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## Review

## Phytochemical importance and utilization potential of grape residue from wine production

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Grapes are placed at a distinct position, both in the economy and at nutritional and phytochemical levels. The number of studies performed aimed at identifying the various bioactive compounds of grapes, how their cellular structures are linked, efficient methods of extraction and its effect on human health are rather relevant. The use of grapes for wine production is a worldwide activity, therefore a great quantity of residues are generated. These, in turn, are commonly used in soil fertilization and animal feed. Research in the food, pharmaceutical, medical and agricultural industries have revealed a great potential of using grape residue to recuperate phenolic compounds, flavonoids and especially the resveratrol. These have been found by these researches to offer benefits associated to its consumption, such as improvements in glucose tolerance in diabetic patients, decrease in the occurrence of cardiovascular diseases, diminishing the symptoms of menopause, protection against osteoporosis, cancer and Alzheimer's disease. The present work aimed at gathering information about the characterization of the phytochemical content of grapes and its residues, as well as gathering data about the extraction process of such compounds and their application in the food industry.

**Key words:** *Vitis vinifera*, phenolic components, antioxidant capacity, extraction methods, vinification.

### INTRODUCTION

Grape production is one of the most important activities in agriculture. Over ten thousand varieties are known around the world. It is estimated that grape production around the world has increased from 59.74 millions of tons in 1990 to 68.31 million in 2010. In the last twenty years, there was an annual average increase of around

0.5% a year (FAO, 2013).

Grapes (*Vitis* sp.) are one of the most important fruit crops worldwide. There are about 60 grape species in the genus of *Vitis*, and the species *Vitis vinifera*, or European grapes, is most widely cultivated (Liang et al., 2014; Urcan et al., 2017). The three main species of grapes are

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the European (*V. vinifera*), the American (*Vitis labrusca* and *Vitis rotundifolia*) and the French hybrid grapes. The phenolic compounds are the most important phytochemicals in the grapes, as they possess various biological activities and benefits to health (Wada et al., 2007). They are consumed as fresh fruits as well as wine, juice and other processed products (Liang et al., 2011; Liang et al., 2014; Xu et al., 2017). About 27% of the grapes are consumed as fresh fruit (table grapes) and 2% as dried fruit, whereas 71% of the crop is processed, especially for winemaking (Wang et al., 2013).

When grapes are processed to produce its derivatives, great quantities of residues are also generated each year, stimulating the development of other economic viable forms of using the waste. The use of grape residue and its components has an important environmental impact in the reduction of waste and the possibility of creating products with high added value. Various studies have been performed to explore the content of bioactive compounds derived from these residues, besides seeking the best method for extraction and its possible incorporation in food products (Ping et al., 2011a, b; Burin et al., 2014; Machado et al., 2014; Yazykova and Andreevna, 2015; Medina-Meza et al., 2015; Mildner-Szkudlarz et al., 2015; Santos et al., 2016). Polyphenols have been associated with the bioactive potential of grapes due to their antioxidant, anti-inflammatory, anticarcinogenic and antibacterial activities (Burin et al., 2014; Sahpazidou et al., 2014; Li et al., 2015; Casazza et al., 2016). To obtain an assessment of the full range of phytochemicals in grapes, Liang et al. (2011) we recently analyzed 36 phenolic compounds in the berry samples of 344 representative *V. vinifera* cultivars while Liang et al. (2014) investigated and analyzed phenolic profiles, antioxidant and antiproliferative activities of twenty-four selected *Vitis vinifera* grape cultivars. It was evidenced the phytochemical potential of the samples investigated.

The present work aims at gathering information about the characterization of grapes, its residues and derived products regarding their phenolic compounds content, as well as their extraction mechanisms and application in the food industry.

## GRAPES

The three main grapes varieties are the European type (*Vitis vinifera* L.), the American type (*Vitis labrusca* and *Vitis rotundifolia*), and the hybrid French variety. Grapes are classified according to the purpose of their use, as table grapes, wine grapes and dried (raisins). Besides, they can be classified as seeded or seedless. As a tradition, the quality of the products derived from grapes is directly related to aspects of its manufacture, but strongly dependent on the physical-chemical characteristics of the raw material that originated them, that is, the properties originally contained in the grape

(Prozil et al., 2012; Ma et al., 2015; Nogales-Bueno et al., 2017).

The European grapes (*V. vinifera* L.) produce the fine wines, and are a variety of great importance in the global context, as well as widely known in wine making. Examples are the white grapes Chardonnay, Sauvignon Blanc and Gewürztraminer and the red grapes Cabernet Sauvignon, Cabernet Franc, Merlot and Pinot Noir. The American grapes (*V. labrusca*), also known as table grapes, are those used for consumption as a fruit and used for juicing, table wines and cooking in general (Ping et al., 2011a; Liang et al., 2014; Cerqueira and Machado, 2016).

Regarding the nutritional profile, the fruit has various important elements, such as vitamins, minerals, carbon hydrates, fibers and phytochemicals. The chemical composition of grapes varies especially according to climate, soil, variety and cultivar. The physical-chemical properties of the fruit can determine the characteristics of its derivatives. The acidity in the juice, for example, is an indicative of the presence of the organic acids tartaric, malic and citric, which give the product a low pH, contributing to a balance between the sweet and acid tastes. The total soluble solids content and the rate soluble solids/acidity are indicative of the grape ripeness and are the most employed criteria for choosing the best time to commercialize or process the fruit (Gil and Pszczolkowski, 2007; Santiago et al., 2014).

The nutritional importance of the grapes and the products derived from it, such as juices, wines, jams and raisins has been constantly reported (Djilas et al., 2009). The direct correlation between the consumption of grapes and wines as beneficial to the health is due to the high quantity of phenolic compounds present in the fruit, which have a significant antioxidant power (Djilas et al., 2009; Syed et al., 2017; Dumitru and Antoce, 2016).

Grapes are an important source of different phenolic compounds in high concentrations. Therefore, the subproducts of its processing retain significant quantities of these substances, such as flavonoids (anthocyanins, catechins and flavonols), stilbenes (resveratrol), phenolic acids (especially benzoic and hydroxycinnamic acids) and a large variety of tannins and proanthocyanidins. These compounds are extensively recognized and reported to have beneficial effects on the human health, including antioxidant, anti-inflammatory, anticancer, vasodilator and antimicrobial activities (Kchaou et al., 2013; Ahmed et al., 2015).

## SOLID RESIDUES FROM GRAPES RESULTING FROM WINE PRODUCTION

Wine making involves the procedures and processes applied to transform ripe grapes in wine. The main phases in the production of white, red and rose wine are harvesting, storing and analysis of grapes; stemming and

crushing; addition of sulphite to wort; addition of pectinolytic enzymes; removal of particulate matter; addition of yeast; adjustment of sugar levels or chaptalization; alcoholic fermentation; maceration; separation of solid and liquid phases; pressing of bagasse; malolactic fermentation; racking; attesting; stabilization; filtrations; stabilization in wooden barrels; cuts; bottling and corking; aging in bottles (Prozil et al., 2012; Cirqueira et al., 2014).

Due to the large number of phases, the processing of grapes for wine making generates a great quantity of residues, derived from different phases of the process. It is estimated that 80% of the global production of grapes is aimed at wine manufacturing. Also, for each 100 L of wine produced, 25 to 31 kg of residues are generated, of which 13 to 17 kg are made of bagasse, which explains why it is considered the main product of grape residues (Spigno et al., 2008; Amarin et al., 2015). With such large quantities of subproducts and residues generated, there is an increasing interest in using these products as raw material for other industries, both for economic reasons and environmental concerns (Prozil et al., 2012; Spigno et al., 2008; Oliveira et al., 2012).

The bagasse is made of skin, stems and seeds. These residues have some important characteristics, such as low pH, a high phytotoxic content and phenolic substances, with high antibacterial properties, which lead to resistance to biological degradation. Normally, these residues are used as fertilizers; however, new alternatives are necessary for its utilization, once the high levels of phenolic compounds in the soil are known to cause germination problems (Negro et al., 2003; Soquetta et al., 2016; Casazza et al., 2016).

Another end given to the grape skin and/or bagasse is its use in making animal feed. This purpose is also limited due to its composition, rich in phenolic compounds, which represents an antinutritional factor. Such fact makes the grape bagasse a residue of great interest for the recovery of these potentially bioactive compounds, recognized by their high antioxidant activity (Ping et al., 2011a, b; Prozil et al., 2012; Lima et al., 2014).

The grape's skin is an expressive source of anthocyanidins and anthocyanins, which are natural dyes and have antioxidant properties, such as the inhibition of lipoperoxidation and antimutagenic activity. The rachis has a high quantity of polyphenol, especially tannic compounds, which have a high nutraceutical and pharmacological potential. Its presence in excess in wine can cause a high astringence to the product, therefore its removal during processing is essential (Souquet et al., 2000; Lima et al., 2014).

The lack of applicability of these residues in new areas is mainly associated to the lack of knowledge about the chemical composition and the structure of the main components of the grape's skin, which corresponds to approximately 90% of the total residue. The majority of the studies are more related to different classes of

extracts, such as anthocyanins, hydroxycinnamic acids, flavonoids, flavonols and glycosides (Kammerer et al., 2004; Zhang et al., 2016a) and less concerned with the evaluation of the basic macromolecular components of the grape's skin (Arnous and Meyer, 2008; Zhang et al., 2016b).

The grape's skin that has been through processing for wine production can be a cheap and valuable raw material for the recovery of biologically interesting polyphenolic compounds and products based on these compounds. The flavonoids present in the grape's skin, especially the anthocyanins, flavonols and flavonoids have been widely studied (Yang et al., 2009). However, there needs to be a more accurate investigation concerning the stilbenes, especially resveratrol, extracted from the skin of different grape varieties and found in considerable quantities (Kammerer et al., 2005; Cirqueira et al., 2013; Rocha et al., 2016).

## BIOACTIVE COMPOUNDS OF GRAPE'S SKIN

The red colour and the sensory attributes of a wine's quality, especially red wines, are largely due to the phenolic substances, taste and precursors existent on the cellular wall of the skin. The grapes' skin represents 5 to 10% of the total dry weight of the fruit and acts as a hydrophobic barrier that protects the grapes from physical and other climatic traumas, dehydration, infection by fungi and UV light. During the wine making process, the transference of phenolic compounds of the red grapes for the wine occur mainly from the grape's skin during the maceration phase, which, in the case of red wine making, occurs directly on the crushed fruits (Spigno et al., 2008; Mildner-Szkudlarz et al., 2015).

The phenolic content on the grapes' skin varies from 285 to 550 mg/kg of grapes' skin, depending on the variety of the grapes and on the type of pre-treatment (Pinelo et al., 2005a, 2005b; Rombaut et al., 2014). The potent antioxidant activity of wine and grape extracts on controlling the oxidation of *in vitro* low density lipoproteins were significantly correlated with the action of phenolic compounds present in the samples, especially on the grapes' skin (Tomera, 1999).

Various studies were developed seeking ways of recovering the phenolic compounds from subproducts of wine making and its application as potentially antioxidant food products (Spigno et al., 2008; Pinelo et al., 2005a; Yilmaz et al., 2015).

When the red grapes are processed for wine making, the skin and seeds usually remain in contact with the wine in fermentation for various days. This material is then submitted to a careful extraction, and has a high content of phenolic compounds. This residue still has high levels of phenolic compounds retained in the skin's matrix, which stimulates its use as a source for recovery of phenolic compounds (Kammerer et al., 2005). Various



**Table 1.** Bioactive compounds and function performed in the food matrix.

Bioactive compound	Function in food matrix	References
Condensed tannin	Anti-microbial and anti-parasite	Naczk and Shahidi (2006)
Cyanidin	Pigmentation	Monagas et al. (2006); Gallego et al. (2013)
Polyphenols	Nutraceutical and antimicrobial	Xu et al. (2017)
Delfinidin	Pigmentation	Revilla et al. (1998)
Flavonoids	Antioxidant	Spinelli et al. (2016)
Flavonoids	Antifungal, cellular antioxidant	Cook and Samman (1996)
Phenolics	Antioxidant	Yilmaz et al. (2015)
Kaempferol Malvidin quercetin	Pigmentation	Nile et al. (2013); Monagas et al. (2006); Montana et al. (2007)
Resveratrol	Antifungal, cellular antioxidant	Ahmed et al. (2015)

factors such as cultivar, growing conditions of vines, period of contact between skin and wine in fermentation, temperature of the process and the presence of seeds and rachis, all affect the transference of phenolics to the wort wine, which in turn determines the available quantity of phenolic components in the bagasse (Pinelo et al., 2005a, b; Mohamed et al., 2016).

In various experiments performed with the objective of recovering bioactive compounds, it was a consensus that it was necessary to determine the best conditions for extraction which benefit the release of phenolic compounds from grapes' subproducts in different solvents (Pinelo et al., 2004; Meyer et al., 1998). Besides the complex composition and the connections between phenolic compounds and the different components of the grape's skin, these aspects are important to increase the efficiency of the extraction process (Vidal et al., 2001).

During the maturation of grapes, environmental factors and endogenous enzymes promote changes that affect the composition and structure of the sugars and phenolic compounds contained in the grapes. Research demonstrates that wines made from more mature grapes, in general have a higher content of anthocyanins, a lower rate anthocyanins -flavonol and a higher quantity of some simple phenolic compounds, such as galic acid and siringic acid (Vidal et al., 2001).

Generally, despite the fact that the phenolic composition varies greatly depending on the variety and growing conditions of the grapes, the skin has the higher content of tannins of the fruit. These tannins differ depending on the fractions of the grape, and could present a higher degree of polymerization and a lower quantity of gallates (Souquet et al., 2000). Catechin, epicatechin and epicatechin gallate are the main unities that constitute the tannins of the skin (Sun et al., 1996). However galocatechin and epigallocatechin are also present in lower quantities (Souquet et al., 2000; Chira et al., 2015). Other compounds, such as quercetin 3-glycuronide were also detected in considerable quantities in the seeds, followed by catechin, caftaric acid and astilbin (Souquet et al., 1996; Lima et al., 2014). Table 1 presents other studies that report different functions

performed by the bioactive compounds of grapes in food.

The grape's skin can be divided in three layers. The outer layer, or cuticle, is composed of hydroxilated fatty acids called cutina, and it is covered by hydrophobic wax (Lecas and Brillouet, 1994). The intermediate layer, the epidermis, consists of one or two layers, which appear as a group of regular cells. The internal layer, hypodermis, which is the closest to the pulp, is composed by various layers of cells containing the majority of the phenolic compounds of the grape's skin (Lecas and Brillouet, 1994; Zhang et al., 2015).

The cellular wall of the grapes forms a barrier against the diffusion of various components, including smells and phenolic compounds, being composed of 30% neutral polyssacharides (cellulose, xyloglucan, arabinan, galactan, xylan and mannan), 20% of pectinic acid substances (of which 62% are methyl sterified), approximately 15% of insoluble proanthocyanidins, and < 5% structural proteins (Lecas and Brillouet, 1994). Bioactive compounds that occur in plants can be linked to the polyssacharides of the cellular wall, confined in vacuoles, associated to the nucleous through different chemical chains, or even joined by physical chains, depending on the composition and disposition of both the phenolic compound and the polyssacharide (Le Bourvellec et al., 2005).

The retention of phenolic compounds on the cellular wall depends on compositional and structural parameters such as stereochemistry, conformational weight or molecular flexibility. Besides that, physical characteristics of the cellular wall, such as surface topography, porosity and chemical composition can also influence the eventual aggregation between conformational polyssacharides from the cellular wall and phenolic substances (Le Bourvellec et al., 2004).

The majority of data about complexation of phenolic compounds with polyssacharides of the cellular wall of plants were obtained using cyclodextrines, polyssacharides in solid state or compounds from the cellular wall of apples prepared by certain chemical treatments. Two mechanisms of association have been proposed to explain the complex links between the

phenolic compounds and the polyssacharides. The first corresponds to hydrogen chains between the hydroxyl groups of the phenolic compounds and the oxygen atoms of ether crossed chains. This way, dextrane gels would be able to encapsulate phenolic compounds inside its pores (Le Bourvellec et al., 2005; Freitas et al., 2003). The second mechanism corresponds to hydrophobic interactions that occur as a result of the ability of certain polyssacharides to develop secondary structures, that is, the nanotubes, or gels, which result in hydrophobic regions. The pockets or hydrophobe cavities can be capable of encapsulating the phenolic compounds, as it has been shown between  $\beta$ -cyclodextrin and different phenolic compounds, such as caffeoylquinic and flavonoids (Le Bourvellec et al., 2005).

Studies have reported an association between flavonoids as the cellular nucleous of various plants. The link of quercetin-3-sulfate to the protein of cellular nucleous of *Flaveria* has already been reported (Grandmaison and Ibrahim, 1996). The association of flavonoids inside the cell nucleous was confirmed by other studies where microscopic techniques made possible the detection of this phenomenon in other vegetable species. Therefore, the substantial quantity of catechin, epicatechin and proanthocyanidins were detected in the cellular nucleous of five different tree species, as well as in flower teas (Feucht et al., 2004a).

However, despite the fact that various studies reveal the existence of an association between flavonoids and the nucleous of vegetal cells, little is known about the specific link between flavonoids and the components of the cell nucleous. In any case, the occurrence of flavonoids related to the nucleous gives rise to new relative questions, such as the possible ability of the phenolic compounds to protect DNA against the oxidative stress through removing free radicals (Albersheim, 2006).

## PHENOLIC COMPOUNDS

The phenolic compounds are largely found distributed in plants, and constitute a very diverse group of phytochemicals derived from phenylalanine and tyrosine, including simple molecules and molecules with a high degree of polymerization. In food, the phenolic compounds are responsible for the colour, adstringence, scent and oxidative stability (Alasalvar et al., 2001; Antonioli et al., 2015).

The phenolic compounds are included in the category of neutralizers of free radicals, being very efficient in the prevention of auto-oxidation. Phenolics from grapes and red wines were associated to the inhibition of human LDL oxidation (*low-density lipoprotein*) *in vitro*, to the prevention of atherosclerosis and to anti-mutagenic and anti-viral effects (Kaur and Kapoor, 2001; Jara-Palacios et al., 2014). Chemically, the phenolic compounds are defined as substances with an aromatic ring with one or

more hydroxilic substitutes, including its functional groups. The antioxidant activity depends on its structure, particularly on the number and position of the hydroxyl groups, as well as on the nature of the substitutions on the aromatic rings. There are around 8.000 different phenolic compounds which, according to their chemical structure, are divided in classes: Phenolic acids, flavonoids, stilbenes and tanins (Balasundram et al., 2006).

The phenolic compounds present in the grape's skin are linked and/or attached to lignin and polyssacharides present in the matrix of the cellular wall. These compounds are linked by hydrophobe interactions and hydrogen bridges, and can also be in other areas of the vegetal cell, including vacuoles and cell nucleous. The release of the bioactive compounds on the grape's skin starts with the degradation of polyssacharides on the cellular wall, either in wine-making process to enrich the wine, or for obtaining compounds from the bagasse for various purposes (Fang et al., 2008).

The phenolic compounds contained in the vacuoles of the cellular wall are weakly linked to the structure, and are presumably, the most susceptible to be affected by variables such as temperature, solvent-solute ratio and type of solvent used, all of which can modify the balance and conditions of the solid-liquid extraction. The temperature is one of the most important variables to affect the release of the phenolic compounds of the grape's skin; increases in the temperature of extraction contribute to improve both the solubility of the solute and the diffusion coefficient. Consequently, in high temperatures, there is an increase in the content of extracted phenolic compounds (Pinelo et al., 2005a; Xu et al., 2014a).

Despite the positive effects of using higher temperatures for the benefit of extractions, the temperature cannot be increased indefinitely, as in temperatures above 50°C there can be instability of the phenolic compounds and the denaturation of the membranes. The increase of the solvent-solute ratio has also been suggested to increase the yielding of phenolic compounds. However there needs to be a balance between the use of high and low ratio of solvent-solids, in order for an equilibrium between the high costs and the solvents residues to occur, aiming at avoiding the effects of saturation (Pinelo et al., 2005b; Pinelo et al., 2004).

The type of solvent used is also one of the variables that mostly influences the extraction process. Methanol, ethanol and water are the most used solvents for the extraction of phenolic compounds from the grape's skin and bagasse derived from wine production. Among these solvents, the methanol presents a higher capacity to extract phenolic compounds, followed by ethanol and lastly water (Pinelo et al., 2005a). When alcohols are used as solvent in extraction, a progressive release of phenolic compounds from the grape's skin is observed, relative to time of extraction. When water is used as

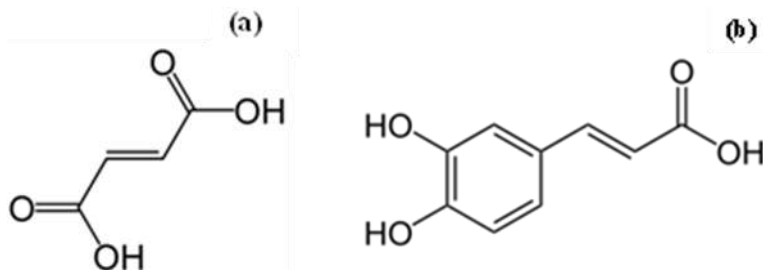


Figure 1. Molecular structure of fumaric acid (a) and caffeic acid (b).

solvent, the time of contact is not so important. Other variables, such as a smaller particle size and higher quantity of sample also benefit the release of phenolic compounds, especially in continuous extraction (Hayouni et al., 2007).

The conditions of extraction can also promote the formation of phenolic compounds that do not occur naturally in the grape's skin. In a study performed (Pinelo et al., 2005b), the formation of polymers of flavan-3-ol was observed in the grape's skin mass, when submitted to a continuous extraction with an ethanolic extractor. Despite observing some of these structural alterations, other factors can occur simultaneously, such as variations in their functional properties, when opting for the limitation in the presence of oxygen during the extraction process of phenolic compounds (Pinelo et al., 2004).

