibrous, showing the formation of Sph-DNA fibrous. D: Sph solution was added to DNA. After mixing, the mixtures were heated. Fluorescent particles were observed, showing the formation of sph-DNA particles. Scale bar is 10 μ m.

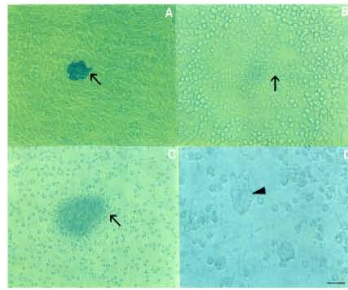


Figure 2: The cytotoxicity of Sph-DNA aggregates

L-M cells were cultured in a plate and confluent cells were used. Sph-DNA aggregates, were prepared and added to the cells. They were then examined under a phase contrast microscope. A: After 15 minutes of the addition, Sph-DNA aggregates (arrow) were observed on the L-M cells. After 3 hours of the addition, the destruction of the cells (arrow) were observed (B).

C: After 24 hours of the addition, L-M cells surrounding the Sph-DNA aggregates were destroyed and formed plaque with cell lysis (arrow) . D: After 24 hours of the addition, the product of the destroyed cells were observed, showing the formation of the cellular particles (arrow). Scale bar is 50 μ m in A, B, and C. and 10 μ m in D.

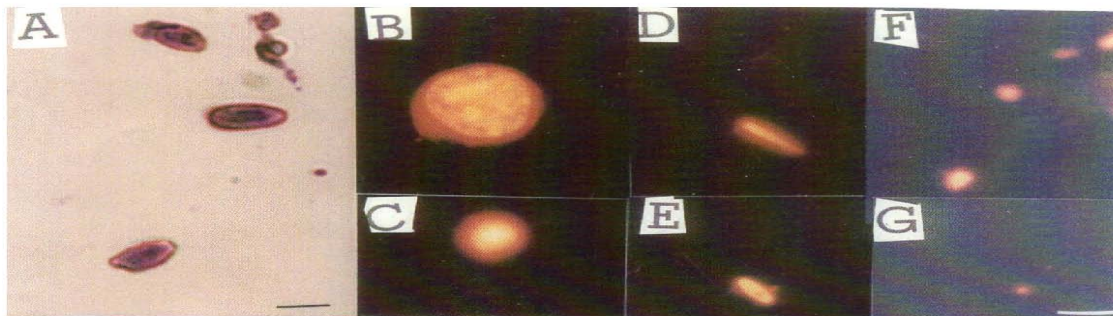


Figure 3: DNA crown cyto-cells

A: Light microscopic observation of DNA crown cyto-cells, showing oval shaped-cells.

B~G: Fluorescent microscopic observation of L-M cells treated with echidium bromide labeled Sph-DNA particles. B: Fluorescence was observed on the surface of cells, showing that Sph-DNA particles were adsorbed on the surface of cells and DNA crown cells were formed. C: shrunken cells D and E; rod-shaped cells F: cocci or rod-shaped cells G :cocci-shaped cells that were approximately $<3.0 \mu$ m in size were observed.

Scale bar is 3.0 μ m in A and 10.0 μ m in B~G.

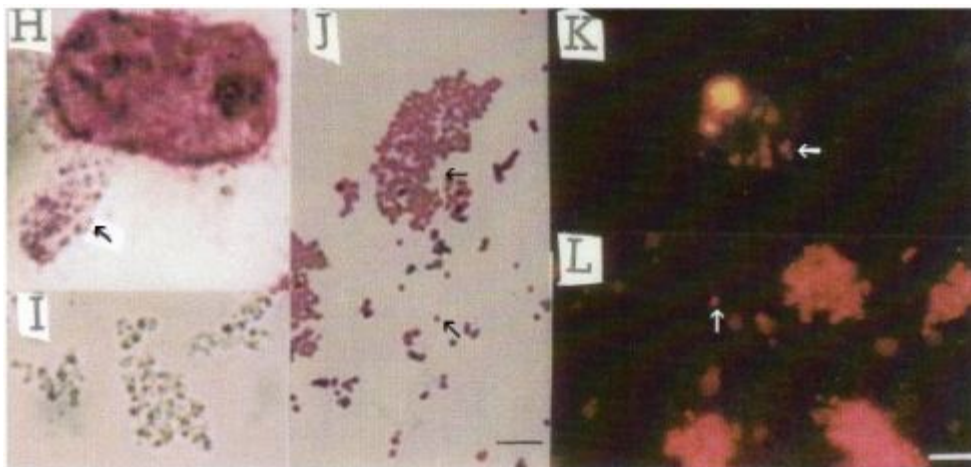


Fig.4: DNA crown cyto-particles

H~J: Light microscopic observation of L-M cells which were treated with Sph-DNA.

H: Numerous small particles were observed within the cytoplasm (arrow)

I and J: An individual or its aggregates of particles were observed in the separated cytoplasm, showing the formation of DNA crown cyto-particles.

K and L: Fluorescent microscopic observation of DNA crown cyto-particles.

Fluorescent-like particles were observed within the cytoplasm (arrow), showing that Sph-DNA particles were incorporated into the cytoplasm (K).

Also, fluorescent-like aggregates were observed, showing that the aggregates of DNA crown cyto-particles were released from the cytoplasm (L).

Scale bar is 3.0µm in H~J and 10.0 µm in K and L.

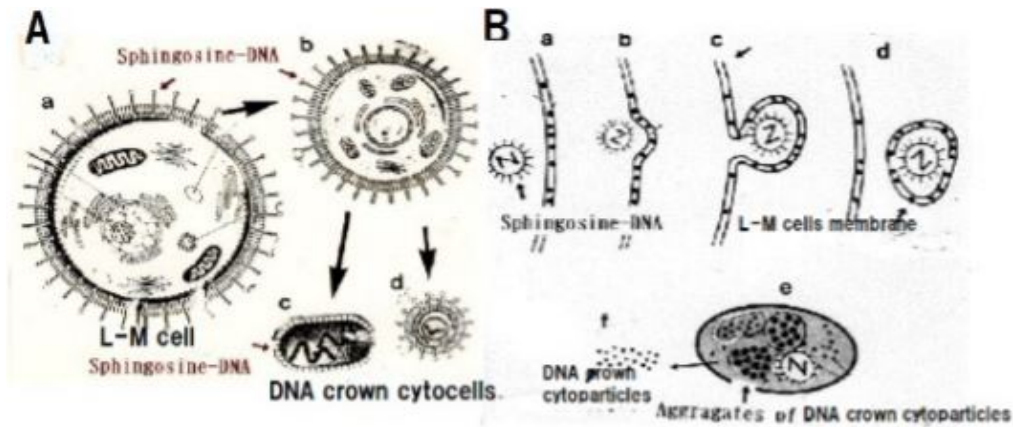


Fig.5: The schematic representation of the possible mechanism by which Sph-DNA will form DNA crown cyto-cells and DNA crown cyto-particles

A: DNA crown cyto-cells

Sph-DNA particles were adsorbed on the surface L-M cells (a) and the cells caused the shrinkage (b), resulting in the formation of rod-shaped (c) or cocci-shaped (d) DNA crown cells.

Therefore, it was considered that its structure was composed of Sph-DNA on the outside, then, plasma membrane, and the nucleus of L-M cells.

The cells had two kinds of Sph- DNA (*quail* DNA) and DNA of L-M cells.

B: DNA crown cyto-particles

Sph-DNA particles were adsorbed into the plasma membrane (a~b) and incorporated into cytoplasm (c). The DNA crown cytoparticles were then formed (d) The cytoplasm that involved cytoparticles was released from nucleus(e) and cell free DNA crown cyto-particles (f), or their aggregates were formed.

Thus, the structure of DNA crown cyto-particles may be composed of Sph-DNA particles enclosed with plasma membrane of L-M cells.

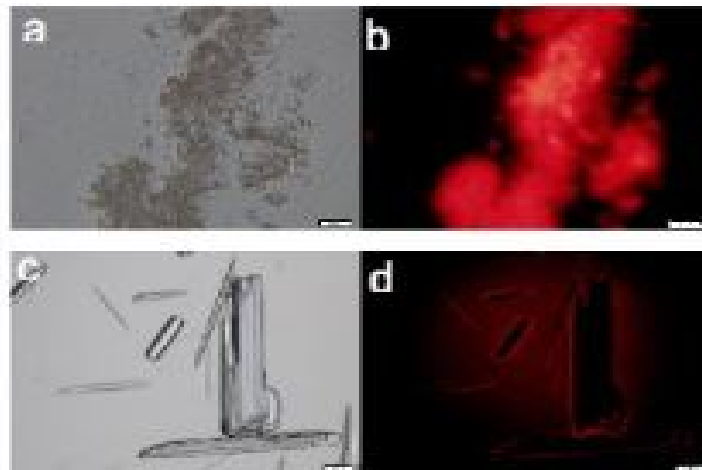


Fig.6: Structural integrity of Sph-DNA aggregates formed with D fraction and the compound formed by mixing of F-fraction and adenosine : (F-A).

Sph was added to DNA, mixed, then, D-fraction (a and b) or F-A compound (use as a solution in distilled water) (c and d) was added. A drop was smeared on a glass slide after the addition of echidium bromide solution. It was observed using phase contrast microscopy and fluorescent microscope.

a) Mucooid type aggregates were observed b) Russel light was observed on the surface of mucooid, showing that the mucooid contained DNA. Scale bar is 20 μ m.

c) Crystal type aggregates were observed. d) Russel light was observed on the edges of the aggregates, showing that the edge consists of DNA. Scale bar is 50 m.

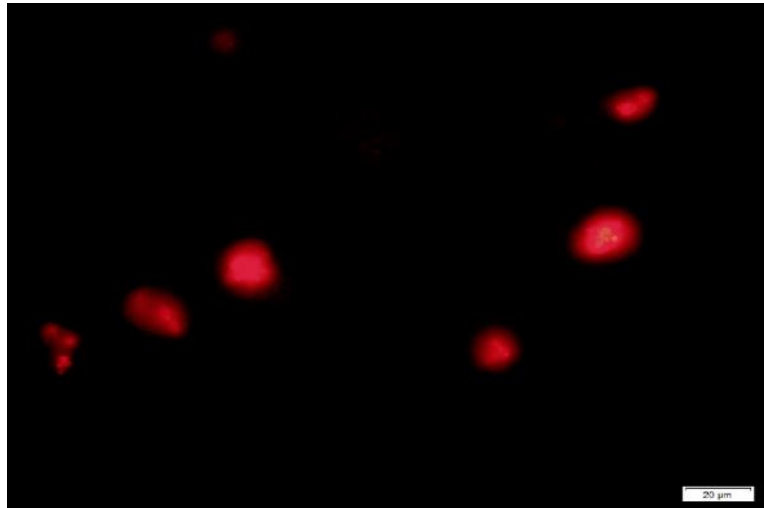


Fig.7: Typical DNA crown cells formed with F-A compound

Sph was added to DNA. After mixing, F-A compound was added and then F-fraction was added. After the addition of echidium-bromide, a drop was smeared on a glass slide and observed under a fluorescent microscope. Russel light was observed on the surface of the cells, showing DNA crown cells. Scale bar is 20 m.

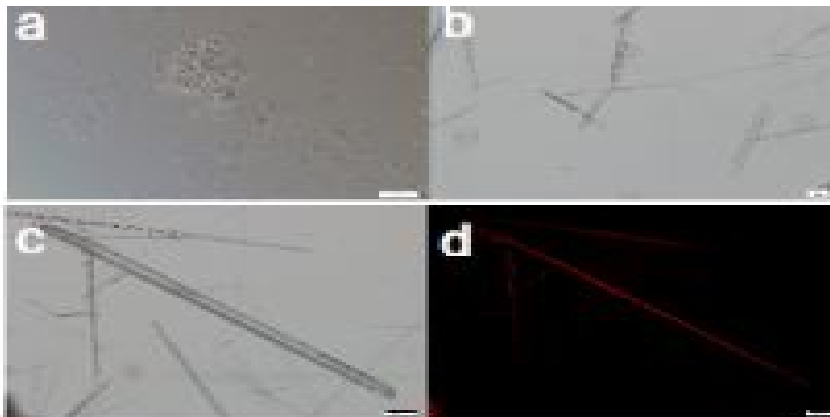


Figure 8: Aggregates of Sph-DNA with the compound formed by mixing of adenosine and monolaurin (A-M).

Sph was added to DNA, then A-M was added to the Sph-DNA mixture. Two types of aggregates formed (mucooid-type in Fig8a and crystal-type in Fig8b). Scale bar 50 μ m.

Typical crystal-type of aggregates was shown in Fig8c

The sample was stained with ethidium bromide. Russert light was observed on the surfaces of the crystal aggregates under fluorescence microscopy (Fig8d), suggesting that the surface contains DNA. Scale bar 50 μ m.

Fig.8c and Fig.8d are the same field of view.



Figure. 9: Construction of DNA crown cells



Figure. 10: A typical DNA crown cell

Sph was added to DNA. After mixing, A-M was added to Sph-DNA mixture, then monolaurin was added to the Sph-DNA-A-M mixtures. The cells of various sizes were observed under a phase contrast microscopy, showing DNA crown cells. Scale bar 50 μm A phase contrast microscopic image of typical cells were shown in Figure.10, The outside layer of cell membrane were observed.(arrow). Scale bar is 20 μm .

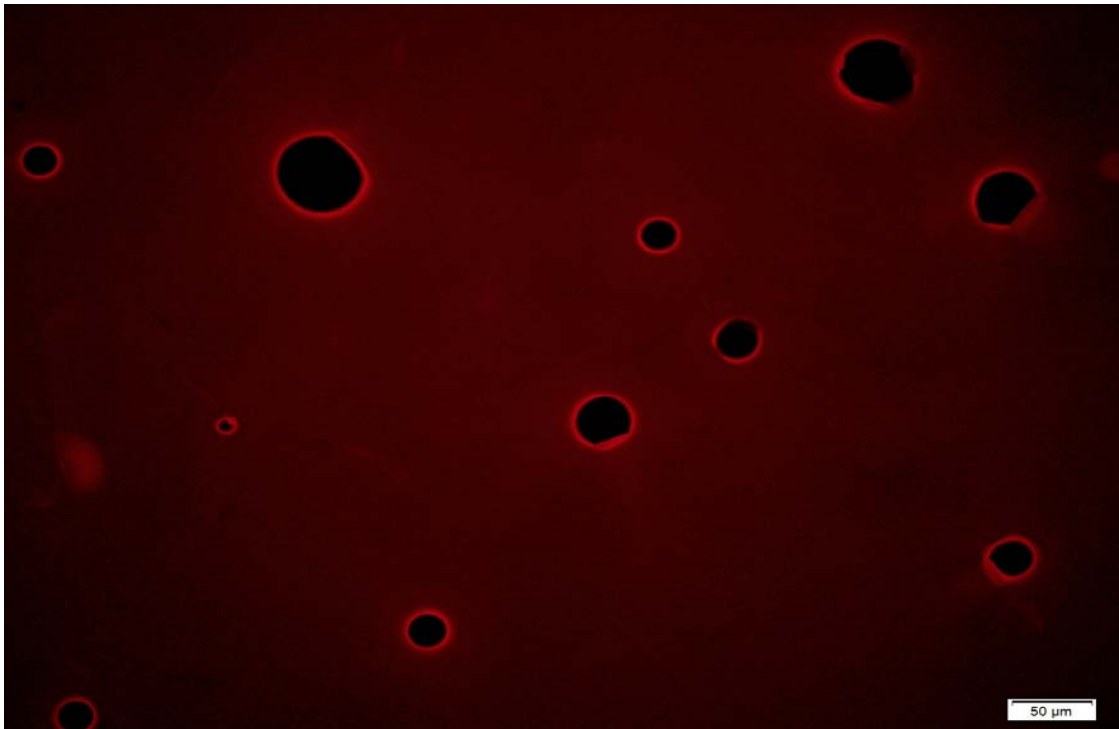


Figure 11: Fluorescence microscopic image of DNA crown cells

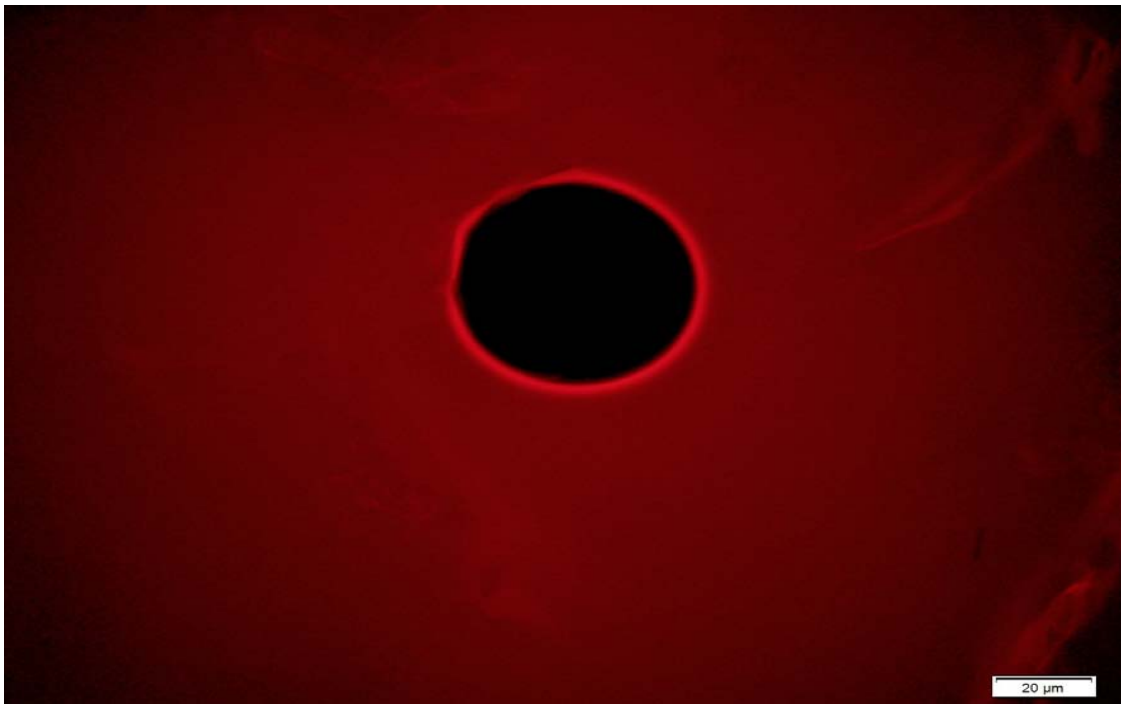


Figure 12: Fluorescence microscope image of a typical cell

DNA crown cells were prepared as described in Fig10 and stained with echidium bromide. Various cells in size were observed. Russett light is observed on the surfaces of the all cells under fluorescence microscopy, indicating that DNA is present on the surface of the cells (DNA crown cells). Scale bar 50 μm .

A typical cell using fluorescence microscopy is shown in Fig.12. Russett light was observed on the wall of the cell, indicating that the wall contains DNA. Scale bar 20 μm .



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Effects of Yeast and Oxygen on Quality Attributes of Wine Produced From Ethiopian Beetroot

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Keywords: beetroot, wine, fermentation, yeast, acid.

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Effects of Yeast and Oxygen on Quality Attributes of Wine Produced From Ethiopian Beetroot

Zinabu Hailu ^α & Derese Mekonnen ^σ

Abstract- The study was focused on the evaluation of chemical composition and quality of wine produced from Ethiopian beetroot such as Ethanol, Volatile Acidity, Titrable Acidity, Specific Gravity and pH. The basic ingredients used for fermentation were water, sugar, yeast, beetroot juice and citric acid. After that, the experimental set up was design through randomized block design with four treatments such as: F-1: beetroot juice mixed with water, sugar, acid and yeast and put under anaerobic fermentation. F-2: conditions similar with fermentation treatment one, rather yeast absence. F-3: this also same condition as treatment condition one, but it put under aerobic conditions, and F-4: with same condition with others under aerobic fermentation with absence of only yeast then maintained for 14 days.

The results obtained showed that treatment F₁-A of Ethanol- 13%w/v, TA- 0.65%v/v, VA- 0.15%v/v, pH- 3.49, SG- 0.99 and F₂-B, F₃-C and F₄-D (15.5,0.735,0.11, 2.89,1.12;7.15,0.6,0.131,3.31,0.983 and 11.17,0.622,0.121, 3.53,0.985) respectively. By using statically analysis and sensory analysis treatment F₁-A was highly acceptable with ($p < 0.02008$) and panelist overall acceptance of 8.8 ± 0.61 . Finally, it can be conclude that beetroot juice treated with pure water, yeast, acid and sugar under anaerobic fermentation is produced wonderful wine.

Keywords: beetroot, wine, fermentation, yeast, acid.

I. INTRODUCTION

Production of wine was first discovered by ancient people using application of fermentation technology of fruits having sugar [1]. Today amateur winemaking is enjoyed by thousands of people throughout the world [8]. Wine consists of flavoring, sugar, acid, tannin and yeast but above all water [6, 3]. The whole production of the wine has to be nurtured to perfection and this winemaking process must not be rushed. Wine is an alcoholic beverage produced through the partial or total fermentation of wine producing fruits. It is the fermented product of the fruit of several species of *Vitis*, mainly *V. Vinifera* [11]. Though, the suitability of fruits other than grapes has been investigated all over the world, like sugar beet, banana, pineapple, beet root etc [3]. Beetroot is a valuable vegetable, which is semi-hardy and biennial. It is grown year-round for its sweet, tender, succulent roots. Beets

contain more sugar than any other vegetable, and its earthy taste and aroma comes from an organic compound called geosmin [12, 9]. Beets of different colors, sizes and shaped have been grown, ranging from red, yellow, white, multi-colored, round, long cylindrical and huge sugar and mangle beets. Beet root has Red color naturally and its color is mostly stable at pH of 4.5–5.5; it is rather unstable outside this range [9, 7]. The composition of beetroot, 100g Nutritional value per 100 g (3.5 oz) Energy 180 kJ (43 kcal) Carbohydrates 9.56 g (6.76 g of sugar) , Dietary fiber 2.8 g, Fat 0.17 g , Protein 1.61 g, Water 87.58g and others 0.72g [7, 11, 12]. Wine production is a technique of great regard and complexity to produce a beverage enjoyed worldwide. The process begins at the vineyard, where wine beetroot using special techniques for cultivating and maintaining the crop, depending upon the species of Beetroot and type of wine associated [6]. Thereafter, the Beetroot are harvested, upon which they are then either extracted of their juices, called the must, in a process called crushing either mechanically or by treading, the traditional method of basically stepping on the Beetroot in a large container. Beetroot wines, as described in this project, are produced by fermented the raw beetroot juice, from which the alcohol that is produced during the fermenting process will begin to give acceptable wine characterize [10, 16]. Fermentation commences by adding the juices, sugar and citric acid and yeast together which then produces alcohol and carbon dioxide [16]. The ethanol content of the beetroot fruit is in between, 7-14% [5]. Thus, the study was focused on the effects of yeast and oxygen in quality attributes of wine produced from Ethiopian beetroot.