## PHENOLIC ACIDS

Phenolic acids are found in higher quantities in the tissues of grape pulp, around 80 to 85%. However, its concentration decreases with the ripening of the fruit and varies according to the cultivar. They are simple compounds formed by an aromatic ring with substitutes capable of sequestering reactive species linked to its structure, such as the radical hydroxyl and the singlet oxygen. The phenolic acids are divided in two groups, the benzoic acids, which have six carbon atoms (C6-C1), such as the gallic acid, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic, and the cinnamic acids (Balasundram et al., 2006). The other group has nine carbon atoms (C6-C3), such as the caffeic, ferulic, *p*-coumaric and sinapic acids (Balasundram et al., 2006). Figure 1 presents the molecular structure of the fumaric acid and caffeic acid.

The relationship between the structures of the phenolic acids and its antioxidant activity has already been established. The antioxidant capacity of the phenolic acids and its esters depends on the number of hydroxyl groups present in the molecule and its position in relation to the carboxyl functional group. Derivates of cinnamic acid are more active as antioxidants than the derivates of

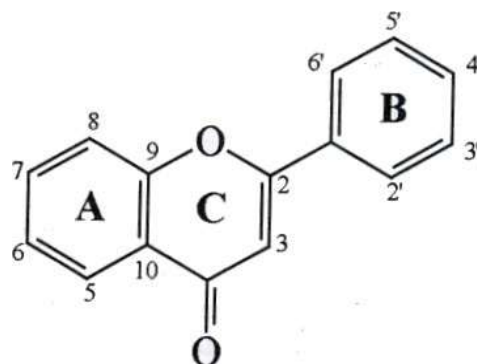


Figure 2. Basic structure of flavonoids.

benzoic acid. This is due to the fact that the first compound presents a higher number of hydroxyl groups in relation to the second, which guarantees a higher ability to donate H<sup>+</sup> ions and stabilize radicals. The introduction of a second hydroxyl group in the *ortho* or *para* position also increases the antioxidant activity of these compounds (Pinelo et al., 2004).

## FLAVONOIDS

Flavonoids constitute the larger group of plant phenolic compounds, being responsible for the colouring of flowers and fruit. They are substances of low molecular weight, composed by 15 carbon atoms. Its general structure is essentially formed by two aromatic rings at the extremities, linked by a bridge of three carbons, usually in the form of a heterocyclical ring (Figure 2). Variations in the substitution configuration of the ring placed between two rings result in the majority of subclasses of flavonoids, which are flavones, flavanones, isoflavones, catechins and anthocyanins (Pinelo et al., 2004).

According to the literature, these compounds have demonstrated activity against allergies, high blood pressure, viral infections, inflammations, arthritis, mutations and carcinogeneses, cancer and AIDS. Its antioxidant potential depends on the number and position

of the hydrogen groups and its conjugations, and also due to the presence of electrons on the benzenic rings. In general, the presence of hydroxyl groups in positions 3, 4 and 5 of the ring at the right extremity has been described as being responsible for increasing the antioxidant activity; however, depending on the flavonoids subclass, the effect could be the opposite (Pinelo et al., 2004).

## ANTHOCYANINS

Anthocyanins are flavonoids which are largely distributed in nature, and are responsible for the majority of blue and violet colours, and almost all tones of reds that appear in flowers, fruit, some leaves, stems and roots of plants. In grape vines, they are responsible for the colour of the red grapes, and they transfer, in part, to the wine during vinification, being also found in the pulp of some grape varieties (Versari, 2008).

The use of anthocyanins as colouring arises great interest, but, at the moment, this happens more due to its antioxidant capacity, which is even greater than vitamin E, butyllated hydroxyanisole (BHA) and butyllated hydroxytoluene (BHT). Highly polar, they can substitute the lypophilic antioxidants, such as vitamin E. It is possible to delay the oxidation of frozen fish by adding grape procyanidins (Kang et al., 2003; Kruger et al., 2014). Besides that, both *in vitro* and *in vivo* studies demonstrate the capacity of anthocyanins in reducing the proliferation of cancerous cells and inhibit the formation of tumours (Kang et al., 2003; Lila, 2004).

## STILBENES- RESVERATROL

The family of the stilbenes is vast. The resveratrol is the main representative of this group, and it occurs naturally in various plant species, such as mulberry, peanuts and grapes, especially red grapes. The interest of the scientific community for resveratrol was originally due to epidemiological studies which indicated an inverse relationship between the moderate consumption of wine and the risk of coronary disease. Besides, the prevention properties of resveratrol were observed both *in vitro* and *in vivo* (Jang et al., 1997).

The resveratrol (3,5,4-trihydroxi-*trans*-stilbene) is a biologically active substance, belonging to the phytoalexins group (Figure 3). It can be found in nature in the aglycosidic or glycosidic forms, the latter having different denominations, depending on the glycon involved and its geometric form, *trans* and *cis*. The isomer *trans*, which is predominant, is generally more biologically active, however it is also thermo- and photo-sensible, being transformed into *cis* in the presence of visible light, which makes its identification difficult depending on the method used (Vitac et al., 2005).

The majority of its cardio-protective properties are

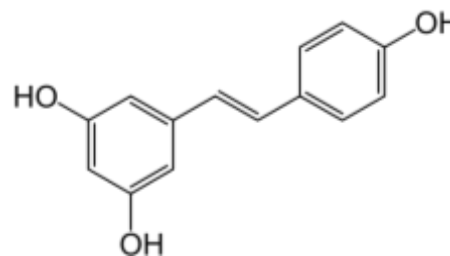


Figure 3. Molecular structure of resveratrol.

associated to its capacity to exert vaso-relaxation and anti-inflammatory response. Among other benefits for the health, the resveratrol has anti-tumoral, anti-diabetic and anti-obesity properties (Li et al., 2012; Jeong et al., 2012).

Generally, the stilbenes are recognized as biological active compounds with anti-fungal action against many pathogens (Jeandet et al., 2002). The most reported anti-microbial effect of resveratrol and other stilbenes is against a common grape vine pathogen, *Botrytis cinerea*, which causes significant losses in grape vines production around the world (Filip et al., 2003). The anti-microbial activity of resveratrol against micro-organisms that cause skin diseases, such as the bacterias *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* and the fungi *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum gypseum*, was evidenced (Jeandet et al., 2002). Recently, other important biological actions of resveratrol were reported, such as the capacity to improve tolerance to glucose in diabetic patients, alleviate the symptoms of menopause and protect against osteoporosis, cancer and Alzheimer's disease (Li et al., 2012).

A growing number of studies have examined the pharmacological properties of resveratrol related to many human diseases, including cardiovascular diseases, diabetes mellitus, neurodegenerative diseases and cancer (Saleem et al., 2005). The antioxidant and anti-inflammatory effects of resveratrol perform a crucial role in the therapeutic treatment, although the action mechanisms need to be evaluated more thoroughly (Jeandet et al., 2002; Ksiezak-Reding et al., 2010; Mossalayi et al., 2014).

The content of resveratrol in grapes decreases drastically during its ripening, being practically undetectable in the ripe fruit. Besides, during the process of wine fermentation, the maceration of the bagass into alcohol transfers a great deal of resveratrol from the grape into the wine, reducing, that way, its availability in the bagass (Jeandet et al., 2002; Oliveira et al., 2015).

In grapes, the resveratrol is synthesized almost exclusively in the peel, and peaks of synthesis occur shortly before the grapes reach maturity. The terminal enzyme involved in the biosynthesis of resveratrol is

stilbene synthase. Its activation happens in response to exogenous stress factors, such as lesions, ultraviolet radiation and chemical signs from fungal pathogenic agents. The peak level of resveratrol occurs in a period of approximately 24 h after exposure to stress and decreases after 42 to 72 h, as a result of the activation of stilbene oxidase. The degree of increase in the levels of resveratrol in grapes depends on the variety and exposure to stress (Adrian et al., 2000). Due to resveratrol being produced in response to external stimuli, it is expected that the grapes and the wine have variations in their levels, according to regions and crops. Besides that, many factors such as the increase in temperature, higher levels of SO<sub>2</sub> and decrease of pH, result in higher levels of resveratrol during the process of vinification (Gambutu et al., 2004).

### PHENOLIC EXTRACTION DURING VINIFICATION

The general variations in the proportion solids-liquids which affect the yield of phenolic compounds extraction, as well as various technical variables, were specifically referred to as having an influence in the phenolic concentration in wines. Some of these refer to the bursting of the grape cell, breaking the links and rigid structure of the cellular wall, allowing for an increase in the release of phenolic compounds. In general, high fermentation temperatures increase the efficiency of phenolic extraction. Previous works have shown that there is an increase in the total yielding of phenol, but there is only a small difference in the content of anthocyanins in wine whose fermentation had temperatures varying from 15 to 30°C. The levels of sulphur dioxide (SO<sub>2</sub>) and the temperatures commonly used for the fermentation of red wine do not affect considerably the extraction of phenolic compounds (Girard et al., 2001; Antonioli et al., 2015).

The use of low temperatures, normally on the range of 10 to 15°C, for many days prior to fermentation does not produce a negative effect on the phenolic composition of the resulting wines. Freezing the wine before fermentation has a potentially bigger effect. Due to freezing, the grapes undergo structural damage and the cellular membranes are broken, thereby releasing the anthocyanins on the (mosto) (Sacchi et al., 2005).

The high temperatures are used in thermovinification, where there the peels are heated up at 60 or 70°C during a short period of time, extracting the phenolic compounds together with the juice, pressing before fermentation. The treatment using heat damages the hypodermic cell membranes, releasing anthocyanins and causing the denaturation of the polyphenoloxidase enzyme, which stops darkening. The pectinolytic enzymes are used to break the (lamella media) between the cells of the pulp and the cellular walls of the peel, releasing the pigments. An improvement in the production of juice and its

colouring has been reported using these enzymes in the extraction process (Ducruet et al., 1997; Rolle et al., 2015).

Regarding maceration, a prolonged period of maceration, of around 4 to 10 days, increases the concentration of anthocyanins and tanins after around a year on bottled wine. It was reported that the reduction in the size of the particles of grape bagass had a positive effect on the recuperation of phenolic compounds of wine bagass. That means that there was an increase in the polysaccharide hydrolysis of the cellular wall, catalysed by several pectinolytic enzymes and mixed preparations of degrading enzymes (Meyer et al., 1998).

### METHODS OF EXTRACTION OF BIOACTIVE COMPOUNDS

#### Use of organic solvents

The use of organic solvents on the extraction of phenolic compounds is one of the most traditional methods of extraction, being greatly used for the obtainment of extracts from various vegetal matrixes (Pinelo et al., 2004).

The conventional techniques of extraction using organic solvents, such as maceration and Soxhlet, are commonly applied in the chemical, pharmaceutical and food industries. This is used for the obtainment of varied extracts and can use a variety of solvents such as methanol, hexane, chloroform, ethyl acetate, acetone, ether, etc. However, these techniques require a high energetic cost and can degrade thermally sensitive substances, as they use high temperatures for extraction or separation of solute-solvent mixture (Castro and Capote, 2010; Kemperman et al., 2013). Assisted maceration by ultrasound is a relatively new technique, based on the use of energy from sound waves, mechanical vibrations transmitted in a frequency superior to the auditive human capacity. In the last decade, its analytical application had a significant increase, particularly in the preparation of samples, rupture of cellular structure and for favouring and accelerate the release of compounds, chemical reactions and physical transformations (Orozco-Solano et al., 2010). There are, basically, two types of device which generate ultrasound waves: water bath and probe. In both, the ultrasound energy is produced by a piezoelectrical ceramic placed between two metallic plates – piezoelectric transducer (Luque-Garcia and Castro, 2003).

The efficiency of this extraction technique is said to be equivalent or higher than that obtained with the Soxhlet extractor. Its advantages are translated into time reduction, temperature of extraction and quantity of reagents, use of different solvents and mixtures, favouring of reactions that would not occur in normal conditions and a consequent increase in yielding (Castro

and Capote, 2010).

### Use of enzymes

One of the tools used in the process of decomposing the structure of the cellular wall are the enzymes such as the cellulases, hemicellulases and pectinases, which are capable of catalysing the hydrolysis of the polysaccharides links of the cellular wall (Kashyap et al., 2001; Xu et al., 2014b). Factors such as the relationship between time and temperature of the enzymatic treatment, relationship enzyme and substrate or the type of solvent used in extraction influence the liberation of phenolic compounds of the grape peel. In using acetone at 70% as a solvent and size of the particles between 125 and 250  $\mu\text{m}$ , extracts were obtained from the grape bagass at a concentration of 6055 mg GAE/L of phenolic compounds after 8 h extraction, using a pectinase Grindamyl. When no enzyme was used, however, the concentration of phenolic compounds on the extract decreased to 4615 mg GAE/L, confirming that the assisted enzymatic extraction is one of the most efficient techniques available to increase recuperation of phenolic compounds (Meyer et al., 1998).

Although recuperations significantly better of phenolic compounds from grape peel bagass have been obtained with previous enzymatic treatment and posterior methanolic or aqueous extraction, the data available indicate that only 5 to 10% of the dry matter weight is degraded, which suggests that the degree of degradation of the polysaccharides from the cellular was low (Landbo and Meyer, 2004).

Pectinases are the enzymes mostly used during vinification. Some studies have demonstrated the positive effect of using pectinases during maceration on the increase of phenolic compounds of wine during the processing and conservation. An increase of approximately 40% in the quantity of anthocyanins, corresponding to 220 for 305 mg/L was observed during vinification and conservation of wines treated with pectinases (Palma and Taylor, 1999).

In another study (Bautista et al., 2005), significant differences were reported in the quantity of total phenolic compounds extracted, when the same wine was submitted to the action of pectinase. It is known that the presence of lignin can delay degradation of polysaccharides, performed by enzymes through the unproductive adsorption of proteic enzyme by lignin. Besides that, the enzymes can also be inhibited by the presence of tanins. The improved knowledge about a particular polysaccharide on the cellular wall and a better understanding of how the lignin links to the phenolic compounds in the matrix of the cellular wall of grapes would bring new perspectives for the use of specific enzymes, and therefore, an improvement in the recuperation of these from grapes' peel (Schofield et al.,

1997).

In recent years, the trend towards improving the efficiency of phenolic extraction have concentrated mainly on using enzymes, independently or combined with other enzymes. As highlighted previously, the knowledge about the specific link and distribution of phenolic compounds can allow the utilization of more specific enzymes. The purification of enzymatic mixtures, as well as the synthesis of new enzymes, can promote an improvement of the phenolic yields during extraction (Kammerer et al., 2004; Mohamed et al., 2016).

Besides that, new extraction principles and optimization of the extraction conditions, such as the relationship temperature, solvent-solid, the use of supercritical fluids and projects of extraction of new cells have shown to be promising to optimize the release of phenolic compounds of the grapes' peel and valorization of bagass derived from wine production (Hayouni et al., 2007).

### SUPERCritical EXTRACTION

An alternative method to the conventional techniques is the supercritical extraction, which uses supercritical fluids as solvents (Pereira et al., 2004; Shao et al., 2014; Machado et al., 2013). The extraction with supercritical fluid consists in the transference of mass based on the use of fluids to temperatures and pressure above the critical values. The critical temperature is the highest one, at which the gas can be converted in liquid through the increase in pressure. The critical pressure is the highest one, at which the liquid can be converted in gas through the increase in the liquid's temperature (Gomez and Ossa, 2002; Machado et al., 2015; Machado et al., 2016a).

The extraction with supercritical fluid stands out for representing a technology that allows the obtaining of high quality extracts and which minimizes damage to the environment, due to the absence of solvent in the final product. That way, the use of supercritical fluids has been considered a great option for the extraction and fractioning of natural products, particularly for the pharmaceutical and food industries (Pereira et al., 2004; Machado et al., 2013).

Thermal degradation of sensitive compounds is avoided due to being operated in lower temperatures, whilst the absence of light and oxygen prevents oxidative reactions. This constitutes a great advantage for the extraction of antioxidants, ensuring the conservation of its biological properties. The materials processed by supercritical fluids do not require a separate phase for sterilization, once the pressure gradient at the exit of the extractor can generate extracts free from live organisms and spores (Adil et al., 2007; Duba et al., 2015b; Machado et al., 2016b).

In practice, over 90% of extractions performed with supercritical fluids are realized with carbon gas for

**Table 2.** Extraction methods used to recuperate bioactive compounds in residues derived from grape processing.

Food matrix	Bioactive compound	Functional activity	Extraction method	Extractive conditions	References
Grape peel	Overall Phenolics	Antioxidant	Extraction with supercritical fluid	CO <sub>2</sub> + ethanol, 170 kgcm <sup>-2</sup> , 43°C	Pinelo et al. (2005)a
Grape peel	Anthocyanins overall	Antioxidant and anti-inflammatory	Extraction with supercritical fluid	CO <sub>2</sub> + ethanol, 170 kgcm <sup>-2</sup> , 46°C	Pinelo et al. (2005b)
Grape bagass	Overall Phenolics	Antioxidant	Ultrasound	Ethanol	Silva et al. (2008)
Grape peel	<i>Trans</i> -resveratrol	Vasodilator and anti-inflammatory	Extraction with supercritical fluid	CO <sub>2</sub> + ethanol, 40 MPa, 35°C	Casas et al. (2010)
Grape peel	<i>Trans</i> -resveratrol	Vasodilator and anti-inflammatory	Ultrasound	Ethanol	Silva et al. (2008)
Grape peel	Polyphenols	Antioxidant	Counter-current chromatography	methanol-water (80/20; v/v) followed by 3.0 L of acetone-water (75/25; v/v)	Luo et al. (2016)
Grape bagass	<i>Trans</i> -resveratrol	Vasodilator and anti-inflammatory	Ultrasound	Ethanol	Silva et al. (2008)
Seed oil	Tocopherols, tocotrienols, chlorophylls, carotenoids and total phenol contents	Antioxidant	Extraction with supercritical fluid	CO <sub>2</sub> + ethanol 50 MPa, 50°C	Mohamed et al. (2016)
Grape bagass	Flavonoids	Antioxidant and anti-inflammatory	Extraction with supercritical fluid	CO <sub>2</sub> + ethanol, 40 MPa, 32°C	Casas et al. (2010)
Grape	Anthocyanin and phenolics	Antioxidant	Radiation	15°C, up to 2 KGy	Gupta et al. (2015)
Grape peel	Flavonoids	Antioxidant and anti-inflammatory	-	Ethanol, 60°C	Katalinic et al. (2010)

\*Grape bagass, Peel, seeds and stems. \* ERPE, Electronic resonance paramagnetic espectroscopy.

a series of reasons. Besides showing relatively low pressure and temperature (73,8 bar and 31,1°C), CO<sub>2</sub> is inert, non-toxic, non-inflammable, of relatively low cost, easily available in high purity, odourless, and can be readily removed from the final product, leaving no residues (Gomez and Ossa, 2002; Diaz-Reinoso et al., 2006; Machado et al., 2013).

The addition of organic co-solvents such as ethanol, methanol, acetone, among other polar solvents, increases the solvation power of CO<sub>2</sub> and the yield of extraction of polyphenols (Adil et al., 2007; Ni et al., 2015). Despite the supercritical water being frequently used for the destruction of risk-prone organics, the high temperature, above 374°C, the pressure above 220 bar, combined with the corrosive effect of water in these

conditions, have a limited practice in extraction of oil from plants. Water in subcritical conditions has shown to be an effective fluid for the extraction of various classes of oils. This practice is called subcritical extraction or extraction with pressurized liquid (Maier et al., 2009).

In a previous work (Louli et al., 2004) the antioxidant activity of extracts from the bagass of grapes obtained with different liquid solvents was evaluated. It was also submitted to different pre-treatments, posterior to the supercritical extraction, to increment the content of phenolic compounds, the antioxidant activity and the organoleptic properties of these extracts.

In another study (Maier et al., 2009), the residue from grapes seed was used, already used for obtainment of oil through pressing, to recuperate

phenolic compounds. The supercritical extraction has also been previously used as an enzymatic pre-treatment of grape seed to increase the rate of burst/intact cells, favouring the extraction and incrementing the yield of oil (Pazos et al., 2006), as well as for the obtainment of grape seed oil, using ethanol as modifying solvent (Silva et al., 2008). Table 2 shows the extraction methods used in different works in obtaining bioactive compounds.

#### APPLICABILITY OF RESIDUES DERIVED FROM GRAPE PROCESSING

Many studies search the applicability of residues from grape processing, especially aiming at



increasing the input of bioactive components in food products. Products based on grape components are already commercialized in the form of supplements, in powder or capsules (Monagas et al., 2006; Duba et al., 2015a; Güzel and Saygılı, 2016; Machado et al., 2014).

A study was developed (Perin and Schott, 2011) with the aim to elaborate a type of flour from the bagass generated from the processing of grape juice. This was intended to be used in the manufacture of a cookie, added with 5, 10 and 15% of this flour, and the bioactive compounds present in the residue, in the flour and the manufactured cookie would be evaluated. The content of total polyphenols detected had a descending value, from the grape bagass, flour and cookie, respectively, with 56, 18 to 12.07 mg of galic acid/100 mL extract. Both the flour and the cookie suffered some processes which used relatively high temperatures for a significant amount of time, which could have led to alterations/degradations of these components.

In the work developed (Özvural and Vural, 2011), the effects of incorporating grape seed flour obtained from subproducts of wine processing in sausages was investigated. The variations in the physical, nutritional and sensorial parameters were observed in different concentrations of flour. The colour values ( $L^*$ ,  $a^*$  and  $b^*$ ) of the sausages decreased, in general, with the increasing quantity of grape seed flour. The use of this flour has also led to a decrease in the level of oxidation of the products, probably due to its antioxidants content. The increment of grape seed flour in sausages reinforced the contents of protein, total dietary fibre and capacity of water retention in the treatments. Although the level of grape seed flour above 0.5% reduced the general acceptability, the sausages with levels of up to 2% had marks above average. The evaluation of the incorporation of residue from vinification in the production of healthier and more functional sausages was reached by the study; however the importance of further research aiming to improve the palatability of the products was mentioned.