II. MATERIALS AND METHODS

Beta vulgar type beetroot was collected from Adama town which is located 98km far away the capital city of Ethiopia, and then transported to Adama Science and Technology University (ASTU), Chemical Engineering department, Food engineering laboratory. Then, the overall experimental work was set as shown in figure below (Fig 2.1). About 2.5 kg of beetroot fruit was cleaned or washed thoroughly and sliced thinly using knife after the fresh beetroot selected. The sliced one

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was placed in juice extractor to be extracted the juice from the beetroot fruit. The chemical composition and quality parameters of wine produced from beetroot were done before and after fermentation. The experimental set up of fermentation treatments were designed in such way: F-1:0.25kg Sugar, 15ml of citric acid (to enhance distinct aroma/flavor), 2.25L of water, 1.25 L of beetroot fruit juice and 0.00017kg yeast was added and mixed together, then placed under anaerobic fermentation condition. F-2:1.25 L of beetroot fruit juice was mixed with 0.25kg Sugar, 15ml of citric acid (to enhance distinct aroma/flavor), 2.25L of water and no yeast; placed under anaerobic fermentation. F-3:1.25 L of beetroot fruit juice was mixed with 10ml citric acid, 2L water and 0.0017kg yeast; put under aerobic fermentation. F-4:1.25 L of beetroot fruit juice was mixed with 10ml citric acid, 2L water and 0.0017kg yeast; placed under aerobic fermentation conditions. The chemical analysis of the product was done before (at time of juice) and after 14 days of fermentation time. Total soluble solid (TSS) was measured by using Model-RF18 (India) handheld sugar refractometer. Titrable Acidity (TA) of the fruit was measured by means of an

acid base titration method using a juice sample (10 ml juice + 10 ml distilled water) and 0.1N Sodium hydroxide (NaOH) with phenolphthalein color indicator. Volatile Acidity (VA) within the distillate samples also expressed in terms of acetic acid mg/100ml and pH was measured using digital pH meter. The specific gravity (SG) and alcohol content of the product (wine) were measured by hydrometer (AOAC2000 method). For the sensory evaluation tests, fifteen panelists was selected from the university and the tests involved individuals in isolated tasting conditions. All panelists were asked to give their individual ratings on all quality attributes of stored fruits including color, aroma, taste, flavor, firmness and overall acceptability of the wine produced from different fermentation conditions using a 9-point structured hedonic scale to conduct the preference test: 1- dislike extremely and 9- like extremely. The scores marked by panelists were collected and an average was calculated for each parameter. One way Analysis of variance (ANOVA) was performed on the data collected using Microsoft excel and Origin Pro8 soft wares.

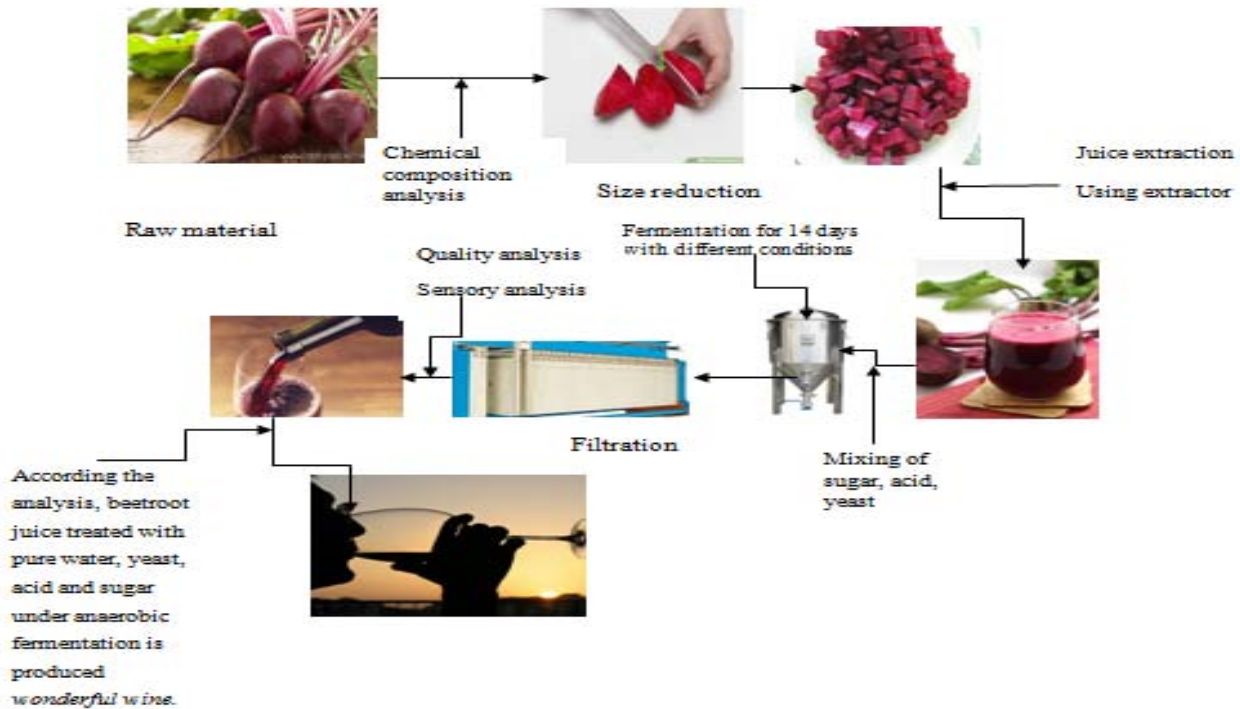


Fig 2.1: Over all experimental set up to conduct the study

III. RESULTS AND DISCUSSION

The effects of oxygen and yeast on quality and chemical composition of the product was presented in tables and figures.

a) Effect of oxygen on quality of wine

Wine produced from anaerobic (with no oxygen in excess) condition (F1-A) had high residual sugar and total soluble solid (24.5%w/v, 18% respectively) before fermentation; F4-D 15%TSS, 21%RS (Table 3.1), and then after fermentation the total sugar convert in to 13% ethanol as well as for F4-D 9.7% ethanol (Fig 3.1). The conversion of reducing sugar in to ethanol and carbon dioxide is due to the activity of microbes [14]. The highest juice yield was recorded F1-A (640ml/ kg) followed by 'B', C and D. The main prerequisite character of juice for fermentation is sugar content. Titrable acidity determined as tartaric acid range from 0.33 to 0.45% (w/v). The pH of the musts was 4.2 to 4.8. The low pH (4.8) was recorded in 'D' treatment condition, this is because lack in microbial activities. Therefore, the effect of oxygen on converting the sugars was effective under an aerobic fermentation.

b) Effect of yeast

The initial environment that affects the microbial makeup of a wine fermentation is that of the vineyard. Although a drastically different environment than juice or wine, the types of microbes present on grapes will have an impact on the ensuing ecology in the wine fermentation, particularly in the early stages. Microorganisms appear to colonize around the grape stomata where small amounts of exudates are secreted

[13]. The spontaneous fermentations were shown to contain several different strains of *S. cerevisiae* that competed within the fermentation, while the inoculated fermentation was dominated by the inoculated strain [15, 17]. The result showed that the effect of yeast in conversion of the simple carbohydrate to Ethanol, pH, TA, RS and VA was (13%, 0.65, 3.45, 2.1 and 0.15 respectively) under F1-A fermentation condition and for the other see on table 3.2. The pH of the wine produced by aerobic fermentation type is low; this is due to the conversion of all the ingredients (activity of yeast) to the alcohol and has more acidic property. The pH of the wine produced by anaerobic fermentation type is likely moderate; this is due to the reason of fermenting without oxygen that means the nutrients contain in the Fermented cannot be easily converted to alcohol, which shows the wine has weak acid characteristic. The percentage of ethanol percentage was higher with treatment F₃ beetroot fermented wines (15.7%w/v) (Table 3.2) and highest content was in the wine produced from 'F₃' and lowest in 'F₄'. Preparation of fruit beetroot fruit influenced the synthesis of higher alcohols during fermentation. Higher alcohols may influence certain sensory characteristics although they constitute a relatively lesser quantity of the total Substances. Fermentation changed the aroma of fruit juice, because of the production of yeast volatiles and the metabolism of original fruit volatiles. And it was affected by many factors like variety of fruit, clarification and fermentation conditions [13, 16].

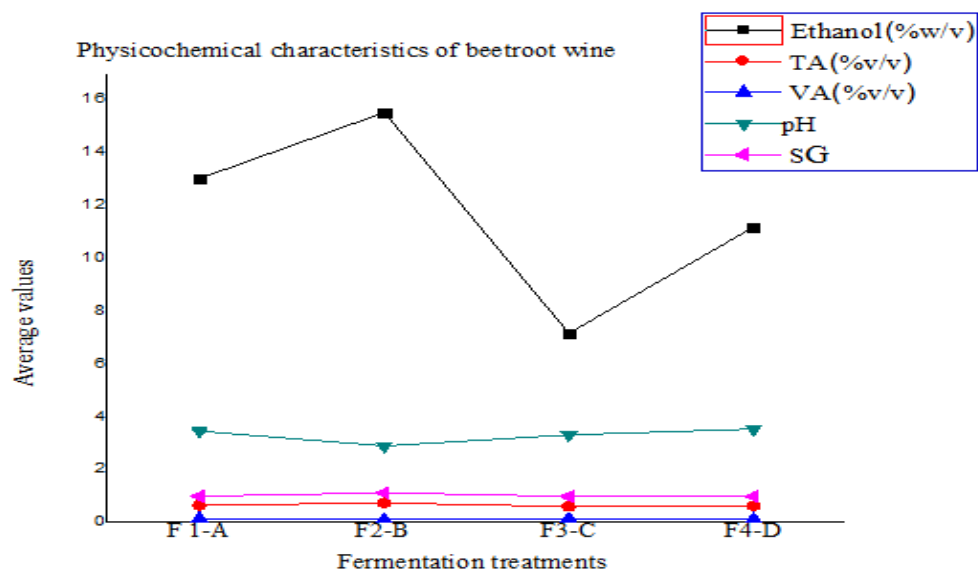


Table 3.1: Beetroot juice chemical compositions

F-wines	Juice (ml/kg)	RS (% w/v)	TA (%)	pH	TSS* %
A-J ₁	640±10	24.5±1.22	0.33	4.2±0.43	18.0±1.24
B-J ₂	510±13	18.5±1.21	0.45	4.6±0.86	16.2±1.87
C-J ₃	550±17	20.5±1.24	0.36	4.3±0.65	17.5±0.79
D-J ₄	500±22	21.0±1.15	0.38	4.8±1.10	15.5±1.23

Table 3.2: Chemical compositions of the product/beetroot wine

Wines	Ethanol (%w/v)	T.A (% v/v)	V.A (%V/V)	PH	SG	RS
F ₁ -A	13.0	0.65	0.15	3.45	0.99	2.1
F ₂ -B	12.0	0.735	0.11	2.89	1.12	2.4
F ₃ -C	15.7	0.6	0.131	3.31	0.983	2.0
F ₄ -D	9.7	0.622	0.121	3.53	0.985	2.0

T.A = Titrable acidity V.A= volatile acidity

c) Sensory evaluation

Wines produced by cells immobilized on grape skins have a better fruity aroma [4]. Similar results were also reported by [2] who found a considerable improvement in the wine sensory profile when fermentations were carried out in contact with the skins of Airen white wine grapes because of the transfer of the precursors of volatile compounds like esters, aldehydes, and alcohols into the wine. The wines produced by

immobilized yeast biocatalyst showed fine clarity at the end of fermentation with low free cell concentrations as well as characteristic pleasant soft aroma and fruity taste [12]. The result showed that, wine produced under anaerobic fermentation with the inoculation of yeast (*S. cerevisiae*) had high over all acceptances (8.8 ± 0.6) than that of produced without inoculating yeast (Fig3.2).

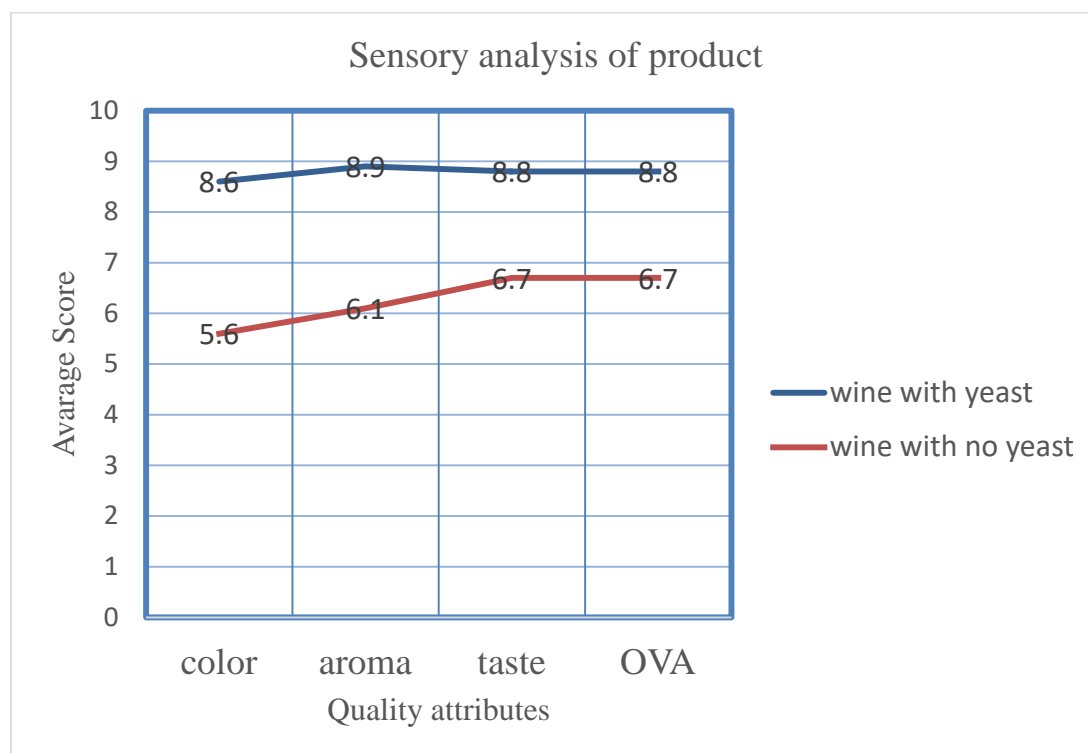


Fig 3.2: Sensory analysis result of the product

IV. CONCLUSIONS

Beetroot is a valuable vegetable and rich in nutrition, mainly it is rich in carbohydrate. By fermenting

the mixed beetroot juice and then purifying the product from by product obtained final analyzed wine. The quality of wine depends up on the compositions of the juice and fermentation conditions. The study was

evaluate the quality and compositions of wine produced from beetroot by setting different fermentation treatments; mainly anaerobic condition with and without yeast and aerobic fermentation with and without yeast. Thus, the result showed that, wine produced from fermentation treatment one (F1-A) was best product based on its quality analysis and it was confirmed by sensory analysis. Finally, it can be conclude that beetroot juice treated with pure water, yeast, acid and sugar under anaerobic fermentation is produced wonderful wine.

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Keywords: cv. keitt, controlled atmosphere storage (CAS), temperature, list significance difference.

GJRE-C Classification: FOR Code: 090499



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Effects of Controlled Atmosphere Storage and Temperature on Quality Attributes of Mango

Zinabu Hailu ^α & Teshome Worku ^σ

Abstract- This study was conducted to analyze the effects of Controlled Atmosphere Storage (CAS) and low temperatures on quality attributes of cv. Keitt mango. Mango is one of the choicest fruits in the world and popular due to its delicious taste, pleasant aroma and nutritional value. To date, only 3% of the total world production is being exported due to the perishable nature of the product, lack of proper storage and other postharvest related diseases. Post-harvest loss of mango in Ethiopia is estimated to be between 25-40% and would occur during handling, transportation and storage. Storage loss could arise owing to atmospheric conditions and temperature. CAS is agricultural technologies which monitor and adjust constantly the level of CO₂ and O₂ within storage to reduce the rate of physiological and biochemical changes, ethylene sensitivity and incidence of decay development of mangoes and also inhibit pathogen growth. Thus, this study was under taken to determine the optimum storage conditions of Ethiopian mango in order to reduce considerable postharvest mango losses. In this work, randomized block design experimental was employed for optimization of the influencing factors mainly CAS and temperatures. Accordingly, experiments were carried out at constant storage temperature of 7°C with different atmospheric conditions of A (10%CO₂ + 6%O₂+84%N₂), B (8%CO₂+ 6%O₂+86%N₂), C (5%CO₂+5%O₂+90%N₂) and Control (0.03% CO₂ + 21% O₂+79%N₂). Besides, fruits also stored at different constant temperatures of 7 °C, 10 °C, 13°C and control (21-24 °C) respectively. The result obtained showed that effect of CAS on quality attributes (weight loss and firmness) of cv. Keitt mango significantly improved the storage life as compared to that stored at ambient atmosphere with list significant difference values being at 0.05=3.12 and 1.708 respectively. In addition, the effect of temperature on Total soluble solid, Titrable acidity, pH, weight loss, firmness and skin color was found to be significant in prolonging the storage life of the fruit with List significant difference values of 0.05(0.317, 6.58, 3.46, 4.148, 6.50 and 2.41).The study revealed that the fruit stored at 7°C under 10%CO₂ +6%O₂storage condition, as confirmed by sensory analysis, was given better preference to extend cv. Keitt mango up to 6weeks. On the other hand, the fruits stored at 13°C with no CAS conditions showed better storage time of up to 21 days without chilling injury effect. Finally, it can be conclude that application of CAS technology can significantly enhance the quality and storage life thereby minimizing post-harvest losses of mango fruit.

Keywords: cv. keitt, controlled atmosphere storage (CAS), temperature, list significance difference.

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I. INTRODUCTION

Mango (*Mangifera indica* L.) is native to south Asia and known as king of the fruits due to its excellent flavor, delicious taste and high nutritive values. There are over thousands varieties around the world, the most common are Keitt, Kent and Tommy Atkins and they are also grow in Ethiopia. Mango is produced mainly in-west and east of Oromia, SNNPR, Benishangul and Amhara (Desta, 2005), which are specific areas of Ethiopia and they have a potential to produce wide variety of mango fruit. According to Ethiopian central statistical agency report in 2011/12 report about 12-14 % of total fruit production is mango. But, it is exported less than 2 % (Joosten, 2007). This is due to perishable nature of the product, lack of proper handling and lack of proper storage (Leon et. al, 1997). Besides, the post-harvest loss of mango is 26.3 %.(Tadesse 1991) Thus, Maintenance of fruit quality for a specific period of time before its consumption is important factor in the post-harvest life of the fruit. The physiological changes may occur in harvested fruit due to unfavorable atmospheric conditions especially fluctuation in temperature and humidity. Fresh mango fruits have a short storage life of 4 to 12 days at room temperature and can also suffer low temperature injury (chilling injury) during refrigerated storage (Lakshminarayana, 1973). Therefore, it is necessary to develop improved methods of mango storage in fresh state that can enhance its shelf life without any detrimental effect on the quality. Various methods of extending the storage life of mangoes have been tested but little research has been conducted on controlled atmosphere (CA) techniques. Controlled atmosphere Storage (CAS) has been shown to be beneficial technique in reducing the rate of physiological and biochemical changes, ethylene sensitivity and incidence of decay development of perishable fruit products (Medlicott & Jeger, 1987) and as a result of it senescence is delayed. The response of mangoes to CA condition reported in the literature has been shown to vary. Nakamura et al., (2004) reported that CA storage having 5-10% CO₂ is effective to suppress the respiration rate of ripe mango. Lalel et al., (2003) found that CA comprised of 2% O₂ and 2% CO₂ is better for maintaining the aromatic compounds of ripen fruit. Similarly Mitra & Baldwin (2005) stated that low temperature, hypobaric and CA storage can keep the

mango fruit for about two to four weeks however, they suggested further research in this area. Lalel et al.,(2005) reported that CA treatments reduced respiration rate, de-greening of the skin and fruit softening of mangoes but yellowness of the skin, total soluble solids (TSS), total sugars content and taste of the ripe fruit were maintained. In another research Palding and Reeder (1977) recommended that shelf life of keitt mangoes can be increased up to 35 days without any significant quality effect by storing them at 13°C in 5% CO₂ and 5% O₂. Different varieties of mangoes showed different responses to CA storage (Rattanapanone, 2001). Therefore, the study focused with effects of controlled atmosphere storage and temperature on quality attributes of Ethiopian mango to reduce the post-harvest loss and maintain desired quality. The experiment was carried out to evaluate the effects of CAS and temperature, determine sensory evaluation and optimum storage condition of cv. Keitt mango.

II. MATERIALS AND METHODS

The mango cv. Keitt sample used in this experiment was collected in plastic bags from Addis Ababa Et-fruit. The fruits were sorted according to their size, weight and color. Then the sorted fruits were washed by tap water, and dry through blowing dried air stored in CA storage, and ambient storage condition. To determine the effects of temperature and CAS, Completely Randomized Design (CRD) was used with below treatments: Storing fruits at constant temperature of 7°C under CAS conditions of A-(6% O₂ + 10%CO₂), B-(6%O₂+ 8% CO₂), C-(5%O₂+ 5% CO₂) and D-Control(21% O₂ + 0.03% CO₂). Besides, the effect of temperatures without considering CAS effect was also studied fruits stored at different storage temperature of 7°C, 10°C, 13°C and Control (21-24°C) respectively. About 112 uniform cv. Keitt mango fruits samples were used for studying out of which 42 samples were stored at CAS, with gas tight plastic bag in order to control the atmosphere by using closed system, 14 samples were stored in ambient atmosphere, while the remaining 56 samples were investigated for temperature effects. Each Sample was randomly taken within five day intervals to evaluate the effect of Controlled Atmosphere Storage (CAS). CAS chambers were calibrated to establish the specified gas composition by a gas blending flow system. The gas blending system generated CA conditions using external supplies of gases from, pressurized gas cylinders fitted with double-stage regulators and outlet controlling devices. These outlets were connected to the inlet flexible pipes that were inserted into the gas tight plastic containers in which the mangoes were stored. Fruits were then stored at specified temperature with different controlled atmosphere which were evaluated every 5 days for total

soluble solids (TSS), Titratable acidity (TA), pH, weight loss, firmness, and skin color. To investigate effects of different temperatures, sample was taken within 7 days interval using I Button. The One-Wire Viewer a Java™-based software package is used to explore Maxim's 1-Wire and I Button devices with a personal computer. The 1-Wire and I Button devices communicate over a single data line plus ground reference, using the 1-Wire protocol. The skin color was measured by using skin indicator chart 1-deep green 2-light yellow-green, 3-yellow-light green, 4-yellow-orange 5-golden orange. Flash firmness was measured (kg/mm²) using FACCHINI-48011-Model (Italy) penetrometer with 8mm probe diameter. Total soluble solid (TSS) was measured by using Model-RF18 (India) handheld sugar refractometer. Titrable Acidity of the fruit was measured by means of an acid base titration method using a juice sample (10 ml juice + 10 ml distilled water) and 0.1N Sodium hydroxide (NaOH) with phenolphthalein color indicator and pH was measured using digital pH meter. Weight loss also measured using digital weight balance. For the sensory evaluation tests, ten panelists was selected from the university and the tests involved individuals in isolated tasting conditions. Five of panelists were asked to give their individual ratings on all quality attributes of stored fruits including color, aroma, taste, flavor, firmness and overall acceptability of the stored fruits with different conditions while the other five panelists were asked to evaluate quality of the effects of temperatures with a 9-point structured hedonic scale to conduct the preference test: 1- dislike extremely and 9- like extremely. The scores marked by panelists were collected and an average was calculated for each parameter. One way Analysis of variance (ANOVA) was performed on the data collected using Microsoft excel and Origin Pro 8 soft ware's. Comparison between treatment means at 5% level of significance was calculated.