The input of bioactive compounds from food matrixes has been profusely reported in the scientific area, due to its beneficial importance to health. In the last few years, scientific studies have focused on the use of residues derived from food processing. These residues have great quantities of significant bioactive compounds, concomitantly pushing forward advances regarding extraction techniques and improvements in yielding.

The content of the bioactive compounds obtained from food residues shows great potential for incorporation in food products. It can offer a functional character to products originally poor in such compounds, an important aspect in the attempt to stimulate a healthy life style.

In summary, information reported in this work shows a growth in the number of studies performed in the last few years, as well as the use and possible combination of new techniques.

## Conflicts of Interests

The authors have not declared any conflict of interests.

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*Perspective*

# Nanoscale development and its application in multidisciplinary area: An African perspective

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The need to fulfil and address the United Nations millennium goals in developing countries is essential and recently nanotechnology has proved and shown potential to change and improve many sectors of African industry, from consumer products to health care to transportation, energy and agriculture. In addition to these societal benefits, nanotechnology presents new opportunities to improve how we measure, monitor, manage, and minimize contaminants in the environment. This article then provides an extensive review of research needs for both environmental applications and implications of nanotechnology in Africa countries. The review evaluates water purification technology, nano health, food security and risk assessment. It was concluded that, nanotechnology is the future solution, however, countries must embrace the emerging technology with symbiotic involvement with scientific, industrial and institutional sectors.

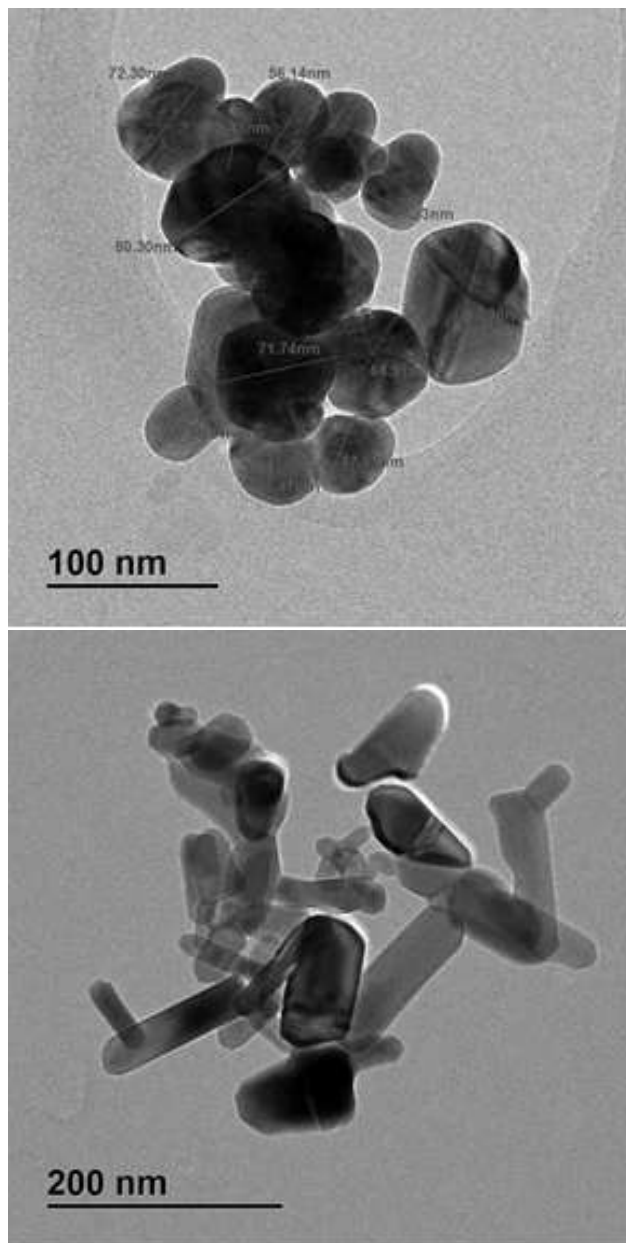
**Key words:** Nanotechnology, nanoparticles, African countries, risk assessment.

## INTRODUCTION

Nanotechnology promises significant improvements of advanced materials and manufacturing techniques with vast range of applications, which are critical for the future competitiveness of national industries (Miyazaki and Islam, 2007; Masoka et al., 2012; Naidoo and Kistnasamy, 2015). The manipulations and productions of materials, whilst controlling the optical properties and surface area to a nanosize scale (Schutte and Focke, 2007; Blatchley, 2013) enabled a birth of a new field known as nanotechnology. Furthermore, it provides the technological platform for the investigation and

transformation of biological systems, with biology offering inspiration models and bio-assembling components to nanotechnology (Roco, 2003). Nanomaterials (NMs) have different strength, conductivity, colour and reactivity, thereby, can be 'tuned' to build faster, lighter and stronger (Schutte and Focke, 2007; Shetty, 2010). It is therefore not astounding to see the commercialised NMs increasing exceptionally from 54 in 2005 and possible to 3400 by 2020 containing mainly silver materials (Figure 1) (WWICS, 2011; Lopes et al., 2016). At the same time, the global production and usage of nanomaterials is

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**Figure 1.** Scanning electron microscope image of silver nanoparticles.

estimated to increase to be thousands of tons in 2004, and projected to increase over half a million tons by 2020 (Maurer-Jones et al., 2013). These predictions therefore, show that nanomaterials will be released into the environment.

The ability to alter these materials' physical and chemical properties to have an enhanced large specific surface area, smaller size and high chemical reactivity has enable NMs to be manufactured and marketed in a wide variety of sectors such as electronics, cosmetics, pharmaceutical, information technology, agriculture, food,

medical, water treatment and environmental protection (Naidoo and Kistnamav, 2015; Lopes et al., 2016).

As highlighted by Naidoo and Kistnamav (2015) that most of the pressing issues faced in Africa practically in the fields of health care, food agriculture, electronics, environment, water, industrial and energy can be addressed with the potential uses of nanotechnology. Furthermore, the major concern in the United Nations (UN) is to meet the Millennium Development Goals (MDGs) by 2015, five goals can significantly deal with the applications of nanotechnology for example eradicating extreme poverty and hunger, reducing child mortality rates, improving maternal health, combating HIV/AIDS, malaria and other diseases to ensure environmental sustainability (Singer et al., 2005). For the sake to revolutionise social, economic and environmental as well as scientific and technological sense, developing countries have realized the need to address their pressuring problems, therefore, a massive lead has been taken in developing, usage and marketing of the nanotechnological products (Singer et al., 2005; Schutte and Focke, 2007; Naidoo and Kistnamav, 2015). The multidisciplinary field is becoming an indispensable component of the global development discourse with focus being set on the expectations that the technology will be an instrument in alleviating poverty and promoting sustainable development especially in water, health and food sectors (Saidi, 2009; Grimshaw et al., 2014).

Freshwater resource is essential to be managed sustainably in order to maintain economic, social and environmental functions due to its contribution to the livelihoods of society (Hillie and Hlophe, 2007; Naidoo and Kistnamav, 2015). Even though the available water resource is approximately 1% freshwater and 97% salt water, the drinkable resources is still labeled as "scarce resource" and most of African countries are considered water scares or water stressed. Unfortunately, only 0.01% of water is available geographically for human usage (Hinrichsen and Tacio, 2002; Fenwick, 2006; Schwarzenbach et al., 2006). However, even though population growth and climate change has an effect on rapid rising withdrawals and increased demand per capita. It is the anthropogenic activities which greatly make detrimental effect on the quality and quantity of drinking water, for instance, partially treated industrial waste with toxic substances, agricultural practices and untreated domestic wastewater. The shortage and inefficiency of drinkable freshwater has significantly paralyzed health, social and economic aspects in most countries (Naidoo and Kistnamav, 2015).

Children under the age of five years are mostly the victims of death attributed to unsafe water, inadequate sanitations or insufficient hygiene (Prüss-Üstün et al., 2008). Water insufficiency and poor water quality leads to food shortage due to scenario such as drought and insufficient supply. With human, the population is expected to increase by 34% with respect to the present

situation by the year 2050. As a consequence of that, there will be an increase in global demand for foods, water and energy (Gutiérrez et al., 2012; Naidoo and Kistnamav, 2015). Therefore, nanotechnology is capable to improve the productivity and efficiency of crop and livestock production (Naidoo and Kistnamav, 2015; He and Hwang, 2016), remove, minimize pollutants and purify water (Grimshaw et al., 2014; Naidoo and Kistnamav, 2015), reduce drug loading and efficacy in medicine (Abuduxike and Aljunid, 2012), whilst increasing the safety, bioavailability, nutritional value, and affordability to meet with future population growth trends. The advantages of various NMs and their application in water technology, health care and food security including their possible risk associated with their usage will be evaluated. Moreover, the involvement of sectors and the current African countries activities and involvement with regards to nanotechnology.

## **NANOMATERIALS IN WATER PURIFICATION AND POLLUTANTS REMOVAL**

Most of the developing countries are facing formidable drinking water challenges and rising demands (Tiwari et al., 2008). There are 1.5 million deaths each year, 4.3% are water related deaths due to repeated diarrhea or intestinal nematode infections and other diarrheal diseases resulting to a total number of deaths to be 860 000 deaths (Prüss-Üstün et al., 2008). Regardless of the efforts to alleviate poverty, two fifths suffer consequences of unacceptable sanitary condition and one-sixth of the world population lack access of safe water (UNESCO, 2003; Li et al., 2008). In 2002, 1.1 billion people lacked access to a reliable water supply, in other words, 40% of people in Africa lacked access to a reliable water supply (Hillie et al., 2006). It is also reported that most children often miss school because their schools do not have adequate drinking water and/or sanitations facilities (Hillie and Hlophe, 2007). Municipal wastewater and industrial processes often discharge their undesirable effluent which impact the aquatic environment, while the latter requires staggering amount of water to pre-treat the waste prior discharging (Tansel, 2008). However, the main issue related to water pollution is not the cumulative of one contaminant, but it is the synergistic effect of two or more contaminants that become toxic to both the environment and human. Disinfection is not only paramount in the final effluent in wastewater or at the potable treatment but invention such as synthesized dendrimers comprising of antimicrobial agents that inhibits growths of microbes to prevent or reduce biofilm formation in water distribution and storage systems (King and Hill, 2006).

There are conventional treatments that are currently being used for water and waste treatment such as but not limited to ceramic filters, distillation, biosand filtrations, UV radiation, chemical treatment, reverse osmosis and

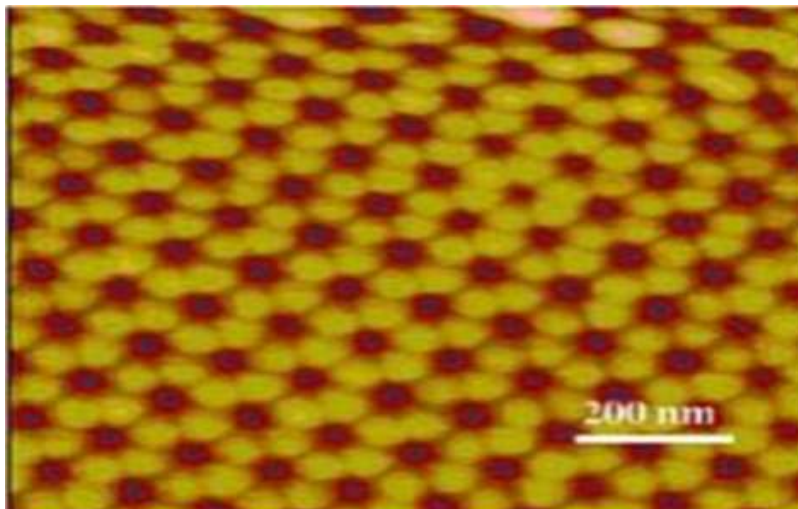
activated carbon filters. However, these limitations ranges from high maintenance, low flow rates, leaching of toxic chemical into water, time consuming and some produces undesirable taste and odors (Meridian Institute, 2006; Naidoo and Kistnamav, 2015). Furthermore, these treatments are unable to remove dissolved salts, organic and inorganic substances (Hillie et al., 2006). Research is underway to use nanosized advanced new materials of nanotechnology to provide potential application of nanoscience to solve technical challenges associated with the removal of water contaminants (Tiwari et al., 2008; Mpenyana-Monyatsi et al., 2012). This method is inexpensive, portable, more effective, convenient to use, efficient, durable and easily cleaned systems which purifies, detoxify, and the desalinate water more efficiently than conventional bacterial and viral filters (Singer et al., 2005; Qu et al., 2013). Researchers have developed technologies that aims to offer a variety of techniques and applications which most have been extensively used and/or tested in developing countries to purify water, for instance, nanofiltration membranes, nanoadsorbents, nanocatalysts, granules, flakes, magnetic nanoparticles, nanopowder, and nanotubes (Hillie et al., 2006; Tiwari et al., 2008). Nanosensors are used to detect chemical and biological contaminant substances including metals (for example cadmium, copper, lead, mercury nickel, zinc), nutrients (for example Phosphate, ammonia, nitrate, nitrite), cyanide organics, algae (for example cyanobacterial toxins), viruses, bacteria, and parasites (Tiwari et al., 2008).

## **NANOPOUROUS**

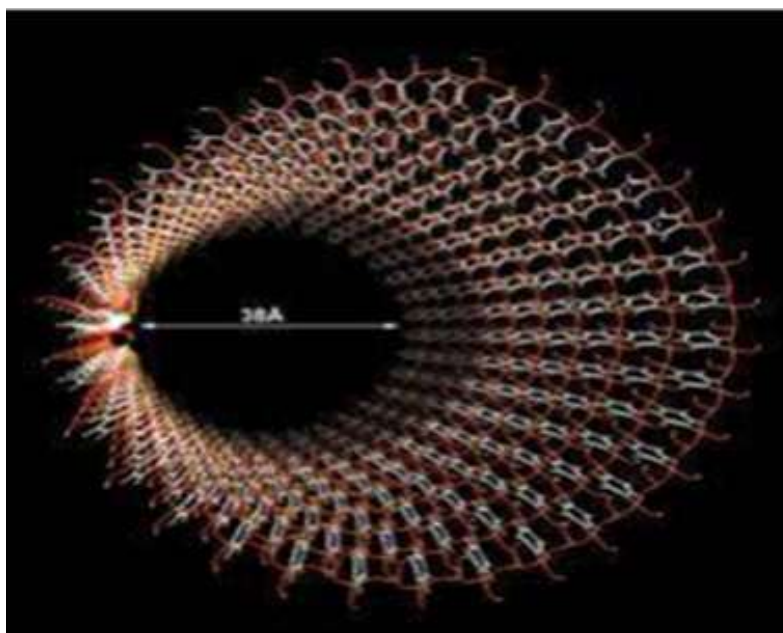
Nanoporous materials such as zeolites, attapulgite clay and nanoporous polymers can all remove contaminants in polluted water with high efficiency of greater than 80% (Singer et al., 2005; Water Wheel, 2008). For instance, as illustrated in Figure 2, when ceramic materials are impregnated with reactant materials, they are able to harvest aerobic bacteria which biologically convert pollutants into nontoxic substances and remove phosphate, lead, arsenic, and other contaminants (Meridian Institute, 2006). Nanoporous function can be compared to nanosorbent using zero-valent and nano iron that are proficient in removing solvents from pumped groundwater and are well capable onto adsorbing organic and inorganic molecules (Lukhele et al., 2010; Water Wheel, 2008). Zero-valent iron absorbs and reduce organic contaminates causing them to breakdown into less toxic simple carbon compounds and heavy metals to agglomerates and stick to soil surfaces (Meridian Institute, 2006).

Zeolites nanoporous are inorganic crystalline porous materials with a highly ordered structure (Figure 3). They are comprised of silicone, aluminium and oxygen. These materials are effective sorbents and ionic exchange





**Figure 2.** Nanoporous membrane (Holister et al., 2003).

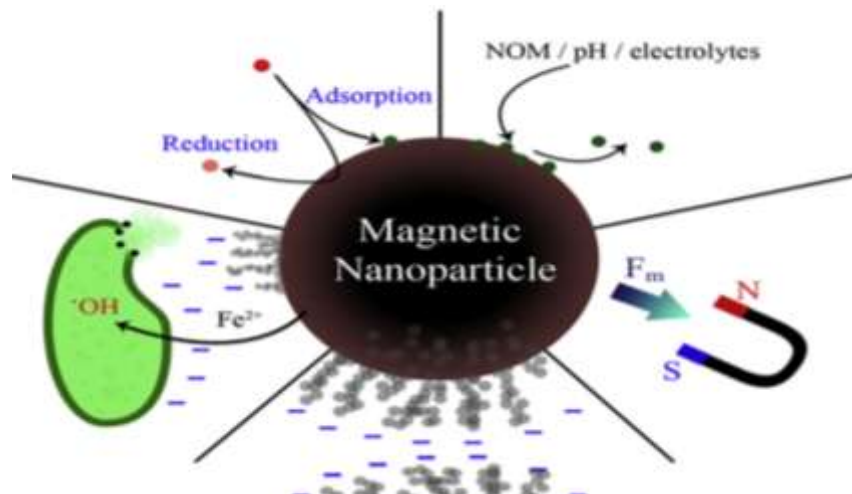


**Figure 3.** Ordered mesoporous organasilica hybrid material with a crystal like wall structure (Holister et al., 2003).

media for ions separation and catalysis (Tiwari et al., 2008). Consequently, nanoporous zeolites incorporated with fly ash has been used in the removal of heavy metals such as chromium, copper, nickel, mercury, zinc, silver and arsenic from acid mine waste and wastewater (Meridian Institute, 2006; Tiwari et al., 2008; Lukhele et al., 2010). In addition, they are effective absorbent, capacity of which is dependent on composition, pH, concentration and type of pollutant intended to be removed (Meridian Institute, 2006). Research study

conducted in Japan demonstrated that ceramic containing silver zeolite had antimicrobial activities that inhibited several functions in the cell and consequently damages cells (Matsumura et al., 2003).

Nigeria, South Africa, and Israel are currently studying nanoporous materials for water filtration, while Algeria is studying the locally available attagulgite clay to filter wastewater from milk factory to offer an economic and effective solution by reducing most organic matters (Hillie et al., 2006).



**Figure 4.** Contaminants removal mechanisms of magnetic nanoparticles (Tang and Lo, 2013).

## NANOCATALYST AND MAGNETIC NANOMATERIALS

Catalyst is a substance that promotes the chemical reactions of other materials without becoming permanently involved in reaction (Hillie et al., 2006). Nanocatalysts and magnetic nanoparticles coated with different compounds for instance, natural chitosan have been used to remove pollutants from aqueous environment and ground water due to the presence of selective affinity for diverse contaminating substances (Figure 4) (Singer et al., 2005; Meridian Institute, 2006; Grimshaw, 2009; Hillie et al., 2006).

Nanocatalysts have been found to have a very good degradation and reduction potential, more reactive, remove a wider range of environmental contaminants, easier to inject and achieve deeper subsurface penetration (Meridian Institute, 2006; Lukhele et al., 2010; Qu et al., 2013). Furthermore, additions of chemicals are not required, easy to operate and maintain and it is selective on the substances to be removed (Zhang et al., 2013). They however, require supporting substrates for their application in water treatment (Lukhele et al., 2010). Several research conducted photocatalytic and photodisinfection by Beckbölet and Araz (1996), Matsunaga and Okochi (1995), and Wei et al. (1994) reported inactivation of *Escherichia coli* and other microbial cells with the presence of  $\text{TiO}_2$

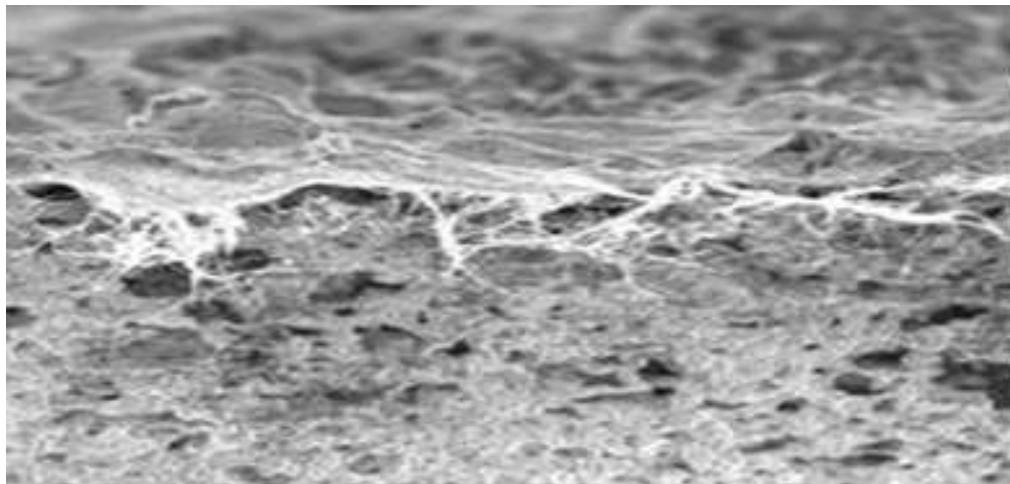
It has been reported that, combining nanomagnetic materials with citric acid allow metallic ions binding and produce a high affinity, therefore, making able to remove heavy metals from soil and water (Figure 4) (Singer et al., 2005). Furthermore, these materials are recycled and reusable, hence, reduces treatment cost, eliminate energy related cost (Singer et al., 2005; Meridian Institute, 2006; Water Wheel, 2008). Magnetic NMs sorption capacity is higher, therefore, are considered

effective adsorbents at low pH and irreversible sorption providing an efficient storage sink for collection of waste (Hillie et al., 2006).