III. RESULTS AND DISCUSSION

The effects of controlled atmosphere storage (CAS) and low temperatures analysis for quality attributes of cv. Keitt mango and the optimum conditions for long storage were studied. In addition, sensory analysis for the final product using nine hedonic scale also carried out. The results of samples analyzed in this work are presented in tabular and graphical form.

a) *The Effect of CAS on Quality Attributes of cv. Keitt Mango*

Skin color: as the skin indicator chart showed that(1-deep green, 2-light yellow-green, 3-yellow-light green, 4-yellow orange, 5- golden orange), Skin color of fruits stored at ambient atmosphere changed their color from deep green to yellow orange after 15 days and to golden-orange after 30 days of storage (see on appendix Table 4.2B), whereas the skin color of fruits

stored at controlled changed their color after 25 days from deep green to yellow orange and to golden orange colors after 40 days of storage (see on appendix Table 4.1A). The loss of green color was the most obvious change in mango which was probably due to the physiochemical change by degradation of the chlorophyll structure and increased in carotenoids pigments during storage. The principal agents

responsible for this degradation might be oxidative system, pH change, and enzymes like chlorophyllase (wills et.al. 1982). The experimental result observed, showed in significant of CAS on skin color (new LSD at $0.05=1.36$) between fruits stored in CAS and ambient atmosphere as presented in (Fig 4.1) and the effect of CAS and ambient atmosphere had similar mean result (5-golden orange) after 40 days storage.

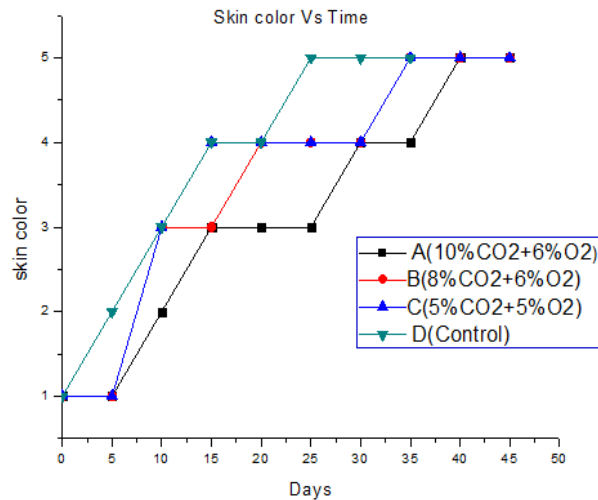


Fig 4.1: Skin color of mangoes at 7°C after storage under controlled atmosphere

Flash Firmness: Mangoes exposed to CAS used in this work remained greener and more firm than those stored in ambient atmosphere (Fig 4.2). The current investigations confirmed the view that CAS conditions delay fruit ripening and softening (Kader, 1986). Ripening and senescence rates in many climacteric fruits like mangoes, can be affected by control of the availability of O₂ and CO₂ to the fruit during respiration and that these two compounds can have a significant inhibitory effect

on ability of ethylene to initiate ripening (Ben-Yehoshua et al., 2005). Thus, flash firmness of fruits kept in air significantly decreased very rapidly from 14.1 kg/mm² to 0.099 kg/mm² after 35 days of storage (see on appendix Table 4.2B) and new LSD at $0.05=1.708$. The flesh firmness of fruits which were kept under (CAS) with treatment of (10% CO₂ + 6% O₂) had high firmness than the other treatments after 6 weeks storage with mean firmness result being 5.541 kg/mm² (Fig 4.7)

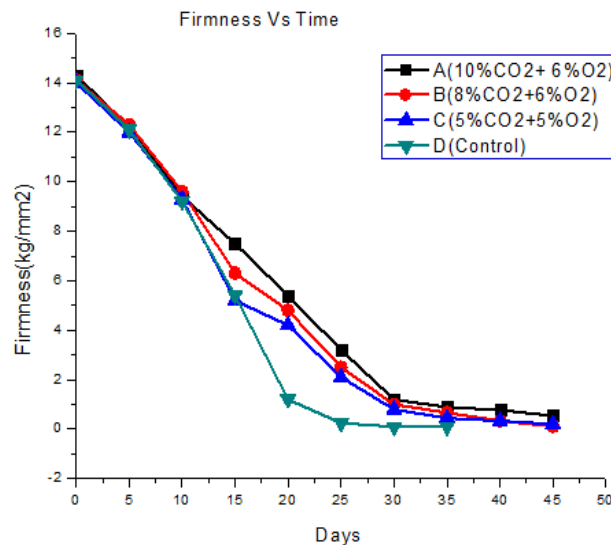


Fig 4. 2: Flesh firmness of mangoes at 7°C after storage under controlled atmosphere

Total Soluble Solid (TSS): it was observed that, TSS of fruits stored in ambient atmosphere were increased from 4.1 to 18.86°Brix after 30 days and decreased to 14.8°Brix after 35 days storage (see on appendix Table 4.2B). The increase in TSS might be due to alteration in cell wall structure and break down of complex carbohydrates into simple sugars. As showed the graph below fig4.3 the total soluble solid of fruits stored at ambient atmosphere sharply decreased in between 30 to 35 storage time, this is due to metabolic process as the temperature become high microbial and respiration activities become increased ,conversion of sugar into alcohol(ethanol)become exist. The acidity of the fruit also becomes high. This increase and decrease in TSS are directly correlated with hydrolytic changes in starch and conversion of starch to sugar being important index

of ripening process in mango fruits and other climacteric fruits and further hydrolysis decreased the TSS during storage (kittur et.al. 2001).Beside, fruits stored at controlled atmosphere storage also increased the TSS content from 4.1 to 18.25°Brix after 45 days of storage (see on appendix Table 4.1A). As the result showed, it would stay in CAS further days without significant quality effects. From the CAS treatments; treatment- A (10%CO₂+6%O₂) had less TSS content with average (13.5°Brix) than of the other treatments as shown in Fig 4.2. Hence, this treatment has a potential to extend the storage life of fruits beyond 45 days of storage time. No significant change was observed in TSS between the fruit samples kept at ambient atmosphere and CAS (new LSD at 0.05=4.406).

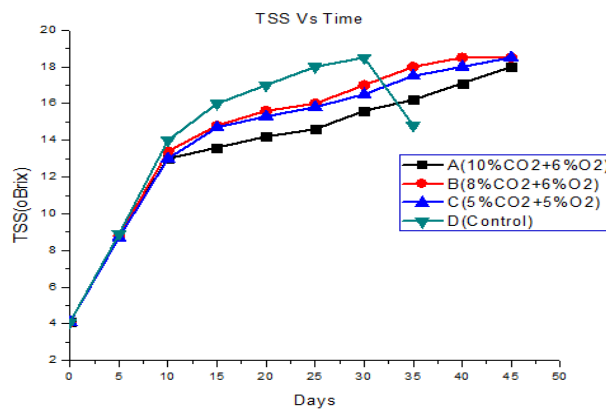


Fig 4.3: Total soluble solid content of mangoes at 7°C after storage under controlled atmosphere.

Titrateable Acidity (TAA): The change in Titrateable acidity of the mango (cv. Keitt) recorded during storage at ambient temperature of (21-24°C) is presented in appendix Table 4.2B. The results revealed that percent Titrateable acidity of treated cv. Keitt mango ranged from 1.665% to 0.0234% with an average of 0.52% (Fig 4.7) during storage and the pH also increased from 2.4 to 5.16 after 35 day of storage. the results observed, indicated that percent of Titrateable acidity showed decreasing trend during 35 days of storage period might be due to the degradation of citric acid which could be attributed to ripening or reduction in acidity

may be due to their conversion into sugars and their further utilization in metabolic process of the fruit. These results coincided with those of Doreyappa-Gowda and Huddar (2001) who reported similar pattern in different varieties of mango fruit stored at 18-34°C under gone a series of Physico-chemical changes during ripening and the major changes were considerably increased in pH from 2.85 to 4.38 and decreased in acidity from 2.71 to 0.04% during ripening. Thus, no significant change in TAA and pH; between fruits kept at ambient atmosphere and CAS were observed (new LSD at 0.05=0.536 and 1.118) respectively (Fig4.4).

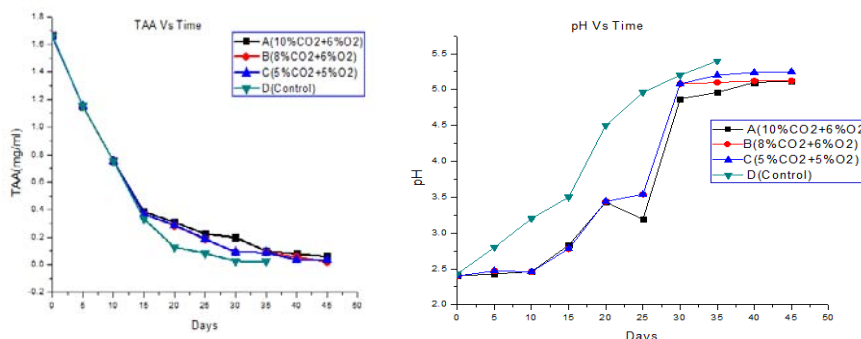


Fig 4.4: Titrateable acidity and pH of mangoes at 7°C after storage under controlled atmosphere

Weight loss (%): The reduction in weight is attributed to the physiological loss in weight (PLW) due to respiration, transpiration of water through peel tissue and other biological changes taking place in the fruit. The effect of CAS was highly significant on weight loss of stored fruit (new LSD at 0.05 = 3.12). The mean weight loss

percentage of fruits stored with treatment A (10% CO₂+ 6%O₂) was less (0.75%), whereas fruits stored at ambient atmosphere was high (5.87%) after 4-6 weeks of storage (Fig4.6).

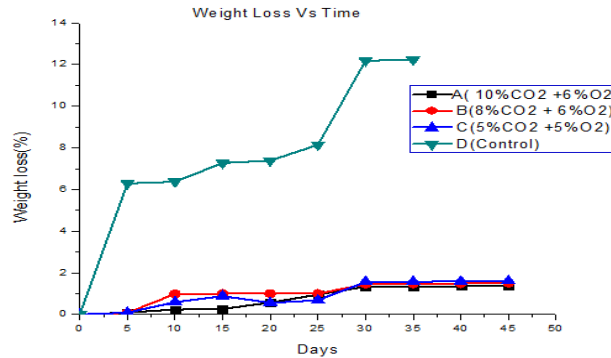


Fig 4.6: Weight loss of mangoes at 7°C after storage under controlled atmosphere

b) *Effect of Temperatures on Quality Attributes of Stored cv. Keitt Mango*

Total Soluble Solid (TSS): Significant increase in sucrose content of mango has been observed during ripening and this has been attributed to an increase in total soluble solids during ripening. This is due to transformation of starch into soluble sugars as the carbohydrates in the fruit are broken down under the action of phosphorylase enzyme during ripening into simple sugars (Mitra S et.al., 1997). Storage temperature significantly (new LSD at 0.05=0.317) affected the TSS of fruits. As the mean storage temperature decreased TSS values obtained were 14.07 °Brix, 12.87 °Brix, and 9.87 °Brix for the fruit stored 21-24

°C, 13°C and 7°C respectively after 3weeks storage time (Fig4.7).

Titrateable Acidity (TAA):Titrateable Acidity of the stored fruit were significantly decreased as the temperature of the storage increased (new LSD at 0.05=6.58) after three weeks of storage (see Table 4.3C appidx) with the mean values of 1.2865 mg/10mg juice at temperatures of 7°C and 0.937 mg/10mg juice at ambient temperature(21-24°C) after 3 weeks storage period. The decline in acidity could be due to susceptibility of citric acid to oxidative destruction as impacted by the ripening environment, and also it is a consequence of starch hydrolysis leading to an increase in total sugars and a reduction in acidity (Aina JO, 1990).

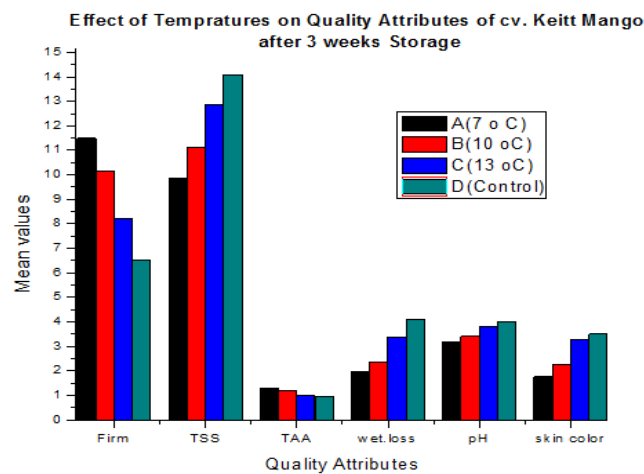


Fig 4.7: Effects of temperatures on cv. Keitt mango quality attributes after 3 weeks storage

Increase in pH during ripening of mango fruits has been reported by other authors (Tovar B.et.al.2000)

and was similar to what was observed in the present study. According to the authors, there is an inverse

relationship between titratable acidity and pH. The increase in pH (decline in acidity) could be due to utilization of acids as respiration substrates (Dadzie BK et.al.1997).The pH of the fruits also significantly affected by storage temperatures (new LSD at 0.05=3.46); as the storage temperature increased from 7°C to ambient temperature (21-24°C). The pH of the fruits also increased from 3.18 to 4.01 after three weeks storage as shown in (see on appdixTable 4.3C).

Skin color: the average skin color of fruit samples stored at 7 °C, 10 °C, 13 °C and 21-24°C were light yellow green light yellow green, yellow-green and yellow orange respectively(see on appdixTable 4.3C). According to the result, the effect of temperatures on skin color of the stored fruits was not significance changes fruits which was exposed to 7, 10, and 13°C, whereas fruits stored in between 7 °C and ambient temperature(21-24°) was significant (new LSD at 0.05=2.41).

Flash Firmness: Effect of storage temperatures on firmness of cv. Keitt showed significant difference between fruits stored at temperature of 7 °C and ambient (21-24°C) temperature (see on appdix Table4.3C)with new LSD at 0.05=6.50)

Weight loss (%): Maximum average weight loss percentage loss observed for fruits stored at ambient temperature (4.44%) after three weeks storage (new LSD at 0.05=4.148). As temperature of storage increased the average weight loss also increased (Table4.3C).

It was observed that Mangoes stored at low temperature leads to certain physiological disorder observed such as Chilling Injuries (CI). The primary cause of CI is thought to be the damage of cell membrane that initiates a cascade of secondary reaction. CI is a time and temperature problem. Mango fruits are subjected to CI when stored below 10°C. The symptoms include grayish scald- like discoloration of the skin, skin pitting, uneven ripening, and reduction in the level of carotenoids, aroma and flavor during ripening (Thomas,. P et.al a1983).Most of mangoes cultivars shows injury below 10°C (50 °F), especially if fruit have just reached maturity. Tolerance to chilling injuries increases during ripening (Medlicott et al. 1990). Chilling injury (CI) has been reported to occurs in mango fruit at temperatures below about 10-13°C, although some cultivars (Dasheri, Langara) were reported to be safely stored at 7-8°C for up to 25 days. Storage at 10 to 13°C (50 to 55°F) with 85 to 90% relative humidity should give a shelf-life of 14 to 28 days for mature green fruit, depending upon variety (Jobin Decor 1988).The result of the experiment showed that fruits stored at temperature of 7°C after three weeks storage time, were exposed to CI starting from 7 days storage, for the fruit stored at 7°C with symptoms of un ripening, skin pitting, discoloration, poor aroma and flavor during ripening and is in agreement with Jobin Decor result (Fig4.9).



Fig 4.8: Photograph of Keitt variety mango fruit before storage

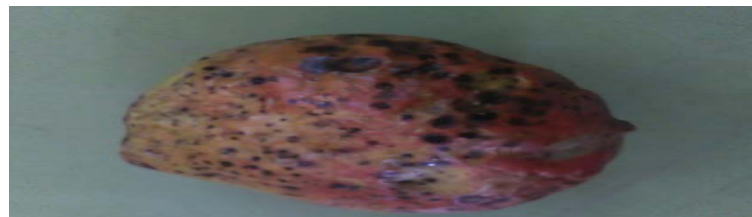


Fig 4.9: Photograph of Keitt type mango fruits stored at temperatures of 7° C

c) Effect of CAS and Temperatures on Sensory Quality Attributes (SQA)

Color changes in mango fruit are due to the disappearance of chlorophyll and appearance of other pigments (Lakshminarayana at.el.1980). The observed results of skin color of fruits stored with treatment (10%CO₂ + 6%O₂) showed maximum preference with it mean and standard deviation result was 8.4 ± 0.89 (Table4.4). Fruit flavor depends upon taste (balance

between sweetness and sourness or acidity, and low to no astringency) and aroma (concentrations of odor-active volatile compounds). Taste development is due to a general increase in sweetness, which is the result of increased gluconeogenesis, hydrolysis of polysaccharides, especially starch, decreased acidity, and accumulation of sugars and organic acids resulting in an excellent sugar/acid blend (Grierson et al., 1981) Sourness or acidity is determined by the concentrations

of the predominant organic acids, which are citric acid, malic acid, and tartaric acid. Some amino acids, such as aspartic and glutamic acid may also contribute to sourness. Several factors affect sugar and acid contents in mango, including cultivar (Kapse et al., 1989) The flavor of Mangoes stored under treatment conditions of A (10%CO₂ + 6%O₂) and B (8%CO₂ + 6%O₂) with average values of 7.4 was preferred by panelists.

But, the overall acceptance was high for fruits stored with treatment A (10%CO₂ + 6%O₂) mean result of 8.0 (see on appendix Table 4.4 Similarly, the effects of temperatures on quality of stored fruits was evaluated by sensory panelists, the color of the fruits stored at temperature of 13 and ambient (21-24°C) had high preferences with their mean values got 7.6 (see on appendix Table 4.5E). Fruit samples stored at temperature of 13°C got maximum overall acceptance, while fruits stored at temperature of 7°C got minimum overall acceptance (Table 4.5E)

IV. CONCLUSIONS

Mangoes are highly nutritious and favorable fruits. However, post-harvest losses and quality deterioration due to exposure to temperature and improper handling management challenged the productivity and wider usage of the fruit. In Ethiopia, the post-harvest loss (transporting, handling and storage) of mango is estimated as to be about 23.6% from this total loss about 7-10% is storage loss. The major storage loss factors are atmospheric conditions and temperature. Thus, study was carried out by using CAS technology which is constantly monitor and adjust the CO₂ and O₂ levels within gas tight stores or containers to reduce storage loss and extending the storage life of mangoes for better duration without loss of its quality.

The Controlled Atmosphere Storage (CAS) positively affected the quality parameters and significantly improved the shelf life of mangoes. It extends storage life of cv. Keitt mangoes for up to 6 weeks without significant quality loss with treatment at 7°C under 10%CO₂ + 6%O₂+84%N₂. This condition is better to store the mango for long time without significance loss relative the other conditions and quality of the fruit after storage also confirmed by sensory analysis, thus the above condition is best. On the other hand, the effects of storage temperature on quality attributes of mangoes were significant. The cv. Keitt mangoes stored at 13°C had better quality after 3 weeks storage time as confirmed by sensory analysis than of fruits stored with other treatments. In general, extending storage duration more than 6 weeks is extending marketing period and maintained fruits availability.

Declaration

This work was our original work; there is no conflict interest among authors.

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APPENDIXES

Table 4.1 A: Mean results of mango quality attributes stored in CAS for 0- 45 days

Days	Firmness (kg/mm ²)	TSS(°Brix)	TAA (%)	pH	Weight loss (%)	Skin color
0	14.13	4.1	1.66	2.40	0	1
5	12.16	8.8	1.151	2.45	0.0869	1
10	9.43	13.13	0.754	2.46	0.603	2.6
15	6.33	14.36	0.373	2.79	0.533	3.3
20	4.8	15.03	0.293	3.43	0.457	3.6
25	2.6	15.46	0.198	3.42	0.623	3.6
30	1	16.36	0.127	5.01	1.452	4
35	0.67	17.23	0.091	5.08	1.457	4.6
40	0.48	17.86	0.056	5.15	1.475	5
45	0.29	18.33	0.0391	5.16	1.491	5

NB: 1-deep green, 2-light yellow-green, 3-yellow-light green, 4- yellow orange,5-golden-orange

Table 4.2 B: Mean results of mango quality attributes stored in ambient atmosphere for 0-35 days

Days	Firmness (kg/mm ²)	TSS(°Brix)	TAA (%)	pH	Weight loss (%)	Skin Color
0	14.1	4.1	1.665	2.40	0	1
5	12.1	8.9	1.152	2.83	6.289	2
10	9.2	14	0.7541	3.24	5.370	3
15	5.4	16	0.3328	3.54	3.272	4
20	1.2	17	0.128	4.56	4.384	4
25	0.25	18	0.0828	4.96	3.144	5
30	0.1	18.5	0.0256	5.27	12.182	5
35	0.099	14.8	0.0234	5.48	12.234	5

NB: 1-deep green, 2-light yellow-green, 3-yellow-light green, 4- yellow orange,5-golden-orange

Table 4.3C: Mean effect of temperatures on mango quality attributes after 3 weeks storage.