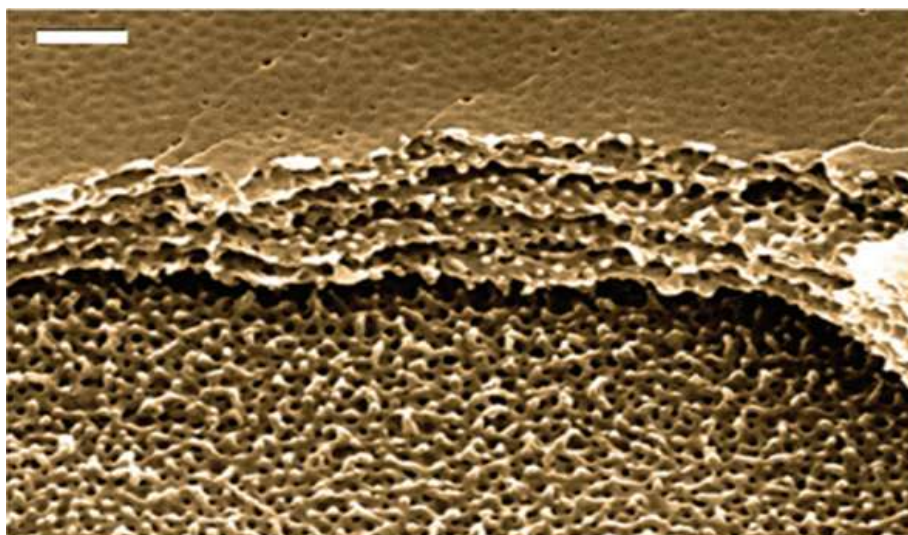
Nanosensors and nanofabrication are some of the technology developed to purify polluted waters. Manipulating nanosized materials to detect and identify the pollutants which allow the nanosensors to progressively remove dissolve salts, degrade broad aspects of water contaminants, capture particles and presence of pathogenic microbes to achieve cleaner water and treat wastewater. It is by the innovation of such technology that gives a real-time detection of the presence of contaminants and pathogens without laboratory testing sample and time consuming technique. Sensors using nanometer scale detectors will enhance and improve health, maintain safe food and water supply, whilst allowing for use of otherwise unusable water resources (Hillie et al., 2006; Qu et al., 2013). Multiwalled carbon nanotubes and single walled carbon nanotubes have been functionalized with cerium dioxide, hydroxyl, phosphate functional groups to remove heavy metals and organics from water (Lukhele et al., 2010). Other NMs that are bioactive, such as silver and magnesium oxide, can kill bacteria and might be used in place of chlorine to disinfect water (Lukhele et al., 2010; Water Wheel, 2008).

## NANOFILTERS

The intelligence of nanofiltration (NF) membrane also known as polymeric permeable membrane has made a breakthrough in drinking water production for the removal of pollutants, provides possibility of refining, improving water, speed and accuracy (Figure 5) (Van der Bruggen and Vandecasteele, 2003; Mousavi and Rezaei, 2011). Nanofiltrations are capable to filter both bacterial and viral



**Figure 5.** Plasma treated nano filters cross-sectional image of the membrane (adapted from Image courtesy of CSIRO Australia).



**Figure 6.** The microstructure of collective osmotic shock generated perforated multilayers (Zava-River et al., 2011).

pathogens (Van der Bruggen and Vandecasteele, 2003; Singer et al., 2005; Grimshaw, 2009), hard, natural organic material (NOM), micropollutants such as pesticides and VOCs, salt, nitrates, and arsenic can be simultaneously or partly removed (Van der Bruggen et al., 2001; Van der Bruggen and Vandecasteele, 2003). These membranes are cheaper to operate, energy saving, have fast flow rate, durable, easy to clean, reusable, and heat-resistance when compared to reverse osmosis membranes (Figure 6), making them to be the latest innovation in the membrane technology and promise to have a huge potential in the desalination of brackish waters (Sonune and Ghate, 2004; Meridian

Institute, 2006; Hillie et al., 2006; Lukhele et al., 2010). Research conducted by the Stephen and Nancy Grand Water Research Institutes of Israel on the treatment and conversion of salt water to freshwater for drinking and irrigation purposes using reverse osmosis (Hillie et al., 2006). Indian research have illustrated that the advanced treatment of wastewater used for additional removal of organic and suspended solids, nitrogenous oxygen demand, nutrient and toxic materials (Sonune and Ghate, 2004). Recent development by Green Turtle Technologies Limited has designed to treat acidic wastewater and allows better control over the effluent pH. This innovative system has replaced the need for large

tanks, caustic injectors and mixers with a very small-automated system. Similarly, disinfection/filtration systems invented by Lindqvist and Sparrman, demonstrated effectiveness as the primary unit comprising of zeolite filter bed that pass through to UV lamp for further disinfection.

NFs are able to retain nutrients present in water that are essential and required for normal functioning of the body (Hillie et al., 2006). Uniformly vertically aligned and tightly packed carbon nanotubes coated with silicon wafer are able to remove almost all kinds of water contaminates. This technology is currently manufactured by university of Stellenbosch and University of North-West University in South Africa (Hillie et al., 2006). Also, these technologies are suggested to be an alternative for reverse osmosis and can be used for desalination (Meridian Institute, 2006). National University of Science and Technology in Zimbabwe conducted waster filter research using ceramic magnetite from abundance iron ore which was excavated from Buchwa mine in Zimbabwe at Dorowa. These researchers inverted communal ceramic water filters that addressed not only the poor quality water issues but also the social impact (South African Department of Science and Technology, 2003).

Nanotechnology has lessened some of the labour intensive and chemical required techniques to clean water. As a result, more people are expected to access clean drinkable water in African countries and more countries are warning up to water technology. Investment calculation study done by Van der Bruggen et al. (2001) indicated that the final price for treatment of ground water is realistic, showing that nanofiltration is a valuable option for ground water treatment. With regards to water quality and quantity it affects people livelihood and also their health status should be taken into consideration.

## NANOMATERIALS IN HEALTH IMPROVEMENT

People depend on water in four key ways: as an input into-production to sustain livelihoods, to maintain health and welfare, and to ensure ecosystems integrity. Lack of drinking water and sanitation kills 4,500 children a day, mostly as a result of waterborne diseases (Hillie et al., 2006). Diseases among others are continuously dealt with in developing African countries such as human immunodeficiency virus infections and acquired immune deficiency syndrome (HIV/AIDS), malaria, and tuberculosis (TB) are major causes of mortality and morbidity (Hauck et al., 2010). The main concerns with the modern medicine are the inability of the human system to absorb the entire dosage of a drug due to low bioavailability (Shetty, 2010).

The effective and efficient nanotechnology has enlighten and encouraged the development of drugs at a nanoscale that are cost effective, more bioavailable,

longer lifespan, improved delivery, reduces their toxicity, dosage and drug loading. Furthermore, it improves the patients' compliances to successfully complete treatment and uptake of fewer drugs per treatment. Diagnosis and screening of disease are achieved more rapidly and sensitively through the assimilation of manipulated engineered NMs that are capable to recognize, screen diseases at a molecular and cellular level and henceforth, produce a reliable visual imagining of the affected organs (Musee et al., 2013).

## DIAGNOSIS AND SCREENING

There is an urgent need to diagnose infectious diseases at their primary stage in developing countries (Shetty, 2010). The use of gold NMs makes the detection of diseases simpler, whilst fluorescence quantum dots, carbon nanotubes and other nanowires are used as biosensors tagged to antibodies that target infected cells with TB or HIV could also be used for detecting malaria (Hauck et al., 2010; Shetty, 2010). Hauck et al. (2010) postulated that NMs have been used to construct sensors in three different platforms for simple infectious diseases diagnostics: (1) NMs labels in immunochromatographic tests (ICT) assays, (2) NMs aggregation assays and (3) NMs labels of whole pathogens. Diagnosis and screening of infectious disease is followed by drug delivery.

## DRUG DELIVERY

There are sensitive aspects taken into consideration with most of the drugs intended to deliver drugs in the human systems. For example, the NMs must be able to degrade naturally at the target site and should not become a biopersistant in the system. Then, the excess drug should be able to be excreted out of the body system. Nanosized drug delivery systems developed to improve and treat varies, namely, polymeric NMs, solid lipid NMs, liposomes nanoemulsion, dendrimers, clydextrins and inclusion, nanosupensions, nanocapsule, buckyballs and drug conjugates (das Neves et al., 2010; Santos-Magalhães and Mosqueira, 2010; Shetty, 2010).

Encapsulated drugs with biodegradable polymers for enhanced and sustained delivery of medicine of TB, HIV and malaria are being studied in South Africa at DST/CSIR at one of the African Network for Drugs and Diagnostics Innovation (ANDI) Centre of Excellence for Health Innovation in Africa, and now is called ANDI Centre of Excellence in Nanomedicine Research (Cele et al., 2009; Musee et al., 2012). Moreover, the Nigeria, Egypt, Botswana, Kenya, Tanzania, and Swaziland are among the African countries that are planning to have national strategies and government funding, having research group engaged or pursuit in nanotechnology (Shetty, 2010). Zimbabwe's government allocated about



60% of new program budget to nanomedicine (Makoni, 2012).

## NANOMATERIALS IN FOOD SECURITY

Water is nexus and inter-connectivity with food security as it is imperative to achieve a sustainable use of natural resources, improve the environment and sustain ecosystem functions and services, which addressed the livelihood and malnutrition status (Lal, 2015; Belal and El-Ramady, 2016). Additionally, Belal and El-Ramady (2016) also highlighted that the close relationship is between soil security, water security, energy security, climate security, economic security and political security. Therefore, agriculture is a fundamental aspect connected with all human societies and securities (economic and political) that are characterised more than ever with increasing world population and are closely linked with food security (Mousavi and Rezaei, 2011; Belal and El-Ramady, 2016).

According to Prüss-Üstün et al. (2008), childhood underweight causes about 35% of all deaths of children under the age of five years worldwide. Freshwater withdrawals for agricultural purposes amount to about 88% which is higher compared to other water users of all annual withdrawals (Hinrichsen and Tacio, 2002). Conversely, the food safety and security is still an issue due to the supply and sufficient supply without demeaning soil health and agroecosystems (Shephard, 2003; Kashyap et al., 2015).

The battle to eradicate malnutrition and prevent the toll deaths of children can be resolved by incorporating nanotechnological materials as agrinotechnology (Belal and El-Ramady, 2016). Nanotechnology in agriculture and food production is intended to improve soil retention; prevent extinction and destruction by means of genetically modified plants and animal species. This technology will provide the efficiency of the agricultural products for higher population growth that is imprisoned by poverty (Mousavi and Rezaei, 2011). Such as, production of the nano-encapsulate for nutrient components alteration, enhance flavours in consumers' food to suits their tastes, food packaging that will detect pathogens, assist in soil retention, soil fertility and production with nano- seeds that have built-in pesticides that is only released on favourable environmental conditions (Scrinis and Lyons, 2007; Belal and El-Ramady, 2016).

Mousavi and Rezaei (2011) postulated that the use of nanotechnology in agriculture and food industry can revolutionize the sector with new tools for disease detection, targeted treatment, enhancing the ability of plants to absorb nutrients, fight diseases, and withstand environmental pressures and effective systems for processing, storage and packaging. Conversely, nanotechnology is rapidly moving from the laboratory and

onto the farm, supermarket shelves and the kitchen table (Scrinis and Lyons, 2007), and will be increasingly available to consumers worldwide with large international interest and investments from governments and global corporations (FAO/WHO, 2012; Belal and El-Ramady, 2016).

## NANO AGROCHEMICALS FOR SOIL

Soils contains complex matrix such as mineral particles and colloids in pore water, therefore, the adsorption and binding of pollutant with the matrix is of concerns (Sharma et al., 2015; Belal and El-Ramady, 2016). Thus, continuous application of variety of chemical in the fields leads to a loss of UV degradation, hydrolysis, microbiota interaction or leaching. As a result, it negatively affects the environment in the form of soil degradation and water pollution (Gutiérrez et al., 2012). Therefore, the invention of an "intelligent" pesticides which are intended to increase their toxicity, better efficacy, use less solvent in spraying and better control of dose (Scrinis and Lyons, 2007; Chaudhry and Castle, 2011) is more appropriate. Encapsulating the pesticide, herbicides, and fungicides will enable to increase the availability, target and deliver in controlled manner by environmental conditions such as heat levels and moisture. But the mobility of NMs is dependent on the attachment between the soil molecules, shape of the NMs, collector and the different properties that change environment surrounding the particles (Belal and El-Ramady, 2016). Nanopesticides adsorb easily by root systems, increase their effectiveness and reduce the dosage and usage of pesticides (Scrinis and Lyons, 2007). For instance, metal oxides NMs ( $\text{Fe}_3\text{O}_4$ , CuO, ZnO and  $\text{TiO}_2$ ) mobility is influenced intensively by humic acids, aquifers and ionic strength of resident water (Ben-Moshe et al., 2010).

As stated by Belal and El-Ramady (2016), the generation of co-productivity, but anthropogenic use of primary resources (soil, water, climate) and secondary inputs (fertilizers, irrigation, tillage, amendments) must be optimised. This technology promises a production approach in remediation of contaminated soil and groundwater. Nanoporous materials such as zeolite materials used in food agriculture industries have been shown to more efficiently slow, controlled release of fertilizers, efficient in livestock feeding and delivery of drugs (Singer et al., 2005). According to Gutiérrez et al. (2012), less herbicide is required to achieve the desired weed reduction effects. If the active ingredient is combined with a smart delivery system, herbicide will be applied only when necessary according to the conditions present in the field (Singh et al., 2015; Belal and El-Ramady, 2016). Soils infested with weeds and weed seeds are likely to produce lower agricultural yields than soils where weeds are controlled. Improvements in the efficacy of herbicides through the use of nanotechnology

could result in greater production of crops and less injury to agricultural workers who are physically removing the weeds if herbicides are not used.

Nanotechnology plays a role in recycling the residual materials of agricultural products to energy and industrial chemicals. With the use of newly-developed solvents and a technique called electro spinning, cotton nanofibers can be used as a fertilizer or pesticide absorbent for encapsulating chemical pesticides, to prevent the scattering of chemical pesticides in the environment, water and soil. Porous nano-polymers have a very similar function to the pollutants molecules, and considered as the most suitable means for separating organic pollutants of soil and water. Nanoporous materials capable of storing water and slowly releasing it during times of drought, therefore, it can be expected to increase yields (Gruère et al., 2011). For example, nanoporous zeolites are used for slow release and efficient dosage of water, fertilizers for plants, release of nutrients and drugs for livestock (Hong et al., 2013). Furthermore, zeolite NMs improves water-retention capacity of soils to increase crop production in areas prone to drought, such as sub-Saharan Africa (Gruère et al., 2011). Similarly, nano fiber-based fabrics are being used as a detection technology platform to capture and isolate pathogens embedded with antibodies against specific pathogens (Mousavi and Rezaei, 2011). Moreover, silica porous membranes are used for filtration to disinfect water and unwanted components in plants extraction and clarifying wines and beers (Chaudhry and Castle, 2011); also, silver and iron nano particles are used in the treatment and disinfection of livestock and poultry (Chaudhry and Castle, 2011; Mousavi and Rezaei, 2011).

## NANO FOOD PACKAGING

Nanocomposites, defined as polymers bonded with nanomaterials to produce materials with enhanced properties, have been in existence for years but are recently gaining momentum in mainstream commercial packaging use (Butschli, 2004). Flexible packaging consumption's rapid growth represents a \$38 billion market in the global community (Thibeault, 2004). As the demand in the industry continues to rise at an average of 3.5% each year, flexible materials need to meet and exceed the high expectations of consumers and the stressors of the supply chain (Butschli, 2005). The longevity and freshness of foods in shelves are essential; therefore, the composite in nanoscale will be applied on the packaging as a barrier. These materials will increase the strength and quality in packaging (Scrinis and Lyons, 2007). NMs applications incorporated for packaging barriers using silicate NMs, nanocomposites, and nano-silver, magnesium- and zinc-oxide and nano-silver, magnesium to increase the packaging quality, improved mechanical and functional properties, improve flexibilities,

temperature and moisture, enhance stability, with incorporating metal and metal oxides for antimicrobial agents (Chaudhry and Castle, 2011). Oxygen is a problematic factor in food packaging, because it can cause food spoilage and discoloration (Mousavi and Rezaei, 2011). Food tends to produce the chemical fumes called ethylene gas, which lead to the freshness wearing off and increases the decay, then introduce pathogens and decrease their life span before reaching the retailer shelves and consumers. Therefore, food packaging is essential for national and global transportation distribution. Whilst, delivering fresher, safe and pathogenic free food products, nanotechnology applied in packages will be able to repair small holes/tears, respond to environmental conditions (for example temperature and moisture changes), and can alert the customer if the food is contaminated (Scrinis and Lyons, 2007; Mousavi and Rezaei, 2011). Nanotechnology furthermore, promises to develop and manufacture packaging which are cost effective and that aim to improving the quality, durability and shelf life longevity (Scrinis and Lyons, 2007).

Nanomaterials and polymers are formulated to a broader variety that exists in forms of nano-textured, nano-carrier, organic NMs, inorganic NMs and liposomes, etc. Processed nano-structured food products are soluble with less fats and emulsion with enhanced taste and it is healthier coupled with antimicrobial effect to pathogens. For instance, nano-encapsulated liposomes or biopolymers and nano-carriers with supplements and nutrients are able to mask and protect tastes of ingredients/additives during processing, improve optical appearance, improve bioavailability, uptake and increase adsorption (Chaudhry and Castle, 2011).

Nano radio frequency with identification tags also known as RFid, tracks the deterioration in food quality before the food is sold, it is eco-friendly device that can be consumed (Scrinis and Lyons, 2007). Nanobarcodes are cheap, efficient, rapid, easy decoding and can be used for detection of diseases, moreover, can tag multiple pathogens in a farm which can easily be detected using any fluorescent-based equipment (Mousavi and Rezaei, 2011). RFid can ultimately increase the efficiency of management and buying arrangements for the large-scale retailers able to absorb the costs of these nano-monitoring and identification techniques and, hence, enable tracking food products in supply chain (Scrinis and Lyons, 2007; Chaudhry and Castle, 2011).

On the other hand, nanosensors devices are one of the technologies that will help the agricultural industry to combat viruses, crop pathogens and pests, chemical contaminant, chemical, physical and biological, nutrient content, and plant stress; due to drought, temperature, and lack of nutrients by using the integrated sensors working at the nanoscale meter (Scrinis and Lyons, 2007; Gruère et al., 2011; Mousavi and Rezaei, 2011;

Neethirajan and Jayan, 2011). Additionally, the sensors can detect chemical odours produced by fungi and insect which promote food spoilage in storage; also it is able to measure changes in carbon dioxide to detect incipient and ongoing deterioration (Bouwmeester et al., 2009; Mousavi and Rezaei, 2011). The study conducted by Gonzalez-melendi et al. (2008) explores the possibilities of using carbon coated iron nanomagnetic materials to track infectious disease on plants and crops. Their findings reveal that magnetic nanomaterials can be used for fruit trees and green house plants. The accumulation and guidance of materials to a specific area of interest can be achieved by magnetic gradient in plants.

Nanosensors have the potential to allow farmers to utilize inputs more efficiently by indicating the nutrient or water status of crop plants over fine spatial and temporal scales, by transmitting to a satellite through a signal towers to the farmer's cellphone to alter the present condition in the storages, allowing the farmer to apply nutrients, water or crop protection measures (insecticide, fungicide, or herbicide) only where necessary (Gruère et al., 2011). GPS connected with nuclear links systems to a satellite have put another step closer for farmers to harvest and plant without uncertainties of weather changes. System controller provides information on each growth factor such as nutrition, light, temperature, planting and harvest time to avoid encountering bad weather conditions. Best time to achieve the highest yield, best use of fertilizers, irrigation, lighting and temperature are all controlled by these systems (Mousavi and Rezaei, 2011).

As a result, it increases the security of manufacturing, processing, and the shipment of food, by enabling early detection of contaminants, and the removal of infected products from the food chain. These 'smart' sensors will then alert the retailer by causing the sensor strip to change colour as a result, giving a clear visible signal of whether the food is fresh or not (Scrinis and Lyons, 2007; Neethirajan and Jayan, 2011). Henceforth, providing solutions to modify the permeation behaviour of soils, increase barrier properties (mechanical, thermal, chemical, and microbial), improve mechanical and heat-resistance properties, develop active antimicrobial and antifungal surfaces (Mousavi and Rezaei, 2011).

## **NANO VETERINARY**

Production of materials that are able to protect animals and livestock and poultry is paramount to food manufactures, animal domestication and veterinary. Nanotechnology has the potential and ability to provide appropriate solutions for providing food items, veterinary care and prescription medicines and vaccines for domesticated animals (Mousavi and Rezaei, 2011; Hong et al., 2013).

Mousavi and Rezaei (2011) stated that nano capsules are used to cap and protect some particular enzymes and

proteins to render them effective in the livestock and poultry food rations in order to increase yield and effectiveness in the specific context. Taking certain medications such as antibiotics, vaccines, and probiotics, would be more effective in treating infections, nutritional and metabolic disorders, when use in the nano level. Medicine used in the nano level has multilateral properties to remove biological barriers for increased efficiency of medicine. Appropriate timing for the release of drug, self-regulatory capabilities and capacity planned are the main advantages of using nanotechnology in the drug treatment. However, it is paramount that the quality assurance in food and bioprocessing industry because consumers demand safe and wholesome food as well as governments impose stringent regulations to ensure food safety and feed hygiene (Neethirajan and Jayan, 2011).

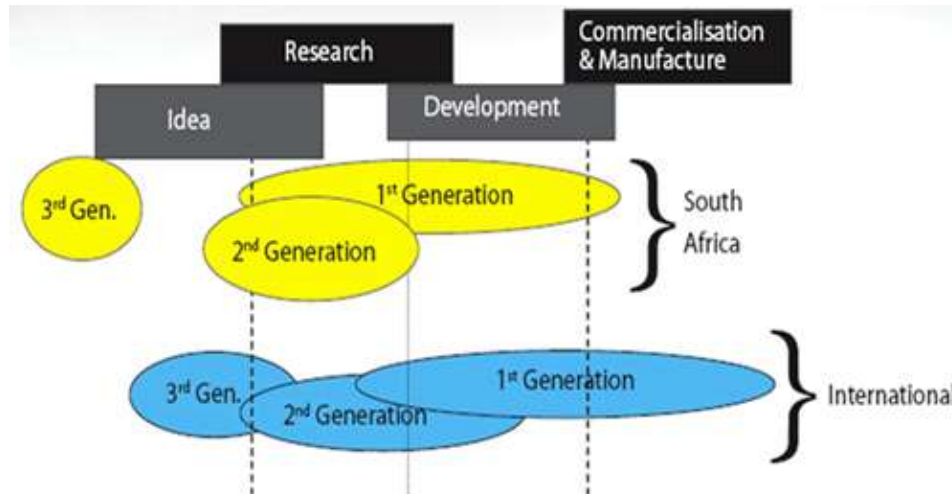
## **THE GOVERNMENTAL AND INSTITUTIONAL INVOLVEMENT ACROSS AFRICA**

Necessary conditions to the development of adapted and appropriate technologies are the presence of sufficient research and development investments, whether in the public or private sectors (Gruère et al., 2011; Fonseca and Pereira, 2014). Lack of support for nanotechnology science and applications in developing countries could force these countries to either abandon nanotechnology options or import (transfer) existing technologies from other countries (Suri, 2006; Cozzens and Wetmore, 2010; Cao et al., 2013). There is lack of emphasis on societal issues and risk assessment principles yet economies and public sectors have heavily invested in nanotechnology.

Meanwhile, Saidi (2009), emphasizes the point on strengthening world-wide capacities in science, technology and innovation provide prospects for human development of which nanotechnology is expected to play an instrumental role. Technology developed is paramount as it can be transferred to countries that needs it the most; however, appropriate and effective approach such as, technological adaptations, technical capability, infrastructure and market potential to prevent an unforesee failure for the technology are needed (Hillie and Hlophe 2007; Gruere et al., 2011).

The involvement of private sector is tricky because their main concern is whether there is sufficient infrastructure, basic capital, and sufficient economic incentives to invest into nanotechnology. The uncertainties of investment and technologies make it a little impossible for domestic companies to invest into long-term research and development programs and lack of access to capital (Gruere et al., 2011). However, African countries such as Egypt, South Africa, Botswana, Nigeria, Morocco, Tunisia, Kenya, Sudan, Algeria, Ethiopia, and Zimbabwe have invested in local research teams involved in nanotechnology.





**Figure 7.** Progress of nanotechnology in South Africa related to the world (South African Department of Science and Technology, 2003).

### South Africa

By far South Africa is one of the first African countries that has received the introduction of nanotechnology well (Figure 7). The government entity funded most of the stakeholders, research institutions and academic institutions for more research for its benefits and risk associated with the usage; moreover, Department of Science and Technology (DST) had placed a 10 year plan for nanotechnology that had given birth to other bodies. South African approach to nanotechnology shows how the country's policy is influenced, albeit timidly, by national interests (for example risk assessment, occupational health and strategic social focus) (Musse et al., 2013). The researchers are broader from water purification, energy, health and agricultural practices and mining.

In 2002, South African Nanotechnology Initiative (SANi) was launched, followed by National Nanotechnology Strategy (the Strategy) published in 2006, with the aim to support and promote nanotechnology R&D, develop human capacity in this field and promoting flagship at national level (South African Department of Science and Technology, 2003). Later on, National Innovation Centers (NICs) were developed containing two centres: (i) National Centre for Nano-Structured Materials (NCNSM) at the Council for Scientific and Industrial Research (CSIR), (ii) Mintek, and serve as national multi-user research facilities. Both NICs are financially supported from the DST and have established partnerships with private companies, for example, SASOL, ECO-Structure International, Biomass Corp., and De Beers. CSIR at the Natural Resources and the Environment (NRE)/DST in 2007, collaborated with academic institutions, Water Research Commission, International Academic Institutions to assess the environmental risk assessment

and management associated with NMs (Cele et al., 2009).

Researchers based at Mintek NIC researched on the development of nanobased targeted drug delivery systems, nanocomposites materials, nanomaterials for the rubber industry, and modelling. They formed partnerships with the Water Research Commission and the Medical Research Council, Goldfields, 180 degrees, Real World Diagnostics, and other South African academic institutions. It is during the year 2008, when DST launched the Nanotechnology Public Engagement Program (NPEP) implemented by the South African Agency for Science and Technology Advancement (SAASTA) (Musse et al., 2013). South African Bureau of Standards (SABS) approves the International Standards Organization (ISO) and International Electro technical Commission (IEC), while the National Institute for Occupational Health (NIOH) is more concerned with the human and occupational health of workers handling and working with NMs.

South Africa has also signed a number of international agreements related to nanotechnology; above all, an active agreement is a trilateral joint venture consisting of India, Brazil, and South Africa (IBSA) (Musse et al., 2013).

### Nigeria

Poverty is a major problem in many developing countries in the world, including Nigeria with reportedly over 70% of the population deriving their livelihood from agriculture. Hunger and malnutrition are aggravated by rapid population growth, influences the food insecurity-adequate quantity and quality of food (Fasoyiro and Taiwo, 2012). Despite socio-economic challenges,

Nigeria has implemented a project on environmental remediation carried out as a joint collaboration of the African University of Science and Technology and the Sheda Science and Technology Complex since 2006 (Masoka et al., 2012; Musse et al., 2013). It also has a project for a national nanotechnology initiative and is currently implementing a pilot project of the United Nation Institute for Research and Training (UNITAR).

### **Egypt**

In Egypt, the Nanotechnology Research Centre was funded by the Information Technology Industry Development Agency and the Science and Technological Development Fund in partnership with IBM, and was launched in 2009. This project is probing the possibility of developing devices for enhancing health provision, and water purification, mostly targeting the rural areas. While the UN Department of Economic and Social Affairs (DESA), for example, is engaged in providing support to the construction of high tech R&D and industrial parks in Ghana and Senegal, while Egypt and Kenya already have techno parks and Ethiopia is currently developing a similar project (Masoka et al., 2012; Musee et al., 2012).

### **Morocco**

In Morocco, projects on the implementation of an international laboratory for molecular chemistry, creation of a Euro-Mediterranean competence pool in micro-technology and nanotechnology, purification and preservation of Moroccan water resources, and urban waste treatment have already been started (Khachani, 2005; Bouoiyour, 2006). In the year 2006, National Initiative for Nanosciences and nanotechnologies was launched; an industrial high tech park was built in Rabat (Technopolis) (Masoka et al., 2012).

### **Algeria**

Although, the field of nanotechnology is nascent in Algeria, incorporated nanomicro-electronics in the Microelectronics Division of the Advanced Technologies Development Centre, and a National Centre for Research on Nanomaterials and Nanotechnology was established at the University M'Hamed Bougara of Bourmerdes in 2011 (Musse et al., 2013).

## **RISK ASSESSMENT FOR ENVIRONMENT, FOOD, AND HUMANS**

Researchers report that the worldwide use of engineered nanoparticles can lead to their release into the environment and their effect become a public health concern (Gottschalk et al., 2013). Therefore, assessing

and managing the possible risk associated with the production and usage of NMs will reduce risk of toxicity exposure to the environment, animals and humans. There are large knowledge gaps that need to be addressed regarding nanotechnology life-cycle in full, from the production, transportation, uses and disposal. According to Wiesner et al. (2009) managing risks associated with NMs exposure will require the ability to quantify and differentiate the relative importance of manufactured, natural, and incidental sources of NMs at each step of the material's life cycle and understand the processes that govern NMs transport, persistence, bioavailability, and toxicity.

Numerous advantages related to nanotechnology and NMs are always highlighted forth; however, it is their ecotoxicological and nanotoxicological information that are still lacking (Savolainen et al., 2010a). In essence, Suh et al. (2009) stated that a fundamental understanding of nanomaterial toxicology (nanotoxicology) is highly desirable both from the material's stand point as well as from the biological system's point of view. This is due to physicochemical characteristics of the material arriving at the receptor; thus, tend to metamorphoses physically and chemically.

The toxicity measure and assessment of NMs are greatly relying on the parameter used for ecotoxicity which give a little insight of the NMs. The main concern with regards to nanotechnology is the uncertainties of exposure and health of occupational, due to, a broad human exposure pathway, through skin, inhalation and digestion is yet not satisfactory to give a holistic view of the danger and hazard (Savolainen et al., 2010b).

Savolainen et al. (2010a) emphasized that the fundamental elements of risk assessment are likely to remain and will continue to include the elements carefully designed for other chemicals and particles, notably (1) hazard identification; (2) hazard characterization; (3) exposure assessment; (4) risk characterization; these are the four steps of the risk assessment process. However, the features of these materials set new challenges for, for example characterization of test materials. Meeting these challenges would also greatly benefit assessment of risks of other materials. Therefore, as much as nanotechnology and NMs are solving most of the problematic issues, the chance of also bringing unforeseeable hazards to human health and depletion of environmental ecosystems is high. Understanding and filling the knowledge gaps will bring more information on handling and quantifying NMs.

### **Water**

The understanding of abiotic interactions between NMs and natural substrates and solutions; interactions between NMs and living organisms and the longevity, reversibility of ecological sources and sinks; and the

resulting consequences of NMs exposure for productivity, organic matter decomposition, and trophic transfer will assist in predicting the ecosystem impacts of NMs (Qu et al., 2013). Manufactured NMs show some complex colloid and aggregation chemistry, which is likely to be affected by particle shape, size, surface area and surface charge, as well as the adsorption properties of the material. Abiotic factors such as pH, ionic strength, water hardness and the presence of organic matter will alter aggregation chemistry; and are expected to influence toxicity (Handy et al., 2008).

While some classes of NMs may be toxic in the lab and inert in natural environments; it is also possible that other classes of NMs will become more lethal as a result of abiotic and biotic processing in complex ecosystems. It has been proposed that passage through the gut of organisms could strip stabilizing coatings of NMs, thus increasing their reactivity and toxicity (for example exposing the Cd-containing core of quantum dots). NMs may also have indirectly detrimental effects on ecosystems through their interactions with existing environmental contaminants (Wiesner et al., 2009).

## Health

Toxicology studies on animals and cells raise the possibility of adverse effects on the immune system, oxidative stress related disorders, and diseases such as cancer (tumour formation). However, the doses needed to produce these effects are generally high and it remains to be seen if such exposure is possible via the environment (food, water or air) or in the work place (Handy and Shaw, 2007).

## Agro-food

Nanotechnology has numerous advantages such as targeting specific infected area in plants and animals, ability to control or delay delivery, increase the efficiency, reduce dosage and drug loading of nano carriers (Mousavi and Rezaei, 2011). However, the potential for nano carries to alter tissues distribution, insolubility, ingestion and biopersistent is highly possible (Oberdorster et al., 2005).

However, uncontrolled use of pesticides, herbicide, and fungicides has caused many problems, such as adverse effects on human health, adverse effects on pollinating insects and domestic animals, and entering this material into the soil and water and its direct and indirect effect on ecosystems (Mousavi and Rezaei, 2011). Occupational health for farm workers and rural residents who are exposed frequently to these nano-pesticides, due to the size and dissolvability of nanoparticles pesticides must be considered (Scrinis and Lyons, 2007; Chaudhry and Castle, 2011). Most of the times pesticides are applied as

a precautionary manner leading to the residual toxicity and environmental hazards and on the other hand application of pesticides after the appearance of disease leads to some amount of crop losses which raised a concern with regards to the controlling the infection diseases (Hong et al., 2013). There is higher possibility that residues of these nanopesticides might be present in products as consumed (Bouwmeester et al., 2009). Meanwhile, the exposure of NMs from animal feeds into consumer's foods (for example meat, milk etc) is also likely (Chaudhry and Castle, 2011). Concisely, the possible toxicological effect of nano-encapsulated pesticides may be soil contamination, environmental pathway and food-chain of living organisms across a wider geographical area (Scrinis and Lyons, 2007). Nanofertilizers have not been linked to increased phytotoxicity or to an increased propensity for eutrophication of surface waters, but little research has been done. It is expected that just as conventional fertilizer products have been associated with some adverse impacts. Conversely, nanofertilizers and nanoherbicides may present some of the same effects (Gruere et al., 2011).

The toxicity studies with regards to active packaging releasing NMs with antimicrobial functions into the food (for example nano-silver and in rare cases zinc-oxide NM), will lead to direct consumer exposure to (free) NMs (Bouwmeester et al., 2009). NMs such as nanoclay that is used as a gas barrier, nano-titanium dioxide for UV protection in transparent plastic and nano-titanium nitrate for mechanical strength bind with polymers to functionalize might migrate into food products (Chaudhry and Castle, 2011).

## CONCLUSION

Nanotechnology on its own is a very important field, with countless advantages that improves the traditional medical, agriculture, electronics, fashion, consumers' products, etc. The discovery to enable, to manipulate atoms and molecules at a size of nanometer has given a promise to alleviate and enhance most of the current issues faced by most African societies which cripple the liberty of social and economy. This technology has proved beyond reasonable doubt that MDGs especially faces by Africans could be eradicated and alleviate poverty situation. It is clear that the only resource that is interconnecting all other is water which is an important commodity that affects the balance of all other factors that supports and services a society, addressing water pollution it addresses water stress and water availability issues, hence, agriculture and health will be resolved.

Yet, on the other hand, nanotechnology is making vibration into potential risks for health or environment and determines their fate and behaviour in the environment, humans and other organisms. However, not all materials

in nano sized are considered toxic, it is the gap of knowledge with regards to the biopersistant NMs that are of great concerns. There is huge debate on the life-cycle of manufactured NMs, their impact and effect on the environment and especially to human. Those who produce or manufacture NMs, those who utilize products assimilated with nanotechnologies, their safety and awareness requires regulatory bodies that will validate information on relevant characteristics of NMs as present in the product is at least known to risk assessors.

It has been seen that the benefits of nanotechnology over power their toxicological effects as most of the investors turn a blind eye and endanger the consumers as the end-users of the life-cycles of the NMs. It is paramount for a continuous open communication and involvement between the government, private, academic institutions, parastatals and stakeholders to develop a strategic plan and risk assessment plans. It is so clear in countries such as South Africa and Egypt, where the government are funding and encourages extensive research on this technology. Even though such initiative has taken place, an active collaboration with international partners is needed. It is a great achievement that there is an improvement in most of African countries involved with nanotechnology. Although some are still skeptic, major strides have been taken already to render their poverty alleviation issues.

### Conflict of Interests

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Plant physiological stimulation by seeds salt priming in maize (*Zea mays*): Prospect for salt tolerance

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Nowadays to lessen the influence of salinity on crop performances such as germination, seedling growth and yield, several actions are undertaken; for example the use of different priming methods, antioxidants and plant growth controllers. Seed priming, best fits in this study on maize crop and is controlled by hydration process followed by re-drying that allows pre-germination which enhanced metabolic activities to proceed rapidly. The objective of this study was to investigate how seed salt priming increases maize crop performance thereby enhancing yields by stimulating the plant physiological processes. Here, sodium chloride and calcium chloride primed seeds germinated earlier than unprimed one. Primed maize seeds had better efficiency for water absorption. Likewise, energetic metabolic activities in germination process commence much earlier than radicle and plumule appearance. Priming of seeds with salt solution enables them to break their dormancy and escape from disease causing agents and competent seeds of weeds. Seed priming with NaCl and CaCl<sub>2</sub> had significant effects on germination, early growth, number branches, number of cobs and grain yield. This increase in growth traits likely helps to reduce the competition for water and nutrients with associated improvements in seed yield. Besides, it makes seed priming practice a viable option for the successful use of maize in irrigation areas. Sodium chloride (NaCl) priming increases shoot length while, calcium chloride (CaCl<sub>2</sub>) priming increases root length. Seeds grown in vertisol soil prefers seed priming for better stand establishment and crop yield whereas; seeds in lithosol soils prefer priming for better seed germination and increase number cob.

**Key words:** Seed salt priming, maize, salt tolerance, germination rate, physiology.

## INTRODUCTION

Crop launching depends on the effects of seed patch of soil and seed good features (Khajeh-Hosseini et al., 2003). Salinity has been recognized as the major seed patch of soil factor, influencing crop establishment in arid and semiarid grass lands (El-kharbot et al., 2003).

Particular salt effect could contribute for significant reductions in the rate and final germination percentage of plants, which in turn may lead to uneven seed emergency and initiation by means of that reduction in crop yields. Speedy, even, and complete germination is a prerequisite

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for successful plant growth, production and stand establishment in grain crops (Demir and Ermis, 2003). In area of short and low rainfall, salt accumulation is common because infiltrating moisture is insufficient to percolate salts added by irrigation. In soils comprising an extra sodium chloride salt, the available water to the plant is limited. This process results in a partial dehydration of the cytoplasm. Consequently, this results in shrinking of the protoplasm of crops from the cell wall resulting to an adverse effect on the metabolism of the cells and the functions of larger molecules which eventually outcomes in the cessation of growth (Le Rudulier, 2005).

Overdue and unpredictable germination and underprivileged preliminary establishment are the major reasons of poor maize crop production under irrigation areas and on saline fields of Ethiopia. Therefore, helping seed germination and stand establishment artificially can benefit to secure good crop yield from salt-affected areas because some findings from primed wheat under saline condition are exemplified as advantageous (Wahid et al., 2010; Jafar et al., 2012). Similarly, as a result of increased salinity problems, the prerequisite to develop crops with higher salt tolerance has increased strongly within the last decade.

To reduce the impact of salinity on crop performance, production and productivity, several treatments are carried out. For example, contrived application of different osmotic protectants (coating and priming), antioxidants, plant growth regulators, and moderate salt treatments are common one (Koyro, 2013). Moderate salt concentration treatment of the promising cash crop, *Panicum turgidum* growing at a low quality soils, enabled the crop to perform better in photosynthetic and growth responses. Its sustainable use can also help in desalinizing and reclaiming degraded land as well as sequestering CO<sub>2</sub> (Koyro, 2013). Furthermore, the addition of organic matter of olive mill waste compost and poultry manure are effective on the availability and uptake of plant nutrients in a highly saline soil (Walker and Bernal, 2008). Likewise, application of compost and sewage sludge increases biological activities of salt affected soils by improved soil physical-chemical properties, especially the carbon and nitrogen balance for the soil microbes (Lakhdar et al., 2010). Similarly, Jasmine rice production in salt affected soils can be improved by the application of gypsum and farmyard manure of the salt affected soils (Cha-um et al., 2011). The addition of supplemental calcium sulphate to nutrient solution containing salt significantly improves growth and physiological variables affected by salt stress (for example plant growth, fruit yield, and membrane permeability) and also increased leaf K<sup>+</sup>, Ca<sup>2+</sup>, and N contents in tomato plants (Tuna et al., 2007). Treatment of seeds with NaCl solution prior to sowing or seed priming induces good stand establishment of crops. Melon seeds primed with 18 dSm<sup>-1</sup> NaCl solution for 3 days at 20°C prior to sowing increased their seedling growth evidences such

that, seed priming prior to sowing improves melon physiological performance. Likewise, seed priming with NaCl induce possible physiological adjustments in pepper seeds and Dekoko seeds, especially in the early stages of development which could be used as a suitable tool for improving germination and growth physiognomies of crops under salt stress conditions (Sivritepe et al., 2003; Aloui et al., 2014; Tsegay and Gebreslassie, 2014) and potassium priming and genetic engineering, which serve as a means to improve plant resistance to salt stress (Cushman and Bohnert, 2000).

All aforementioned treatments enable plants to respond to stress physiology by overproduction of different types of responsive biological solutes (Azevedo Neto et al., 2006). Responsive biological solutes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular concentrations. Generally, they protect plants from stress through different courses which include contribution to cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins. Furthermore, since some of these solutes also protect cellular components from dehydration injury, they are commonly referred to as osmoprotectants (Saadia et al., 2012; Koyro, 2013).

Salinity tolerance of plants can be improved by soaking seeds in solutions of different salts before sowing, as plants from such treated seeds show more adaptation with enhanced physiology to saline conditions than the untreated seeds (Farooq et al., 2010a). Seed priming or osmoconditioning is one of the physiological methods which improves seed performance and provides faster and synchronized germination (Neto and Tabosa, 2000). It is an easy, low cost and low risk technique which is recently used to overcome the salinity problem in agricultural lands. It entails the partial germination of seed by soaking in either water or in a solution of salts for a specified period of time, and then re-drying them just before the radicle emerges (Neto and Tabosa, 2000). Seed priming stimulates many of the metabolic processes (physiological and chemical) involved with the early phases of germination. Moreover, it has been noted that seedlings from primed seeds emerge faster, grow more vigorously, and perform better in adverse saline conditions (Cramer, 2002). Seed priming with CaCl<sub>2</sub> and NaCl could be used as an adaptation method to improve salt tolerance of seeds. Studies conducted by Cano et al. (2001) and Cayuela et al. (2006) with tomatoes, Ahmadvand et al. (2012) with soybean, and Elouaer and Hannachi (2012) with safflower evidenced that seed priming improves seed germination, seedling emergence, growth and yield production under saline conditions. Passam and Kakouriotis (2004) also reported benefits of CaCl<sub>2</sub> and NaCl; seed priming did not persist beyond the seedling stage in cucumber, while Farooq et al. (2005) found that NaCl seed priming had positive effects on mature plants and yield of tomato.