Temperatures	Firm(kg/mm ²)	TAA (%)	TSS(°Brix)	Wet loss (%)	pH	Skin color
7 °C	11.5	1.2865	9.875	2.6	3.18	1
10 °C	10.125	1.202	11.125	2.9	3.3975	2
13 °C	8.2	0.98975	12.875	4.25	3.81	3
Control	6.255	0.9374	14.075	4.44	4.015	4

NB: 1-deep green, 2-light yellow-green, 3-yellow-light green, 4- yellow orange, 5- golden orange

Table 4.4 D: Effect of CAS on Sensory Quality Attributes of stored fruits (Means \pm SD)

SQA	A	B	C	D
Color	8.4 \pm 0.89	7.8 \pm 0.83	7.6 \pm 1.14	7.8 \pm 1.09
Flavor	7.4 \pm 1.14	7.4 \pm 0.89	6.8 \pm 1.30	7 \pm 2.00
Taste	8 \pm 0.70	8.6 \pm 0.54	6 \pm 1.58	5.8 \pm 2.38
Firmness	7.4 \pm 1.14	7.4 \pm 0.54	7.4 \pm 0.54	8 \pm 0.00
Over acceptance	all 8 \pm 0.70	7.8 \pm 0.44	7.2 \pm 0.83	6.8 \pm 1.78

NB: A (10%CO₂+6%O₂), B (8%CO₂+6%O₂), C (5%CO₂+5%O₂) & D (Control)

Table 4.5 E: Effect of Temperatures on Sensory Quality attributes (Means \pm SD)

SQA	7°C	10°C	13°C	Control(21-24°C)
Color	4 \pm 1.58	6.4 \pm 1.51	7.6 \pm 1.14	7.6 \pm 1.14
Flavor	3.2 \pm 1.48	5.8 \pm 0.83	8 \pm 0.70	7.2 \pm 0.83
Taste	3.4 \pm 0.89	5.2 \pm 0.83	7.6 \pm 1.14	5.8 \pm 0.83
Firmness	3.8 \pm 3.8	6 \pm 1.22	7.2 \pm 0.83	5.6 \pm 1.14
Over acceptance	all 3.6 \pm 3.6	6.4 \pm 0.54	8 \pm 0.70	6.4 \pm 0.54

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An Overview on the Production of Microbial Copper Nanoparticles by Bacteria, Fungi and Algae

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Keywords: *metallic nanoparticles, copper nanoparticles, microorganisms, biotechnological processes.*

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An Overview on the Production of Microbial Copper Nanoparticles by Bacteria, Fungi and Algae

Cristiano José de Andrade ^α, Lidiane Maria de Andrade ^σ, Maria Anita Mendes ^ρ
& Claudio Augusto Oller do Nascimento ^ω

Abstract- Bionanotechnology is an emerging field, which involves multidisciplinary areas such as engineering, chemistry, biology, among others. Bionanotechnology encompasses the production of organic and inorganic nanomaterials by living organisms such as vegetable, animal and microbial cells. In this sense, the microbial productions of metallic nanoparticles have drawn much attention mainly due to their alignment with the principles and concepts of green chemistry (no need for organic solvent). A wide diversity of biological organisms, such as bacteria, lichens, fungi, yeasts and algae, produce metallic nanoparticles. This mini-review specifically highlights the main keys to the production of copper nanoparticles by bacteria and fungi. In addition, this report indicates the lack of knowledge on the production of copper nanoparticles by algae, as well as the purification and application of metallic nanoparticles.

Keywords: metallic nanoparticles, copper nanoparticles, microorganisms, biotechnological processes.

I. INTRODUCTION

Nanotechnology is an emerging field. The application of nanomaterials is predicted to reach 58,000 tons by 2020 (Maynard et al., 2006). Nanoparticles are defined to range within 1-100 nm (diameter). The chemical composition, size and shape of nanoparticles have a significant effect on their properties (Singh et al., 2010; Gurav et al., 2014; Shobha et al., 2014).

One of the earliest studies on the production of metallic nanoparticles by microorganisms (bacteria) was reported by Temple and Le Roux, (1964). However, only in the 21st century has the production of metallic nanoparticles been more deeply investigated.

The electro-chemical method is the most feasible to produce copper nanoparticle (short period of time to synthesize large quantities of nanoparticles). In this sense, nChemi - a startup company located in São Carlos, Brazil - has been working on developing, customizing and fabricating metal oxide nanoparticles by the electro-chemical method (nChemi, 2017). However, the production of metallic nanoparticles by living organisms has competitive advantages over the electro-chemical method, such as being eco-friendly

(green chemistry concept) (Shobha et al., 2014; Cuevas et al., 2015).

Among the metallic nanoparticles produced by living organisms, gold, silver and iron are the most well-known (investigated). The metallic nanoparticle producers (living cells) have unique characteristics such as magnetosomes (organelles) that store magnetic nanocrystals composed of greigite (Fe₃S₄) or magnetite (Fe₃O₄) (Singh, 2015). These metallic nanoparticles are produced by both intra and extracellular biocompounds. The wide range of molecules favors the reduction of metal ions - Brust-Schiffrin synthesis (bottom-up approach) (Singh et al., 2010; Usha et al., 2010; Le et al., 2013; Salvadori et al., 2014; Shobha et al., 2014; Singh et al., 2015).

Although researchers have focused mainly on silver, gold and iron nanoparticles, copper nanoparticles have drawn attention due to their unique properties such as electrical, magnetic, thermal, antimicrobial (*Escherichia coli*, *Bacillus subtilis*, *Vibria cholera*, *Pseudomonas aeruginosa*, *Syphillis typhus* and *Staphylococcus aureus*), optical and catalytic, which can be used in electronic devices (lithium batteries), magnetic phase transitions, gas sensors, industrial cooling and heating, mass transfer enhancement, energy storage devices, in the production of cosmetics and pharmaceuticals, etc (Varshney et al., 2010; Gurav et al. 2014; Shobha et al., 2014; Cuevas et al., 2015, Shankar et al., 2016).

Therefore, there is a trend towards nanotechnology; particularly that applying living cells (e.g., the production of copper nanoparticles) becomes increasingly important, due to its competitiveness, effectiveness and low operational cost (Salvadori et al., 2013).

II. MICROBIAL PRODUCTION OF COPPER NANOPARTICLES

The production of copper nanoparticles by microorganisms (e.g., bacteria, fungi and algae) is relatively a novel approach. There is a wide variation in the production of metallic nanoparticles by living cells (e.g., organelles and compounds responsible for production, shape and size of nanoparticles), which

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depends on the mechanisms of metal ions bioreduction (Singh, 2015).

a) *Bacteria*

In general, the production of metallic nanoparticles by bacteria takes advantages of shorter generation times, for instance *Escherichia coli* (bacteria) 18 minutes (Bremer, 1982), versus *Saccharomyces cerevisiae* (yeast) 100 minutes (Hartwell 1974) or *Chlorella vulgaris* (microalgae) 3.35 days (Andrade et al., 2014). Usually, the production of metallic nanoparticles by bacteria occurs during the stationary phase. In theory, when compared to the logarithmic phase, greater metabolic stress is observed during the stationary phase. Consequently, metabolites with greater capacity of chemically reducing other compounds are synthesized during the stationary phase. Thus, these metabolites are able to reduce metal ions, which lead to the production of metallic nanoparticles (Hasan et al., 2007; Shobha et al., 2014; Ammar, 2016).

Theoretically, the metallic nanoparticle production is a very general microbial detoxification mechanism (soluble metals → insoluble nanosized structures), since copper ions lead to change in the helical structure by cross-linking and, consequently, to many biochemical pathways (Abboud et al., 2014). A wide range of bacteria (Table 1) is able to reduce metal ions (metallic nanoparticle production) by their compounds such as proteins, polysaccharides and periplasmic proteins (Singh et al., 2010; Le et al., 2013; Shobha et al., 2014; Singh et al., 2015). For instance, Singh et al. (2010) described the production of nanoparticles from *Escherichia coli* proteins, in which *E. coli* was cultivated in citrate minimal medium. The biomass was recovered (centrifugation) and suspended in an aqueous 1 mM CuSO₄ solution. The secreted proteins were precipitate by trichloroacetic acid followed by dialysis (deionized water) for 24 hours. Then, the proteins were concentrated by membrane (molecular weight cut-off of 3 kDa) and their profile was studied by electrophoresis. In conclusion, the proteins with 22 kDa, 25 kDa, and 52 kDa were related to the production of copper oxide nanoparticles and acted on their stabilization (copper oxide nanoparticle). Belchik et al. (2011) proved that the outer membrane c-type cytochromes of *Shewanella oneidensis* MR-1 played an important role in the reduction of Cr(VI). The authors evaluated the effects on Cr(VI) reduction by deleting the

mtrC and/or *omcA* gene. When compared with non-engineered *Shewanella oneidensis* MR-1 (wild), the *mtrC* knockout led the rate of reduction of Cr(VI) to 43.5%, *omcA* by 53.4%, both *mtrC* and *omcA* genes by 68.9% of reduction. Then, the authors proved that purified MtrC and OmcA reduced Cr(VI).

Mat Zain et al. (2014) produced copper nanoparticles by using ascorbic acid (reducing agent) in the presence of chitosan and microwave heating, in which 40 mL of copper nitrate solution (10, 30 or 50 mM) was mixed with 40 mL of chitosan solution (1, 2 or 3% w/v) and 4 mL of a 10% (w/v) ascorbic acid solution. Chitosan led to the higher stability of copper nanoparticles and avoided agglomeration. The authors defined the synthesis of copper nanoparticle as fast, inexpensive, environmentally friendly and high energy-efficient. In addition, the concentration of chitosan was positively correlated to copper nanoparticle size.

Varshney et al. (2010) reported an easy, fast, and cost-effective production of copper nanoparticles by the non-pathogenic bacteria *Pseudomonas stutzeri*. The copper nanoparticles showed great stability. Thus, the metabolites from *Pseudomonas stutzeri* produced copper nanoparticles besides stabilizing them.

Therefore, many biocompounds are able to reduce metal ions, producing metal nanoparticles.

The initial concentration of copper ions strongly affects the production of nanoparticles by living cells, for instance, Honary et al. (2012) tested three species of *Penicillium*: *P. aurantiogriseum*, *P. citrinum* and *P. waksmanii*, which were cultivated in a fluid zapex dox broth at 28 °C, 200 rpm for 10 days. Then, they were centrifuged and their supernatants were used for producing copper nanoparticles, that is, the authors used the metabolites produced during the fermentation instead of the living cells (directly). In addition, the effects of copper concentration (1, 3 and 5 mM of CuSO₄) and pH (5, 6, 7, 8 and 9) were investigated. The authors reported a direct correlation among pH, concentration of copper, polydispersity index and particle size, that is, the 5 mM CuSO₄ (highest concentration) led to the largest copper nanoparticles (diameter), whereas pH 5 (the lowest pH), led to the production of smallest (diameter) copper nanoparticles. Moreover, the same trend (correlation among pH, concentration of copper, polydispersity index and particle size) was observed among the three species.

Table 1: Production of copper nanoparticles by bacteria

Bacteria	Shape	Diameter*	Copper Source	Reference
<i>Pseudomonas stutzeri</i>	Spherical	8-15	CuSO ₄	(Varshney et al., 2010)
<i>Pseudomonas stutzeri</i>	Cubic	50-150	CuSO ₄	(Varshney et al., 2011)
<i>Pseudomonas</i> sp.	Cubic	84-130	Metallic copper	(Shobha et al., 2014)
<i>Escherichia coli</i>	quasi-spherical	10-40	CuSO ₄	(Singh et al., 2010)
<i>Streptomyces</i> sp.	X	100-200	CuSO ₄	(Usha et al., 2010)
<i>Serratia</i> sp.	Cubic	10-30	CuSO ₄	(Shobha et al., 2014)

Morganella morganii	Cubic	15-20	CuSO ₄	(Shobha et al, 2014)
Serratia sp.	Spherical	10-30	CuSO ₄	(Hasan et al., 2007)

* (nm)

b) Fungi

A wide range of the genera of fungi was already reported as metallic nanoparticle producers (my conanotechnology), such as *Penicillium aurantiogriseum*, *P. citrinum*, *P. waksmanii*, *Fusarium oxysporum*, etc (Table 2). Compared with the biotechnological processes that apply bacteria, algae, cyanobacteria and plants, fungi are more resistant to mutations and have the ability to synthesize silica nanoparticles. Nevertheless, there is no consensus on the biomechanism of the metallic nanoparticle production. In other words, there is no evidence that a specific type of protein, or carbohydrate, or lipid or any other molecule is the major responsible for the production of metallic nanoparticles (Singh, 2015).