Since  $\text{CaCl}_2$  and  $\text{NaCl}$  seed priming have become an important technique to increase salt tolerance of plants, it is necessary to understand the physiological effects which mediate the responses to salinity. According to Cano et al. (2001), the higher salt tolerance of plants from primed seeds seems to be the result of a higher capacity for osmotic adjustment, since plants from primed seeds have more  $\text{Na}^+$  and  $\text{Cl}^-$  ions in their roots and more sugars and organic acids in leaves than plants from non-primed seeds. External  $\text{Ca}^{2+}$  has been shown to ameliorate the adverse effects of salinity in plants (Kaya et al., 2006). According to Hasegawa et al. (2000), this amelioration is most probably, facilitating higher  $\text{K}^+/\text{Na}^+$  selectivity of the plants. Calcium has often been used as a pelleting (seed coating) material. For instance, it has been known that coating rice seed with calcium peroxide increased germination and plant establishment in their various forms. Seed coatings have become an important part of modern agriculture, and some have been shown to improve emergence and seedling growth in agronomic crops like safflower (Elouaer and Hannachi, 2012).

Ethiopia has been reported to possess over 11 million hectares of unproductive naturally salt which affected wasteland (PGRC, 2000). These areas are normally found in the arid and semi-arid lowlands and in rift valley and other areas that are characterized by higher evapotranspiration rates in relation to low precipitation (PGRC, 2000). According to Tamir (2004), salt-affected flats have increased from 6 to 16% of the total land area of Ethiopia in recent years where 9% of the population lives in these areas. About 44 million ha (36% of the country's total land areas) are potentially susceptible to salinity problems.

Claiming back these salt affected areas and the use for agricultural production is very costly and time consuming. The practice of maize production in Ethiopia ranges from large-scale commercial farms to smallholders and subsistence farmers; and consumed as main food, with its crop residues and by-products commonly fed to livestock. Hence, Priming of maize seeds (one of the most important crop in Ethiopia) with  $\text{CaCl}_2$  and  $\text{NaCl}$  could be important in improving the growth performance and yield enhancement of maize in areas that are potentially susceptible to salinity problems and totally unproductive naturally salt affected wastelands. Such study was not done on physiological tolerance of maize seeds, by seed priming in Ethiopian agricultural landscapes and particular in the study sites. Therefore, this study investigated the evaluation on physiological stimulation of priming on improving seed germination, seedling growth and finally yield enhancement of maize under different salinity levels at open field condition.

## MATERIALS AND METHODS

### Geographical location of the assay

The study was conducted in Sirinka Agricultural Research Center

(SARC). Sirinka Agriculture research center is one of the regional research centers under ARARI. It is located in the dryland areas of north eastern Ethiopia (north Wollo Administrative Zone) 508 km from Addis Ababa on the main road to Mekelle and 12 km south eastern of Woldia. SARC is found in Habru district at Sirinka kebele. The center has 2 sub-centers (Kobo at 1450 m.a.s.l and Hayik at 1680 m.a.s.l). With regard to climatic condition, it has an altitude of 1850 m.a.s.l,  $11^\circ 45' 00''$  north latitude  $39^\circ 36' 36''$  east longitude (Figure 1) with major soil type of Eutric vertisol. The average annual rain fall of the study area is 945 mm and average maximum and minimum temperature is 26 and  $13^\circ\text{C}$ , respectively (Administrator, 2014).

### Seed collection and soil sampling

The improved variety of *Zea mays*, Melkasa-2 multiplied in Sirinka Agricultural Research Center (SARC) which was released from this research center was used for this study. Melkasa 2 seeds were obtained from the certified seed supplier of north Wollo zone found in SARC located in south eastern of Woldia. A total of 800 seeds were sown in 80 green plastic pots each with four replications. Soil samples were taken from the Kobo and SARC for nutrient analysis. This was done purposely for the determination of the amount of the salt treatments containing the respective  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ionic nutrients.

Representative soil samples were collected from the fields to estimate their soil fertility through soil analysis using multiple sampling methods. Samples were collected from soil surface layer down to the depth of 15 to 25 cm which is the standard depth in cultivated fields. The samples were taken from five soil cores both in Kobo and SARC to show variations in fertility from one part of the field to another and the value of mean were taken, after mixing and sieving the soil samples thoroughly.

### Seed priming methods

Seed priming is applied preferably in this study. It is a controlled hydration process followed by re-drying that allows pre-germination metabolic activities to proceed rapidly (Farooq et al., 2005a). The addition of  $\text{CaCl}_2$  and  $\text{NaCl}$  approaches was applied for this study prior to sowing of the maize seeds. Melkasa 2 seeds were sterilized superficially with sodium hypochloride ( $\text{Na}_2\text{HCl}$ ) solution on smooth layered flow for 3 min, followed by thoroughly washing for 5 min with distilled water.

Subsequently, the seeds were primed by soaking 5g/l  $\text{NaCl}$  and  $\text{CaCl}_2$  solutions for 12 h, under shade and the ratio of seed weight to solution volume were set at 1:5 (g/ml). After priming, seeds were removed and washed with tap water and then rinsed three times in distilled water. Finally seeds were left in air between two filter papers to re-dry to their original moisture level as a method proposed by Afzal et al. (2009b).

### Design of the experiment assay

The experimental design was three factors factorial, arranged in a randomized block design, with four replications. The first factor was salinity types and stresses due to salt solutions (0, 5, 7, 9 and 11 dS/m  $\text{NaCl}$  and  $\text{CaCl}_2$ ), the second factor was the Melkasa-2 seed types (primed and unprimed seeds) and the third factor was soil types of black and red soils. This experiment was conducted in 80 green plastic pots (40 for  $\text{NaCl}$  treatment and 40 for  $\text{CaCl}_2$  treatments and each divided into 20 pots per the two soil types arranged randomly in a mixed manner).

Ten seeds were sown in each 50 L contain pot (40 cm height and 35 cm in diameter) at 2 cm depth and replicated four times. The

## Sub Centers and Stations Under SARC



**Figure 1.** Map of the study area. Sirinka Agricultural Research Center is found in the Northern part of Ethiopia. Source: (Administrator, 2014).

pots were internally covered with black plastics to maintain constant environmental temperature. The bottom of each pot was lined with drainage sand to keep the soil well-drained and was filled with 2:6:2 sand to local soil (the growth medium) and organic manure fertilizer, respectively, instead of drilling to have bottom water drainage and side aeration holes (Ahmadvand et al., 2012).

All the time, watering was made continually by the respected salt concentrations for various treatments. Each pot was irrigated with 250 ml of saline solution, produced by sodium chloride and calcium chloride of desired treatment during early morning and evening every day. After the 10<sup>th</sup> day seedling emergence, some seedlings were removed per two weeks interval to maintain nutrient nourishment level, aeration, water usage and other environmental factors optimum for those that long last for the continual observation and assessment with good spacing. Three seedlings per pot were left for last measurements. The salinity of water in the experiment was similar to that used by farmers to irrigate their corn fields, even at the end.

Shoot and root length were measured starting from the 2<sup>nd</sup> week of planting and were done for four successive months. After five months from the start of the experiment, the total number of leaves of every plant in each pot was counted. Subsequently, root length, shoot and root fresh weights were determined immediately in each treatment. Seedlings were dried out in drying oven for 48 h at 70°C, shoot and root dry weights were measured (Elouaer and Hannachi, 2012). Then plants were harvested carefully to prevent falling of the seeds. Finally, biomass difference, length variation, yield enhancement and overall performance of the treatments were,

analyzed.

### Parameters assayed during the salinity experiment

Some among the measured growth features of the crop were:

Total germination percentage (TG %) which was measured in 147 day using the formula

$$(TG\%) = \frac{n}{N} \times 100 \quad (1)$$

Where n, number of seeds germinated per day and N total number of seeds sown.

Mean germination is the number of seeds germinated in the intervals of time, established for data collection (The number of days required for seeds to complete the germination process). It was calculated according the formula of Kader and Jutzi (2004).

$$MGT (d) = \frac{\sum(TiNi)}{\sum Ni} \quad (2)$$

Where Ni = number of germinated seeds on the i<sup>th</sup> days and Ti= rank order of day i (number of days counted from the beginning of germination).

Germination index (GI) was calculated using the equation used

**Table 1.** Analyses of variance of different parameters.

Source of variance	TG (%)	MGT	GI	CV	SL	RL	CY	SB	VI
unprimed	80*	9.11*	238.96*	4.87*	299.50*	361.86*	7.40*	8.00*	76.91*
Primed	69.47*	15.24*	183.11*	7.14*	212.00*	344.70*	36.40*	9.00*	70.30*
Unprimed x primed	79.75*	6.53*	116.498*	3.14*	0.14*	0.13*	5.467*	0.18*	0.01*
Error	0.03*	0.07*	6.12*	0.92*	11.72*	2.18*	0.98*	0.56*	0.05*

TG = Total germination of seeds in percentage; MGT = mean germination time; GI = germination index; CV = coefficient of variation; SL = shoot length; RL = root length; CY = crop yield; SB = shoot branch; VI = vigor index. \*Significant at 0.05 according to Duncan test.

**Table 2.** Means comparison of the measured assays under different salt type treatments.

Parameters	TG (%)	MGT	GI	CV%	SL	RL	CY	SB	VI
Control	70.50 <sup>c</sup>	8.15 <sup>c</sup>	118.98 <sup>a</sup>	3.97 <sup>c</sup>	133.25 <sup>b</sup>	289.51 <sup>a</sup>	4.03 <sup>c</sup>	2.5 <sup>b</sup>	42.98 <sup>c</sup>
NaCl priming	77.51 <sup>b</sup>	12.78 <sup>a</sup>	70.69 <sup>c</sup>	5.17 <sup>b</sup>	167.25 <sup>a</sup>	220.27 <sup>b</sup>	19.55 <sup>a</sup>	2.3 <sup>c</sup>	50.06 <sup>b</sup>
CaCl <sub>2</sub> priming	82.5 <sup>a</sup>	9.33 <sup>b</sup>	83.22 <sup>b</sup>	6.11 <sup>a</sup>	124.25 <sup>c</sup>	207.72 <sup>c</sup>	9.65 <sup>b</sup>	3.5 <sup>a</sup>	50.94 <sup>a</sup>

\*Means with the same letters in each column are not significantly different at 0.05 according to Duncan test. TG = Total germination of seeds in percentage; MGT = mean germination time; GI = germination index; CV = coefficient of variation; SL = shoot length; RL = root length; CY = crop yield; SB = shoot branch; VI = vigor index.

by Kader and Jutzi (2004).

$$GI = \frac{\sum TiNi}{Ti} \quad (3)$$

Where, Ti is the number of days after sowing and Ni is number of germinated seeds in the observation day.

Coefficient of velocity of germination (CVG), gives an indication of the rapidity of germination, which was calculated following the method used by Kader and Jutzi (2004).

$$CVG = \frac{\sum Ni}{\sum (TiNi)} \times 100 \quad (4)$$

Where, Ti is number of days after sowing and Ni is number of germinated seeds in an observation day.

The vigour index (VI) was used to assess the power of surviving, growing and thriving of the germinated seeds and was measured using the formula of Elouaer and Hannachi (2012) calculated as follows:

$$VI = \frac{[TGP(\%) \times \text{Seedling Length}(cm)]}{100}$$

## RESULTS

### Salt stress response evaluation of the assayed parameters

Analysis of variance showed that all salinity levels, salt types and soil types have significant effects on the studied parameters of *Z. mays* (Melkasa 2) seeds ( $p < 0.05$ ). Moreover, the interaction of salinity level and soil type with different salt solutions, had significant effect on

all parameters tested at 5% significant level except the early emerged roots measured in the first and second weeks after the emergence of plumules and radicals (Tables 1 and 2).

Priming with NaCl and CaCl<sub>2</sub> increases the germination (total germination of seeds in percentage, mean germination time, germination index and coefficient velocity of germination) and growth parameters (grain yield, root to shoot ratio and vigor index) of maize (*Z. mays*), as compared to unprimed seeds (control) under different levels of saline conditions. For example NaCl and CaCl<sub>2</sub> priming is an energetic supplement for uniform start of germination for maize seedling under saline conditions (Figure 2).

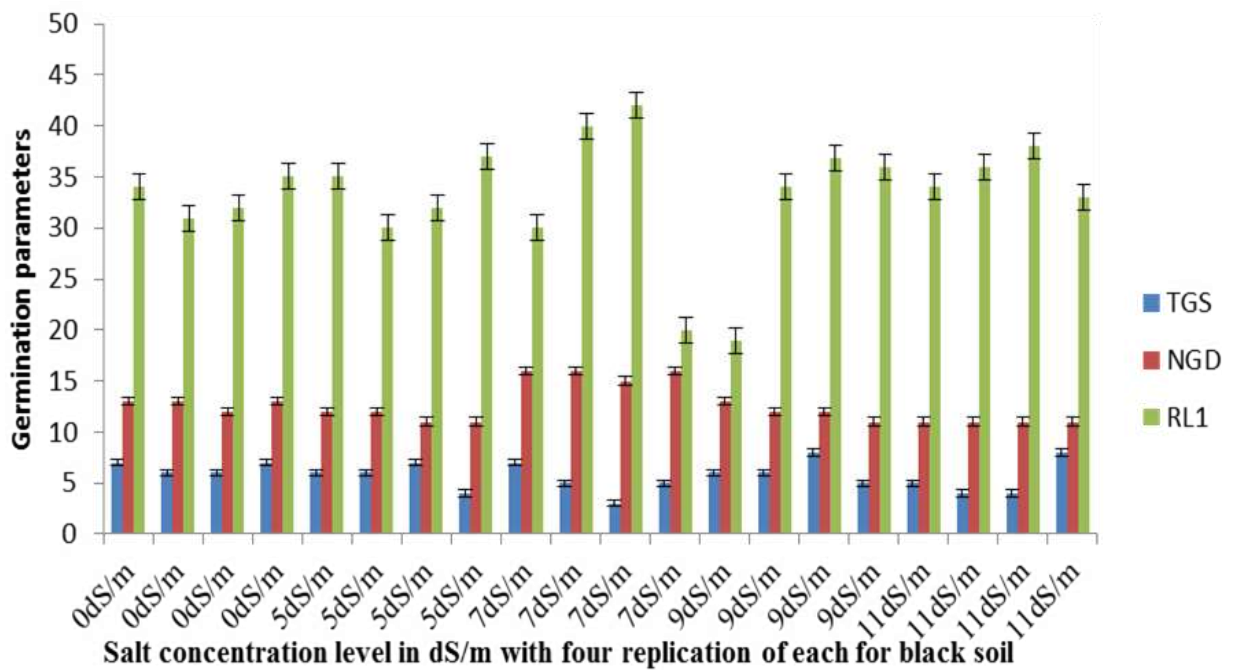
At the lower salinity level (7 dS/m) total germination of seeds decreased as compared with other salinity levels. However, the number of days for the completion of seed germination increased at 7 dS/m salinity level like the other higher salinity levels in black soil type at control (Figure 3).

Conversely, in red soil type the total germinated seeds were almost similar apart from the highest concentration level (11 dS/m) that showed lower germinated seeds of *Z. mays*. When primed seeds of maize are sown in black soil, their root length at the first instances of radical and plumule emergence was less susceptible to salt stress. In all the treatments the root length at the first two weeks was approximately similar, 35 cm except for 7 dS/m showed longer root length. Here unprimed seeds have longer root length than the primed once (Figure 3).

As it can be seen from this figure with four replication and five different salt treatments for Melkasa 2 seeds, total germinated seeds were highly pronounced at 7

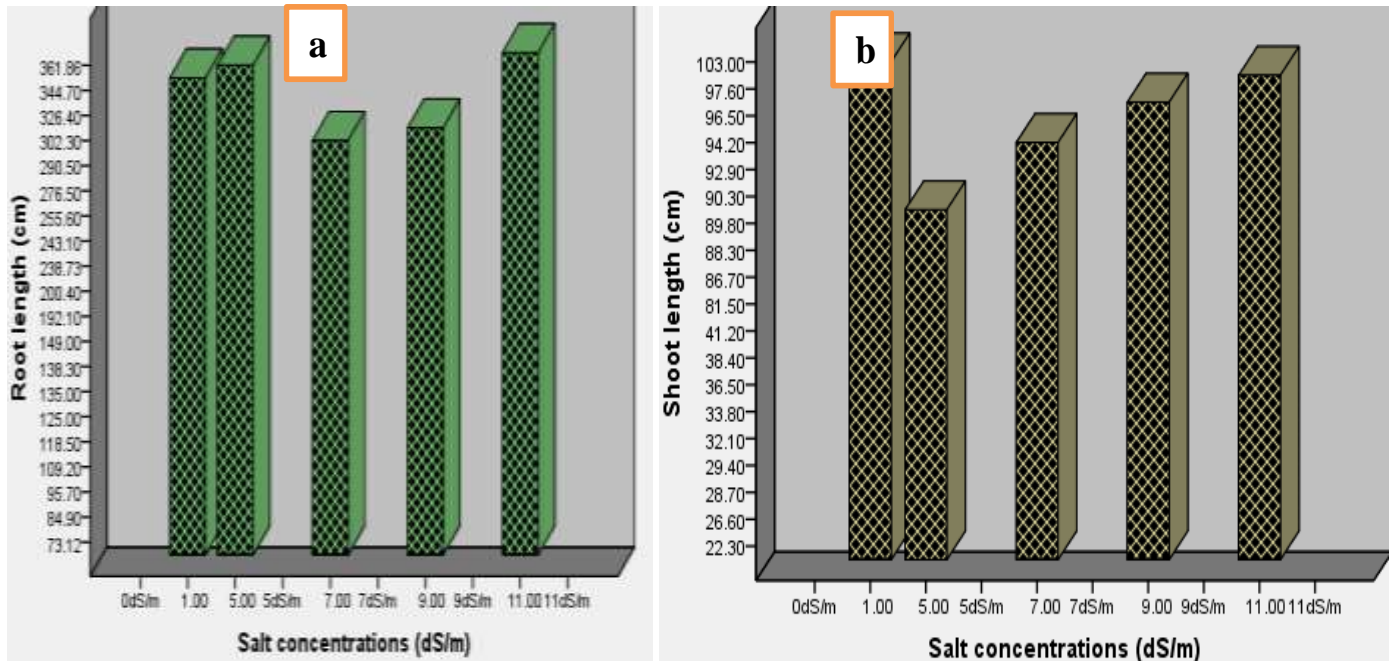


**Figure 2.** Uniform stand establishment of salt (NaCl and CaCl<sub>2</sub>) primed maize seeds.



**Figure 3.** The effect of salinity on total germination of seeds (TGS), number of days for germination compellation (NGD), and root length (RL) of Melkasa 2 seeds.





**Figure 4.** The effect of different salinity levels of (a) NaCl on root length and (b) CaCl<sub>2</sub> on shoot length of maize seeds.

dS/m in the third replication. Moreover, the highest averaged root length was found as 34 cm at 7 dS/m similar to the control (0 dS/m) followed by 33.5 cm for 11 dS/m, 33.25 cm for 5 dS/m, and 30.5 cm for 9 dS/m, respectively. Similarly, the number of days for germination compellation was higher at 7 dS/m.

#### Root length and shoot length susceptibility to salt stress

Priming seeds with NaCl and CaCl<sub>2</sub> prior to sowing, decreased root length at the medium salinity levels (7 and 9 dS/m) as compared with the control. However, the lowest and highest salinity levels (5 and 11 dS/m), respectively indicated longer average root length compared with the control (Figure 4a). There was a 17.16 cm root length difference between the medium salt concentrations and the lowest and highest salt concentrations.

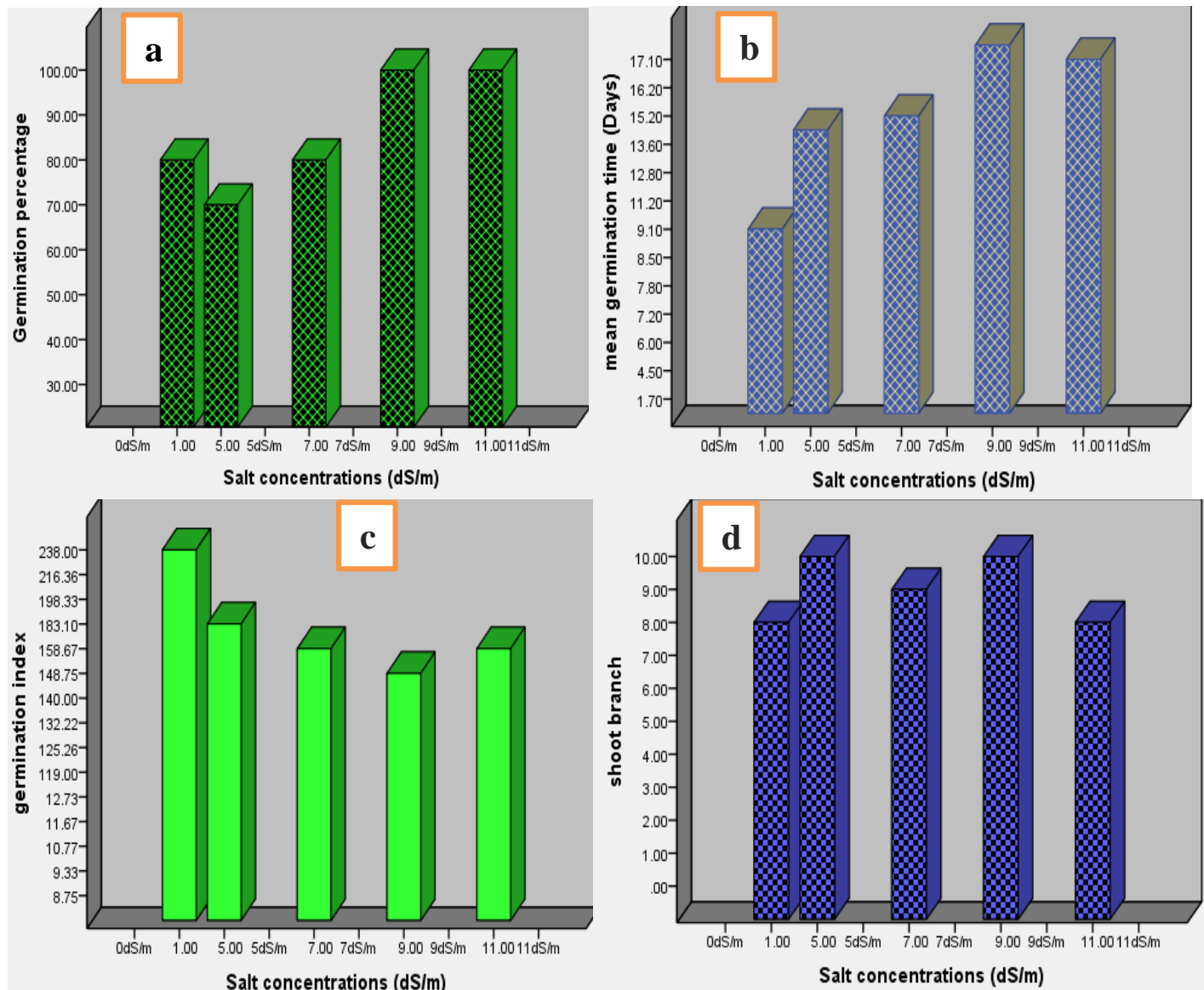
Moreover, *Z. Mays* seeds showed a reduction in shoot length compared to the control as the salinity level of CaCl<sub>2</sub> increases, except the highest salinity level (11 dS/m) that possess similar shoot length with the control. But with salt treatments as salinity level increases, shoot length of primed seeds also increases. At the highest salinity level, primed seeds have similar average shoot length with an unprimed maize seeds (Figure 4b). This may be because external Ca<sup>2+</sup> ameliorates the adverse effects of salinity by facilitating higher K<sup>+</sup>/Na<sup>+</sup> selectivity of the plant.

#### Germination percentage, mean germination time, germination index and shoot branch

Sodium chloride and calcium chloride priming decreased earlier in the population of seeds to be germinated compared with the control though there was a satisfactory increase later. Total germination percentage of primed maize seeds increased significantly ( $P < 0.05$ ) with an increasing NaCl and CaCl<sub>2</sub> salinity level except the lower salt treatment (5 dS/m) (Figure 5a).

Moreover, the rise in total germination percentage was significantly higher for seeds primed with higher salinity levels (9 dS/m and 11 dS/m) as compared to the control and lower salinity level primed seeds (Figure 4a), indicating that priming increases root physiology such as water absorption and nutrient uptake. The higher salt tolerance of the roots from primed seeds seems to be the result of a higher capacity for osmotic adjustment in the root system, since plants from primed seeds have more Na<sup>+</sup> and Cl<sup>-</sup> ions in their roots and more sugars and organic acids in leaves than plants from non-primed seeds. The rise with 20% on total germination percentage due to an increase in salinity level from 0 to 11 dS/m showed that, seed priming increase germination percentage when compared to unprimed seeds except the lowest salt treatment with 10% reduction (Figure 5a).

The lower mean germination time is 10 days for control group, indicating the lower average length of time for maximum germination of seed lots (Figure 5b). Germination index however was lower in salt treated seeds compared with the control. Seed priming prior to



**Figure 5.** The effects of different salt concentrations on (a) germination percentage, (b) means germination, (c) germination index time and (d) shoot branch of *Zea mays*.

sowing with NaCl and CaCl<sub>2</sub> also enhances shoot branch in *Z. mays* crops particularly of Melkasa-2, compared with seeds sown without any salt treatment. There is two more branch increment in 5 and 9 dS/m salt solutions compared with the control (Figure 5d). The addition of NaCl and CaCl<sub>2</sub> might increase leaf size by increasing the membrane permeability which increases the leaf N<sup>+</sup> and Ca<sup>2+</sup> thereby the number of leaf branches.

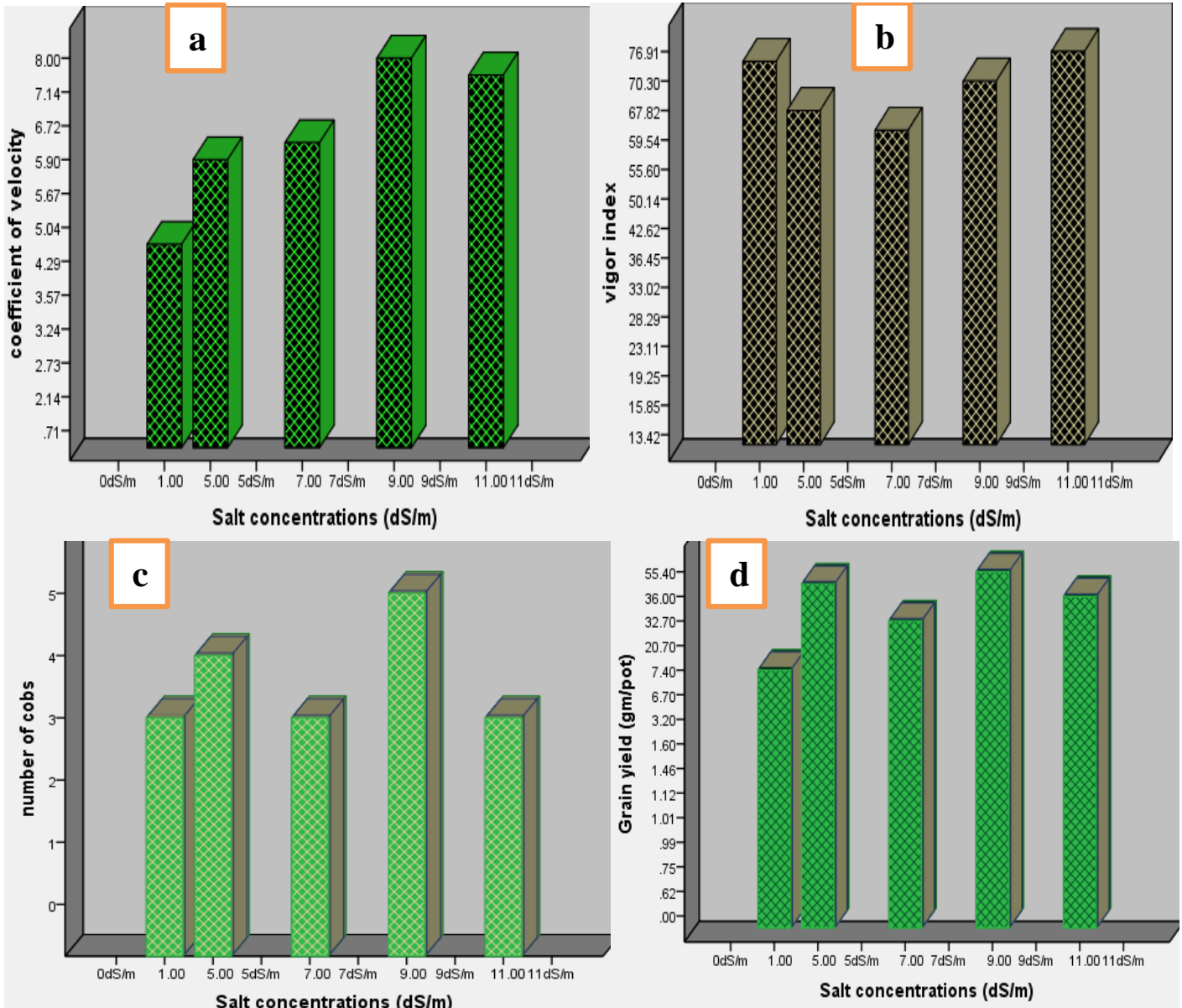
These is a statistically significant ( $P < 0.05$ ) difference in all the growth features of Melkasa 2 maize, where primed with different salt types (NaCl and CaCl<sub>2</sub> (Table 2). Compare with the unprimed seeds, CaCl<sub>2</sub> and NaCl priming respectively in descending order showed better total germination percentage and vigor index. However,

mean germination time, shoot length and crop yield were better in NaCl primed seeds as compared with CaCl<sub>2</sub> primed seeds (Table 2). This is because CaCl<sub>2</sub> is preferable for seed coating and germination and did not persist beyond the early seedling growth whereas NaCl seed priming had positive effects on mature plants and yield.

#### **Coefficient of velocity, vigor index, number of cobs and grain yield per pot**

Salinity causes an increase in the coefficient of velocity which ranges from 5 germinated seeds per day at 0 dS/m





**Figure 6.** Effect of different salinity levels on (a) coefficient of velocity, (b) vigor index, (c) number of cobs and (d) grain yield *Zea mays*.

to reach 10 germinated seeds per day at 11dS/m. Coefficient of velocity was higher in  $\text{CaCl}_2$  seed priming (10%) compared to those with NaCl seed priming (8%) and control seeds (5%) (Table 2 and Figure 6a). In fact,  $\text{CaCl}_2$  seed priming had significantly ( $P < 0.05$ ) increased coefficient of velocity of about 2.96 in comparison with control seeds. Likewise, coefficient of velocity of NaCl primed seed also significantly ( $P < 0.05$ ) increased compared with control. Generally coefficient of velocity of Melkasa-2 indicated a faster germination when exposed to saline environmental condition than in non-saline areas. A similar results are observed from the means where the CV values are  $\text{CaCl}_2 > \text{NaCl} > \text{control}$  (Table 2). From the present findings seeds primed prior to

sowing germinated two times more rapidly than the control.

Vigor index is not greatly significant with regard to salt treatment compared with the other growth parameters. There is no significant difference between the control and the highest salt treatment (Figure 6b). Seeds treated with lower salinity have lower chance for growth and survival. Increasing salinity causes a significant increase ( $P < 0.05$ ) in maize vigor index for primed seeds. This value increases from 67.82 at 7dS/m to gain 78.20 at 11 dS/m (Figure 6b). Besides, NaCl and  $\text{CaCl}_2$  seed priming increases significantly ( $P < 0.05$ ) vigor index of maize (77.01 and 80.06, respectively) with  $\text{CaCl}_2$  having better role compared with seedlings from control seeds (76.91)

(Figure 6b). Numbers of cobs per plant were different in salt treatments 5 dS/m and 9 dS/m as compared to unprimed seeds with 4 and 5 cobs plant values, respectively (Figure 6c).

As salt concentration increases from 5 to 11dS/m, grain yield per pot showed a significant increment as compared with the control. Grain yield of the maize crop were found higher in primed seeds which might be as a result of good stand establishment, speedy and uniform germination of primed seeds (Figure 6d). The salinity treatments 9 dS/m followed by 7 dS/m is better for study crop in terms of cobs per plant and grain yield. This is an indication that this released variety of maize is moderate to salt tolerant crop which can improve growth and physiological variables that can be affected by salt stress. This may be that, crop which can overproduce different types of responsive biological solutes have low molecular weight, highly soluble and usually nontoxic at a high cellular concentrations, contributing to cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins.

#### **Soil type based salt treatment responses of maize crop assayed parameters**

All growth parameters except the early emerging roots, coefficient of velocity and salt type showed a significant effect with respect to the soil types treated with different salinity levels and salts types ( $P < 0.05$ ). Number of days to complete germination, total germinated seeds from the sown one, root length, shoot length, mean germination time, germination index, germination percentage, vigor index, coefficient of velocity, number of cobs per plant and grain yield per pot respond differently in the two types of soils (Figure 7b). Seeds primed with NaCl have wider leaf area and longer shoot compared to the seeds primed with  $\text{CaCl}_2$  (Figure 7a to d).

Moreover, the kernel that contains the seed and husk of the maize is larger in plants primed with NaCl. Salt primed maize seedlings grow in black soil types are more effective than the maize seeds grown on red soils from both salt treatments (Figure 7a and b). The reason may be due to the water holding capacity and moisture content of black soils that help to adjust the osmotic potential of the crop. The results of this study also indicted salt priming of maize seeds grown on red soils, affect negatively at seedling stage though, the lower treatments are indicators of medium salt tolerance of the crop on red soils (Figure 7c and d).

#### **Germination percentage, root length, shoots length, and vigor index**

A decrease in germination percentage of the black soil

and increase in germination percentage of the red soil is observed initially. While on seedlings from vertisol soils, there is a higher germination percentage as compared with lithosol soils (Figure 8a). The results also suggested a reduction by 20% in germination percentage in lithosol soil sown maize seeds germination percentage compared with maize seeds sown in vertisol soil type (Figure 8a). Germination percentage increased in both soil types with increase in salt concentration. The increment was higher at 9 and 11 dS/m salt concentrations for *Z. mays* seeds in black soil and at 7 dS/m for *Z. mays* seeds in red soil which show that, all salinity levels showed a significant ( $P < 0.05$ ) increase in germination percentage of primed *Z. mays* seeds compared with the control (Table not presented here).

Compared with lithosol soil, vertisol soils showed better performance in root length when seeds are sown by priming with different salt solutions. During the early seedling, growth stage seeds on red soils perform well in root length, but after adapting the salt stress condition seedlings from black soil grow well (Figure 8b). Unlike root length, shoot length has a different response at the early seedling growth stage. Lithosol soils have a longer shoot length as compared with the vertisol soils. There was an 86.15 cm root length difference between black soil and red soil and 87 cm difference in shoot length between red soil and black soil, respectively. As salinity level increases shoot length in black soil also increases. The increase in salt stress in the growth medium causes a final increase in root length of black soil and shoots length of red soil.

Contrarily, vigor index of *Z. mays* seeds response better for red soils as compared with the one grown in black soil (Figure 8d). Increasing salinity level causes significant ( $P < 0.05$ ) increases in *Z. mays* vigor index in both the primed and unprimed seeds with both NaCl and  $\text{CaCl}_2$  treated *Z. mays* seeds. The survival and growth power of germinated seeds was highly pronounced in black soil at the higher salinity levels (Figure 7). However, primed maize seeds sown in red soil were highly thriving in medium salinity levels particularly of 7 dS/m NaCl and  $\text{CaCl}_2$  treatments.

#### **Coefficient of velocity, number of cobs per plant and grain yield per pot**

Results from figure 8 revealed that primed seeds grown in black soil have higher coefficient of velocity (Figure 9a), which have more number of cobs per plant and more grain yield than primed *Zea mays* seeds grown in red soil. Compared with the control, NaCl and  $\text{CaCl}_2$  primed seeds showed higher coefficient of velocity, more cobs and better yield than the unprimed seeds. Primed seeds of maize from red soil have two more less number of cobs per plant than primed seeds from black soil (Figure 9b). Similarly, *Z. mays* seeds treated with NaCl and



**Figure 7.** Differences in growth traits of *Zea mays* seedling treated with similar salts (CaCl<sub>2</sub> and NaCl) and effect of the salt priming on cob number and size of red and black soil types. (a) Maize seedlings grown on black soil primed with CaCl<sub>2</sub> (b) maize seedlings grown black soil primed with NaCl (c) maize seedlings grown on red soil primed with CaCl<sub>2</sub> (d) maize seedlings grown on red soil primed with NaCl.

CaCl<sub>2</sub> grown in black soil have 52.2 g more grain yield per pot than the similarly treated seeds from red soil (Figure 9c).

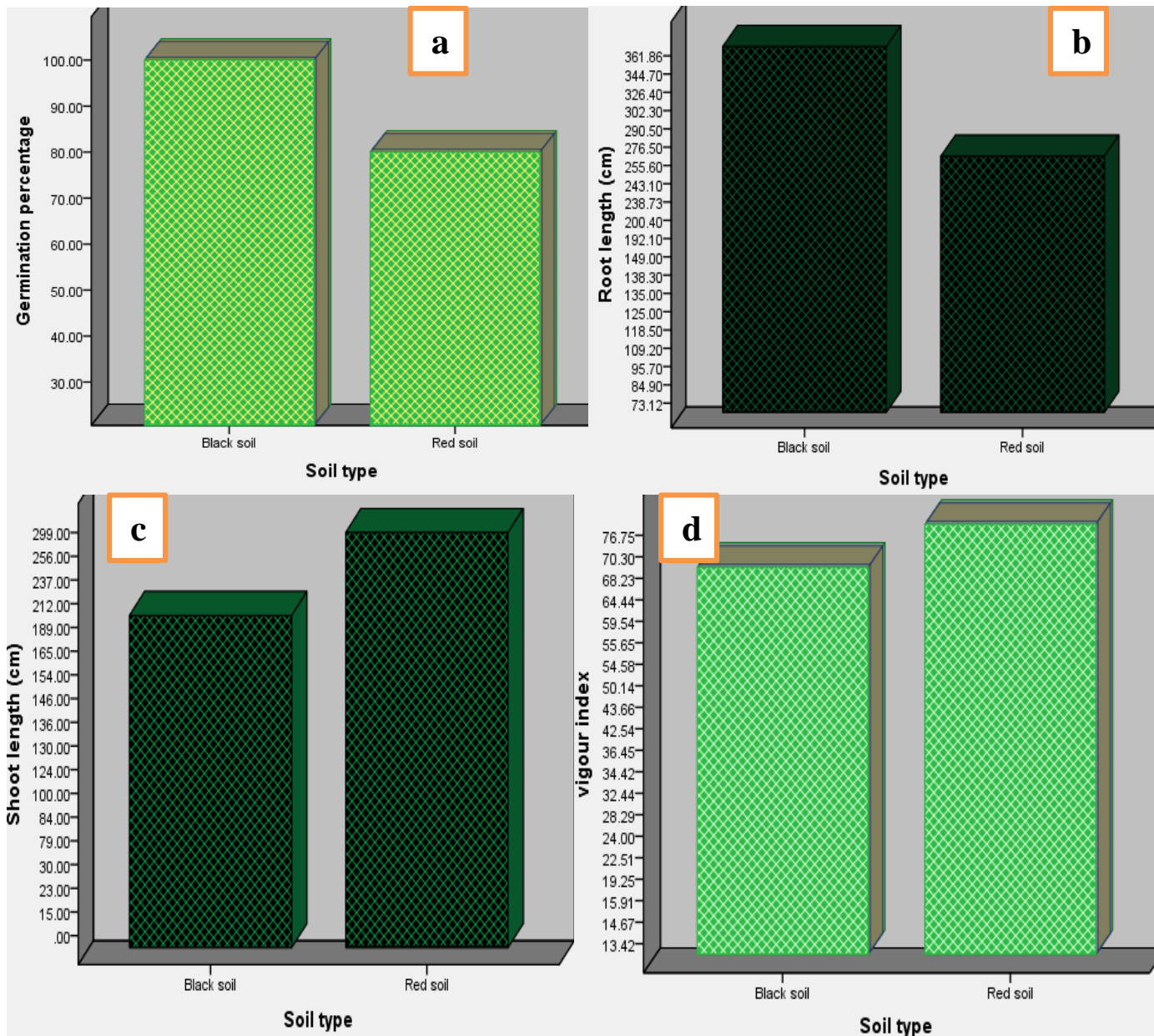
## DISCUSSION

Salt priming significantly affect maize physiology starting from germination up to yield. Germinations from primed seeds were better than un-primed seeds when exposed to different salinity levels and soil types. Similar findings were reported by Kaya et al. (2006), where seed

treatment of sunflower overcomes salt and drought stress during germination. Priming with NaCl and CaCl<sub>2</sub> increases the germination parameters (total germinated seeds in percentage, mean germination time, germination index and coefficient velocity of germination) and growth parameters (grain yield, root to shoot ratio and vigor index) of maize (*Z. mays*), as compared to unprimed seeds (control). The study revealed that seed priming treatments not only improved the stand establishment but also provided physiological improvements.

A significant decrease in emergence time and increase in final emergence count of the plant may be because of



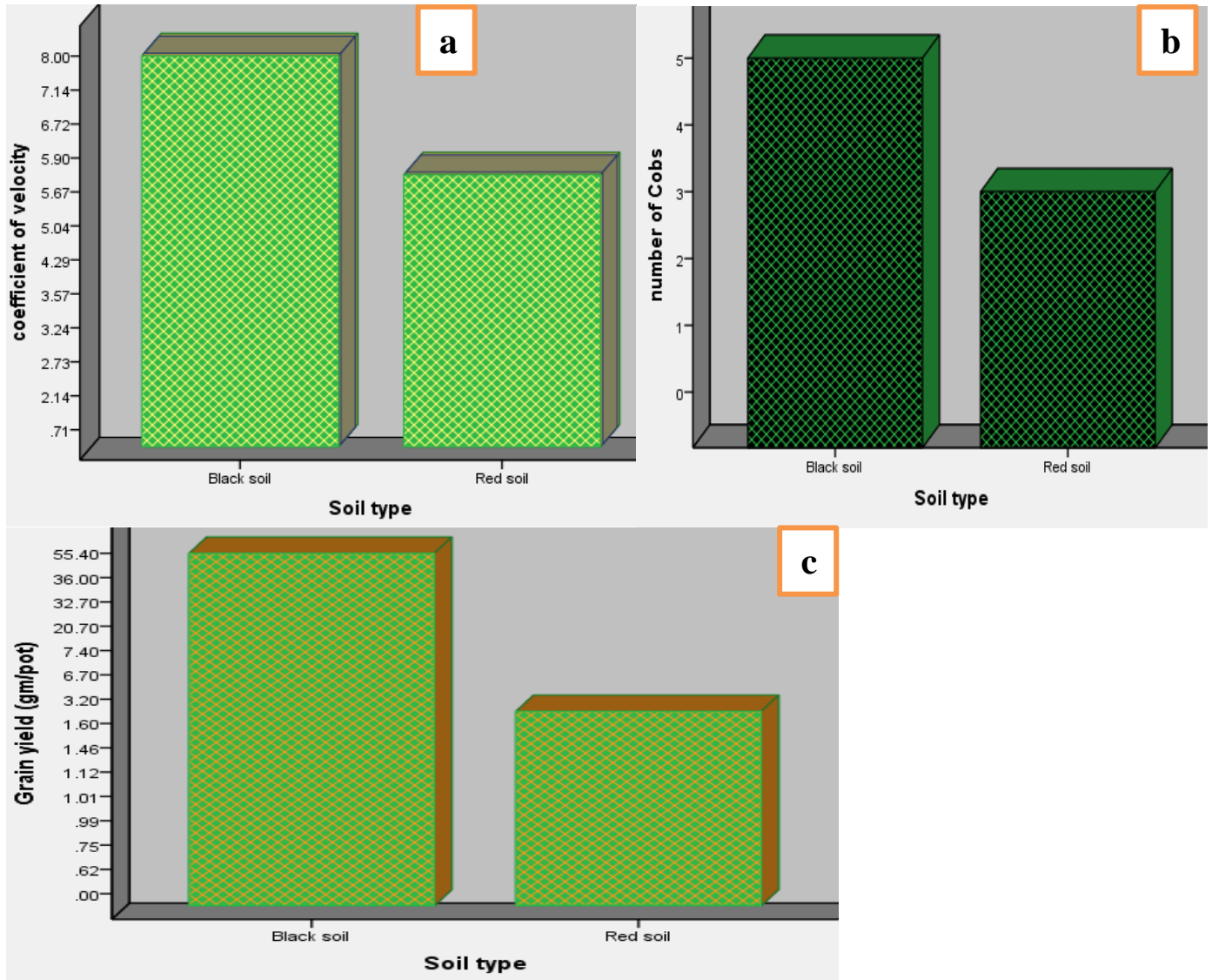


**Figure 8.** Effect of NaCl and CaCl<sub>2</sub> priming on (a) germination percentage, (b) root length, (c) shoot length, and (d) vigour index of *Zea mays* seeds grown on black and red soils.

the fact that seed priming induces a range of biochemical changes such as hydrolysis, activation of enzymes and dormancy breaking (Aziza et al., 2004; Farooq et al., 2010b), which are required to start the germination process, this edge of primed seeds over nonprime (control) resulted in improvement of maize grain yield. Higher salinity decreases maize germination and let seedling growth deteriorate; this might be due to the toxic effects of Na<sup>+</sup>, Ca<sup>2+</sup> and Cl<sup>-</sup> in the process of germination which terminates and starts of seedling growth even with soil types. Also, salinity stress affects seed germination through the restriction of seed water absorption

(Tsegazeabe and Berhane, 2012), imbalanced use of nutrient pool and creation of disorders in protein synthesis (Bordi, 2010).