In this sense, proteins appear to be fundamental to the production of copper nanoparticles, in which the amide groups lead to stability and to capping agents around copper nanoparticles (Shobha et al., 2014). On the other hand, studies have indicated that secreted enzymes by fungi act on the production of metallic nanoparticles (instead of only on the stability) (Cuevas et al., 2015). However, other compound types are also related to the production of metal (silver) nanoparticles such as anthraquinone pigments and their derivatives, which were produced by *Fusarium oxysporum* strains (Duran et al., 2005).

In a very specific study, Jain et al. (2010) detailed the profiles of the extracellular proteins (*Aspergillus flavus*) during the synthesis of silver nanoparticles. The authors investigated mainly two proteins, 32 kDa and 35 kDa, in which the 32 kDa protein acted as reductase (production of silver

nanoparticles) and the 35 kDa protein enhanced the stability of the silver nanoparticles.

The oxidative stress is often related to a high concentration of metals (e.g. Ag, Fe, Cu, Co, Cd and Cu) (Jomova and Valko, 2011). Ramezani et al. (2010) highlighted the correlation between the production of glutathione (glutathione-like) and heavy metal stress (cadmium) in yeasts, in which metallic nanoparticles were produced. In theory, cells feel the decrease in glutathione/oxidized glutathione and then begin to synthesize more glutathione (injurious response). Thus, the glutathione antioxidant defense system is critical for the survival of the microbial cells.

In addition, other factors inherent in any biotechnological production seem to affect the production of metallic nanoparticles. For example, Salvadori et al. (2013) indicated the effect of pH on the production of metallic nanoparticles by *Hypocrea lixii*. On the one hand, at an acid pH (2-4), the membrane of microorganisms is positively charged with consequent reduction of metal biosorption. On the other hand, at pH 5, the cell membrane is negatively charged, which favors the biosorption of copper. Thus, the membrane is expected to be fundamental for the metallic nanoparticles, instead of cytoplasm (Salvadori et al., 2013).

An interesting approach was described by Ahmad et al. (2007) who produced the transparent p-type conducting oxide CuAlO₂ (bimetallic nanomaterial) by *Humicola* sp., exploiting the unique valence-controlled nanosynthesis capability of the *Humicola* sp. biosynthesis. Moreover, the material formed was free of any impurities (e.g CuO, Cu₂O or Al₂O₃).

Table 2: Production of copper nanoparticles by fungi

Fungi	Shape	Diameter*	Copper Source	Reference
<i>Fusarium oxysporum</i>	X	93-115	Metallic copper	(Majumder, 2012)
<i>Pseudomonas</i> sp	X	84-130	Metallic copper	(Majumder, 2012)
<i>Hypocrea lixii</i>	spherical	24.5	CuCl ₂	(Salvadori et al., 2013)
<i>Stereum hirsutum</i>	spherical	5-20	CuCl ₂	(Cuevas et al., 2015)
<i>Rhodotorula mucilaginosa</i> †	spherical	10.5	CuCl ₂	(Salvadori et al., 2014)
<i>Penicillium aurantiogriseum</i>	spherical	89-250	CuSO ₄	(Honary et al., 2012)
<i>Penicillium citrinum</i>	spherical	85-295	CuSO ₄	(Honary et al., 2012)
<i>Penicillium waksmanii</i>	spherical	79-179	CuSO ₄	(Honary et al., 2012)

* nm

† yeast

c) Algae

To the best of our knowledge, Abboud et al. (2014) were the first to report the bioproduction of copper oxide by algae microorganism (*Bifurcaria bifurcata*). The production of other metallic nanoparticles

by algae was also reported (Table 3); for example, (i) iron nanoparticles by *Chlorella* sp. MM3 (Subramaniyam et al., 2016); (ii) gold nanoparticles by *Stoechospermum marginatum* (Rajathi et al., 2012), *Turbinaria conoides* (Vijayaraghavan et al., 2011), *Sargassum wightii*

(Singaravelu et al., 2007), *Laminaria Japonica* (Ghodake and Lee, 2011) and *Kappaphycus alvarezii* (Rajasulochana et al., 2010); and (iii) silver nanoparticles by *Chlorococcum humicola* (Jena et al., 2013).

Table 3: Production of copper nanoparticles by algae

Algae	Shape	Diameter*	Copper Source	Reference
<i>Bifurcaria bifurcata</i>	spherical	5-45	CuSO ₄	(Abboud et al., 2014) †
Other metallic nanoparticles				
<i>Chlorella</i> sp. MM3	spherical	5-50	FeCl ₃	(Subramaniyam et al., 2016)
<i>Stoechospermum marginatum</i>	spherical	18.7-93.7	HAuCl ₄	(Rajathi et al., 2016)
<i>Sargassum wightii</i>	spherical	8-12	HAuCl ₄	(Singaravelu et al., 2007)
<i>Turbinaria conoides</i>	cubic	20-80	HAuCl ₄	(Vijayaraghavan et al., 2011)
<i>Laminaria Japonica</i>	cubic	15-20	HAuCl ₄	(Ghodake and Lee, 2011)
<i>Sargassum myriocystum</i>	spherical	10-23	HAuCl ₄	(Dhas et al., 2012)
<i>Kappaphycus alvarezii</i>	spherical	10-40	HAuCl ₄	(Rajasulochana et al., 2010)
<i>Chlorococcum humicola</i>	spherical	2-16	AgNO ₃	(Jena et al., 2013)

* (nm)

† small percentage of elongated particles

Therefore, the production of copper nanoparticles by algae should be investigated.

III. RECOVERY AND PURIFICATION OF MICROBIAL COPPER NANOPARTICLES

To the best of our knowledge, there is no report on the purification of copper nanoparticles and there is little information about the purification of metallic nanoparticles (biogenic production). However, Vijayaraghavan et al. (2011) cited that gold nanoparticles adhered to the surface of the biomaterial may be recovered by sonication. Thakkar et al. (2010) suspended the fungal mycelia in deionized water then filtered it (Whatman). Silver nitrate was added to the filtrated solution (metallic nanoparticle production). The metallic nanoparticle solution was dried under an infrared lamp. Singh, (2015) indicates that the procedures for recovering extracellularly synthesized nanoparticles are centrifugation or filtration. The nanoparticles should then be stored in the dark at low temperature. Yet, for the intracellular production, prior to the recovery of metallic nanoparticles, the microbial cells have to be lysed (lysis buffer, sonication and detergent solutions).

IV. APPLICATION OF MICROBIAL COPPER NANOPARTICLES

a) Antimicrobial

In 2008, the United States Environmental Protection Agency approved copper as an antimicrobial agent, particularly against harmful bacteria (potentially deadly microbial infections). In this sense, attention has been drawn to the bactericidal effect of copper nanoparticles (Theivasanthi et al., 2011).

The copper nanoparticles produced by ascorbic acid, chitosan and microwave heating were slightly more effective (minimum inhibitory concentration) against *B. subtilis* instead of *E. coli* 0.313 and 0.469, respectively (Mat Zain et al., 2014).

Abboud et al. (2014) detailed the antibacterial properties of copper nanoparticles produced by algae extract against bacteria *Enterobacter aerogenes* and *Staphylococcus aureus* by using the agar disc diffusion method. Regarding copper nanoparticles, the radial diameter of the inhibition against *E. aerogenes* and *S. aureus* were of 14 and 16 mm, respectively. Moreover, the algae extract did not show antibacterial activity. Theivasanthi et al. (2011) produced copper nanoparticles by dissolving CuSO₄ in distilled water and electrolyzing this solution. Then, the authors recovered the copper nanoparticles at the cathode and showed their antimicrobial properties against *Escherichia coli* and *Bacillus megaterium* by using the agar disc diffusion method, in which the diameter of inhibition against *E. coli* mm and *B. megaterium* were 15 mm and 5 mm, respectively. Merin et al. (2010) reported the antimicrobial activity of silver nanoparticles produced by *Chaetoceros calcitrans*, *Chaetoceros salina*, *Isochrysis galbana* and *Tetraselmis gracilis* (microalgae) against *Klebsiella* sp., *Proteus vulgaris*, *Pseudomonas aeruginosa* and against *Escherichia coli* by using the Muller Hinton agar disc diffusion method. The silver nanoparticles produced from *I. galbana* showed the highest zone of inhibition against *Klebsiella* sp. (≈ 20 mm), whereas the silver nanoparticles produced by *C. salina* showed the highest zone of inhibition against *P. vulgaricus* and *P. aeruginosa*.

To the best of our knowledge, despite the broad potential application of copper nanoparticles (biogenic), only their antimicrobial properties were investigated.

V. LIMITATIONS ON THE PRODUCTION OF METALLIC NANOPARTICLES BY LIVING CELLS

The two main limitations on the microbial production of metallic nanoparticles are to achieve the monodisperse size production and the lack of

knowledge on the mechanism of the synthesis of metallic nanoparticles.

VI. CONCLUSION

Compared to copper, the biogenic recovery (production of metal nanoparticles) of other metals such as gold, silver and iron has been much deeply investigated. The biogenic production of copper nanoparticles by bacteria and fungi is relatively well known; on the other hand, the biogenic production of copper nanoparticles by algae is very rare. There is no consensus on which type of biomolecules (e.g. proteins, carbohydrates, lipids, etc) plays a major role in the production/stabilization of copper nanoparticles. To the best of our knowledge, there are no procedures concerning the purification of copper nanoparticles (biogenic). In addition, despite the many potential applications of copper nanoparticles (biogenic), only their antimicrobial properties were described.

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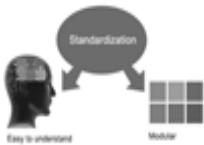
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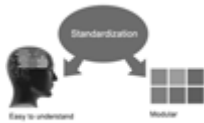
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The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently. You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.



Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
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- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

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INDEX

A

Aldhaydes · 19

B

Beetroot · 16, 19, 20
Benishangul · 21

C

Carotenoids · 23, 27
Cytochromes · 32, 36
Cytotoxic · 2

E

Echidium · 6, 10, 11, 12, 15

G

Geosmin · 16
Gluconeogenesis · 28
Glutamic · 28
Greigite · 31

H

Humicola · 33

I

Inooka · 1, 3, 4, 5

L

Libehaber · 1, 5

M

Mangifera · 21
Monolaurin · 1, 4, 12, 13
Mucoid · 3, 4, 11, 12

N

Noireaux · 1, 5

P

Penicillium · 32, 33, 34, 36

R

Refractometer · 17, 22

S

Salvadori · 31, 33, 34, 37
Sphingosine · 1, 4, 5
Stoechospermum · 34, 37

T

Titrate · 16, 17, 18, 19, 21, 22, 26
Trichloroacetic · 32
Trypsinized · 2

W

Waksmanii · 32, 33



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