At the lower salinity level, 7dS/m total germinated seeds decreased compared with the other salinity levels even though the number of days for the completion of seed germination is increased at 7dS/m salinity level like the other higher salinity levels in black soil type at control. Conversely, in red soil type the total germinated seeds are almost similar apart from the highest concentration level (11dS/m) that showed lower germinated seeds of *Zea mays* (Melkasa-2). When primed seeds of maize are



**Figure 9.** Effect of NaCl and CaCl<sub>2</sub> priming on (a) coefficient of velocity, (b) number of cobs per plant and (c) grain yield per pot of *Zea mays* seeds grown on black and red soils.

sow in black soil, their root length at the first instances of radical and plumule emergence is less susceptible to salt stress. In all the treatments, the root length at the first two weeks is approximately similar 35 cm except for 7dS/m, which showed longer root length. Here unprimed seeds have longer root length than the primed once.

Priming with CaCl<sub>2</sub> and NaCl, improves the performance of maize germination, seedling growth and production. This is similar with the idea osmopriming with CaCl<sub>2</sub> and NaCl priming which improves maize performance on salt treated soils (Ashraf et al., 2003; Nawaz et al., 2013). Cellular membrane integrity can be maintained by Ca<sup>2+</sup> which reduces Na<sup>+</sup> and favours the K<sup>+</sup> absorption (Ashraf et al., 2003). The effectiveness of osmopriming with CaCl<sub>2</sub> has already been reported for

yield improvement in several crops including wheat (Farooq et al., 2008), maize (Ashraf and Rauf, 2001) and rice (Farooq et al., 2006). Primed seeds of maize might had better competency for water absorption from the growing media that enable metabolic activities in seeds during the start of germination process, much earlier than when radicle and plumule appearances (Elouaer and Hannachi, 2012). The earlier and superior germination of a primed maize seeds is correlated with breakdown of dormancy due to priming (Butler et al., 2009). Farooq et al. (2005) found faster emergence of germination, emergence of radicle and plumule in primed seeds. Similarly Afzal et al. (2009) reported that increased solubilization of seed storage proteins like the beta subunit of the globulin and reduction in lipid per-oxidation

and enhanced anti-oxidative activity in primed seeds facilitated germination. This faster germination is due to the synthesis of DNA, RNA and protein during priming. Osmopriming with  $\text{CaCl}_2$  gives higher benefit-to-cost ratio in the soils. The easy availability and relatively non-toxic nature of  $\text{CaCl}_2$  may lead to wider on-farm adaptation of this osmoticum and has been suggested to farmers for usage.

Although, salt priming decrease mean germination time for primed maize seeds on both soil types, the primed seeds have significantly lower mean germination time compared to unprimed seeds. Similar results were reported by Ashraf and Rauf (2001) working with other priming treatments, such as polyethylene glycol (PEG), inorganic salts or even ABA. According to Kaya et al. (2006), seed priming leads to the initiation of primary metabolic processes and the time required for germination decreases. This positive effect is probably due to the stimulatory effect of priming on later stages of the germination process through the mediation of cell division in germinated seeds and rapid synthesis of DNA, RNA and protein during priming (Sivritepe et al., 2003). Farooq et al. (2005) reported the swelling of the embryo inside primed tomato seed speeding up germination by facilitating water absorption. This is due to the uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  ions by the seed, maintaining a water potential gradient which allow water uptake during seed germination (Kaya et al., 2006). Seed priming, upgrades early stand establishment and vigorous seedling growth, earlier flowering, and increased seed yield and harvest index in maize. Priming improved seedling vigor which can be attributed to stimulated starch metabolism triggering earlier emergence and vigorous seedling growth (Farooq et al., 2010b; Berhane and Berhanu, 2016).

Improved performance of  $\text{CaCl}_2$  is related to involvement of  $\text{Ca}^{2+}$  in membrane repairs and activation of enzymes, when starch reserve mobilization and radicle protrusion are in progress (Farooq et al., 2010a). Earlier and vigorous stand might reduce crop weed competition and increased absorption of water and nutrients by vigorous root system producing increased number of branches, tillers/plant, cobs/plant and yields/pot (Berhane and Berhanu, 2016). Increased emergence, tillers number and number of branches by seed osmopriming with  $\text{CaCl}_2$  reported in rice and wheat are consistent (Farooq et al., 2008; Singh et al., 2015) with present study. Increased seed yield was also associated with an increase in yield contributing traits. Increase in biological yield was due to increased plant height from healthy and vigorous seedlings which might be due to, improved crop growth rate and net assimilation rate (Farooq et al., 2006; Singh et al., 2015). Similarly, increase in harvest index from primed seeds might be due to an increase in dry matter, and partitioning towards the growing pods thereby increasing grain yield. Increased growth and yield in sunflower by seed treatment with  $\text{CaCl}_2$  reported by Kaya

et al. (2006), confirms present study findings.

## Conclusions

Seed priming prior to sowing, stimulates the physiological activities of maize seeds which resulted in rapid and uniform seed germination, as a result of the permeability of the roots changed by the treatments. The priming methods improved growth and enhances yield of crops through stimulation and the seeding up of the physiological processes of the plant although the seeds of the plant showed variation in responses to different salt types/concentrations and soil types they grew.

Priming with sodium chloride improves the crop maturity and yield whereas calcium chloride priming serves as seed coat and increases the  $\text{Na}^+$  and  $\text{Ca}^{2+}$  concentration of roots thus speeds up germination process. Black soils require moderate salt priming supplements for better crop production than red soils.

## Conflict of Interests

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Use of biosurfactant surfactin produced from cassava wastewater for anaerobic treatment of effluent from a poultry slaughterhouse

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The use of a biosurfactant surfactin produced by *Bacillus subtilis* LB5a in cassava growth medium (cassava wastewater) was evaluated to treat anaerobically, the effluent from a poultry slaughterhouse. During the effluent pretreatment, effects of surfactin concentration factors were evaluated, considering the ones which were superior and below its critical micelle concentration (CMC = 28 mg L<sup>-1</sup>): 6, 13, 27, 31, 48, 73 and 56 mg L<sup>-1</sup> and temperature (25, 30, 42.5, 55 and 60°C) up to 6 h, using the rotational central composite design. During anaerobic treatment, flasks were filled with anaerobic sludge as inoculum and a pretreated effluent for 4.5 h in its different concentrations of surfactin. Reactors were connected to eudiometers under static system at 34°C. During the pretreatment phase, there was a direct ratio between temperature and surfactin concentrations according to the increase of organic matter solubilization, measured by soluble chemical oxygen demand (SCOD). These results have shown surfactin applicability produced by *B. subtilis* LB5a during the anaerobic treatment of effluent from a poultry slaughterhouse; as for all treatments, there was no inhibition of microbial consortium of the anaerobic sludge. SCOD removal was above 80%, while oil and greases removal was above 70%, plus a propitious specific methane yield.

**Key words:** *Bacillus subtilis*, agro-industrial effluents, anaerobic sludge.

## INTRODUCTION

The slaughter and poultry processes have produced high volumes of effluent with high organic load, mainly due to lipids (Dallago et al., 2012). Lipid fraction is characterized

by oils, greases, fats and fatty acids, and it is one of the most important components in wastewater from food industries (Mendes et al., 2005; Chipasa and Medrzycka,

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**Table 1.** Average values for the characterization parameters of raw effluent from poultry slaughterhouse.

Parameters	Average values
Total alkalinity (TA) (mg L <sup>-1</sup> )	185.25
Volatile acidity (mg L <sup>-1</sup> )	243.63
Chemical oxygen demand (COD) (mg L <sup>-1</sup> )	2360.49
Soluble chemical oxygen demand (SCOD) (mg L <sup>-1</sup> )	590.30
Oils and greases (OG) (mg L <sup>-1</sup> )	535.33
pH	6.17
Total dissolved solids (TDS) (mg L <sup>-1</sup> )	515.00
Fixed dissolved solids (FDS) (mg L <sup>-1</sup> )	217.50
Volatile dissolved solids (SDV) (mg L <sup>-1</sup> )	297.50
Total solids (TS) (mg L <sup>-1</sup> )	1594.66
Fixed total solids (FTS) (mg L <sup>-1</sup> )	208.67
Volatile total solids (VTS) (mg L <sup>-1</sup> )	1386.00

2006), since it may cause operational problems to the effluent treatment system. Limiting the transfer of gas is an example, since it is necessary for biological degradation, fouling in reactors, biomass flotation and the absence of methanogenesis and acetogenesis phases during anaerobic treatment processes (Cammarota and Freire, 2006; Cirne et al., 2007; Gomes et al., 2011).

The physico-chemical pretreatment phase is generally used to remove lipid fraction and ensure efficiency of the subsequent anaerobic biological treatment (Cammarota and Freire, 2006; Cirne et al., 2007). However, the chemical reagents applied are expensive and the produced sludge is difficult to dispose (Semerjian and Ayoub, 2003). Microbial enzymes have been applied and evaluated to hydrolyze and dissolve fats from wastewater during the pretreatment of fish industry effluent (Valente et al., 2010), dairy effluent (Rosa et al., 2009; Mendes et al., 2006; Leal et al., 2002; Cammarota et al., 2001), effluent from a poultry slaughterhouse (Valladão et al., 2007), and effluent from a swine slaughterhouse (Masse et al., 2003).

Gallert and Winter (2005) described that maximum hydrolytic activity, provided by enzymes present in medium, can be achieved when lipid fraction of effluent is emulsified, that is, when there is dispersion of lipids in water like microscopic droplets (Desai and Banat, 1997). Emulsion can be obtained by the action of chemical surfactants or microbial origin (biosurfactants).

A biosurfactant is known as an additive when it stimulates biodegradation (Cammarota and Freire, 2006) as well as remove environmental oily substances due to their high molecular weight and micelle formation that are able to reduce the surface and interfacial tension. So, there is an increase in solubility and bioavailability of hydrophobic organic compounds (Pacwa-Plociniczak et al., 2011).

The use of surfactants produced by microorganisms is also evaluated by environmental sanitation area as well

as for the treatment of contaminated soil and water by fats, oils and derivatives (Nitschke and Pastore, 2002; Nakhla et al., 2003; Kumar et al., 2008).

In order to treat specifically effluents, Damasceno et al. (2012) evaluated combined application of an enzymatic preparation obtained from *Penicillium simplicissimum* and a kind of rhamnolipid biosurfactant, produced by *Pseudomonas aeruginosa* to treat wastewater from a poultry slaughterhouse. Daverey and Pakshirajan (2011) also evaluated the use of a kind of sophorolipid biosurfactant produced by *Candida bombicola* in the treatment of effluent from dairy industry.

Therefore, based on brief discussions, this study aimed at evaluating the application of surfactin as a biosurfactant produced by *B. subtilis* LB5a in a growth medium of cassava effluent (cassava wastewater), during the anaerobic treatment of effluent from a poultry slaughterhouse.

## MATERIALS AND METHODS

### Collection and characterization of effluent from a poultry slaughterhouse and anaerobic sludge

The raw effluent used in this study was collected during slaughtering period in a poultry agro-industry in South of Brazil. This effluent is produced with a 180 m<sup>3</sup>.h<sup>-1</sup> average flow. After collection, it was correctly preserved and aliquots were taken for their initial characterization (Table 1), while the remaining part was stored and frozen.

The sludge, used as inoculum during anaerobic treatment phase, was collected from an anaerobic digester of an agro-industry that produces cassava starch. It showed the average values for the following parameters: series of suspended solids - total (40.301 mg L<sup>-1</sup>), fixed (20.448 mg L<sup>-1</sup>), volatile (19.852 mg L<sup>-1</sup>), and specific methanogenic activity (SMA) (0.12 gCOD<sub>CH<sub>4</sub></sub> gVSS<sup>-1</sup> d<sup>-1</sup>).

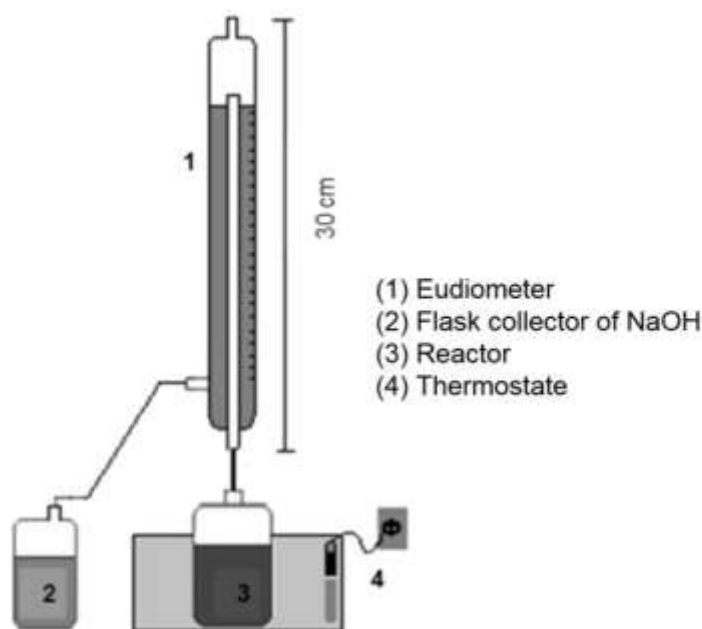
### Biosurfactant production

The microorganism that produces the biosurfactant, surfactin in

**Table 2.** Coded values and real factor: Biosurfactant concentration (BC) and temperature (T).

Variable	Levels				
	-1.41	-1	0	1	1.41
BC (mg L <sup>-1</sup> )	6	13.27	31	48.73	56
T (°C)	25	30	42.5	55	60

BC = biosurfactant concentration, T = temperature.



**Figure 1.** Schematic model of an experimental unit used in anaerobic treatment of wastewater.

cassava wastewater is the bacterium, *B. subtilis* LB5a, which was stored in agar nutrient at 4°C, at Bioaromas Laboratory of FEA/UNICAMP (Nitschke et al., 2004). Cropping of this microorganism for biosurfactant production in cassava was carried out in a pilot bioreactor, New Brunswick Scientific®, model Mobile Pilot Plant fermentor 8000 MP 80 with 56 L of cassava wastewater as growth medium and 4 L of inoculum. The experimental conditions of such bioreactor, procedures for obtaining semi-purification of biosurfactant, are described by Nitschke and Pastore (2003), Nitschke et al. (2004) and Barros et al. (2008).

After three growing days, biosurfactant was obtained and characterized as: emulsification index (in gas: 61%; diesel: 74%); concentration in crude extract (0.3 mg mL<sup>-1</sup>); surface tension (25.97 MN m<sup>-1</sup>); critical Micelle concentration (CMC = 28.33 mg L<sup>-1</sup>) and chemical oxygen demand (COD = 1.26 gCOD/g biosurfactant).

#### Pretreatment of the effluent with biosurfactant

During the pretreatment, surfactin emulsified lipid fraction was evaluated, and thus solubilization increase of organic matter in the effluent was promoted. The effects of temperature and concentration factors concerning biosurfactant on soluble COD increase (SCOD) were evaluated by using rotational central composite design (RCCD) (Rodrigues and lemma, 2009). Value

ranges used for the factors were determined according to the study of Damasceno et al. (2012). The conditions in which the pretreatment tests were carried out are shown in Table 2.

The eleven pretreatment assays were carried out in 250-ml glass beakers containing 140 ml of medium consisting of raw effluent and 10 ml of a medium consisting of surfactin in its different concentrations, totaling 150-ml net volume. In a shaker incubator, a 150-rpm stirring was determined for up to 6 h, and aliquots were withdrawn for analysis at fixed intervals of 1.5 h (Damasceno, 2013). The results were analyzed by the Statistica 8 software and for statistical analysis of variance (ANOVA), the maximum value of soluble COD (SCOD) was used as a variable response.

#### Anaerobic treatment of a pretreated effluent

Based on the results obtained in the pretreatment tests, anaerobic biodegradability of the effluent previously exposed to surfactin was evaluated. So, an experimental apparatus (Figure 1), which consists of: 1) a 62-L plastic box, filled with water and used as a water bath container; (2) Two 100-W thermostats were used to heat and keep temperature at 34 °C water bath; (3) Eighteen glass bottles (reagent grade) of 610 ml volume, with a 450-ml net volume were used as reactors; (4) Eighteen glass eudiometers were filled up with NaOH (20%) and a 50-mm diameter, 300-mm length and

**Table 3.** Coded matrix and soluble COD values during the evaluated periods in response to temperature and biosurfactant concentration.

Assay	Coded		Real		Soluble COD (mg L <sup>-1</sup> )					Maximum Value
	BS	T	BS	T	Incubation period					
					0 h	1.5 h	3 h	4.5 h	6 h	
1	1	1	48.73	55	650.20	1048.55	1282.07	1428.59	1275.20	1428.59
2	1	-1	48.73	30	634.17	670.80	771.54	1135.55	1176.76	1176.76
3	-1	1	13.27	55	583.81	936.37	1144.71	1163.02	1197.36	1197.36
4	-1	-1	13.27	30	570.07	677.67	1087.47	723.46	1014.21	1087.47
5	0	1.41	31	60	553.38	944.48	1108.06	1190.79	1292.33	1292.33
6	0	-1.41	31	25	523.29	820.38	856.10	946.36	950.12	950.12
7	1.41	0	56	42.5	500.73	797.81	873.03	997.12	952.00	997.12
8	-1.41	0	6	42.5	410.48	636.11	747.05	771.49	728.24	771.49
9	0	0	31	42.5	886.01	986.74	1121.81	1005.05	780.69	1121.81
10	0	0	31	42.5	636.46	989.03	1087.47	1050.84	1039.40	1087.47
11	0	0	31	42.5	654.78	998.19	1087.47	1053.13	936.37	1087.47

BS = Biosurfactant surfactin; T = temperature.

output with 8-mm tubes, whose total volumetric capacity was 450 ml were used. They were used to measure, through NaOH solution displacement, the volume of methane produced in effluent biodegradability.

The reactors flasks were sealed with a rubber cork and connected to eudiometers by silicone hoses. This experimental apparatus was operated in accordance with Suarez et al. (2012). The inoculum used in this phase was the anaerobic digester sludge from the starch manufacturer. The volumes of effluent and sludge were calculated in order to obtain an initial ratio of COD : VSS- 1: 1 in the reactor flasks.

Three replications were developed for each of the six effluent compositions containing different concentrations of surfactin (0, 6, 13.27, 31, 48.74 and 56 mg L<sup>-1</sup>), which consisted of a 415-mL volume of each unit regarding the pretreated effluent during 4.5 h. Also, a 35-mL anaerobic sludge was used as inoculum, and the total was 450 ml of net volume in each reactor. The pH of the pretreated effluent (pH = 6.17) was not adjusted prior to mixing with sludge, and each mixture was submitted to purging of oxygen by bubbling N<sub>2</sub> for five minutes.

Daily, there was a reading to record the produced gas volume by 20-cm rules, fixed in glassware previously calibrated as Aquino et al. (2007) methodology states. At the same time, 500-ml plastic bottles with 450-ml volume were used under the same conditions of the treatments observed in eudiometers. They were incubated at 34°C in BOD chamber aiming at evaluating the time profile of the anaerobic treatment for each composition. Weekly, two bottles of each treatment were analyzed to record the effluent biodegradability by removing SCOD, oils and greases (OG), solid series, pH, alkalinity and acidity/alkalinity (VA/TA).

Aliquots were taken to obtain the initial characterization from each composition just before contact with sludge, and the sludge itself. The results obtained during anaerobic treatment phase were evaluated by descriptive statistics and Tukey test with 95% significance with the Statistica 8 software.

#### Analytical methods

Parameters such as total alkalinity (TA), volatile acidity (AV), volatile acidity/total alkalinity (VA/TA) ratio, chemical oxygen demand (COD), soluble chemical oxygen demand (SCOD), oils and

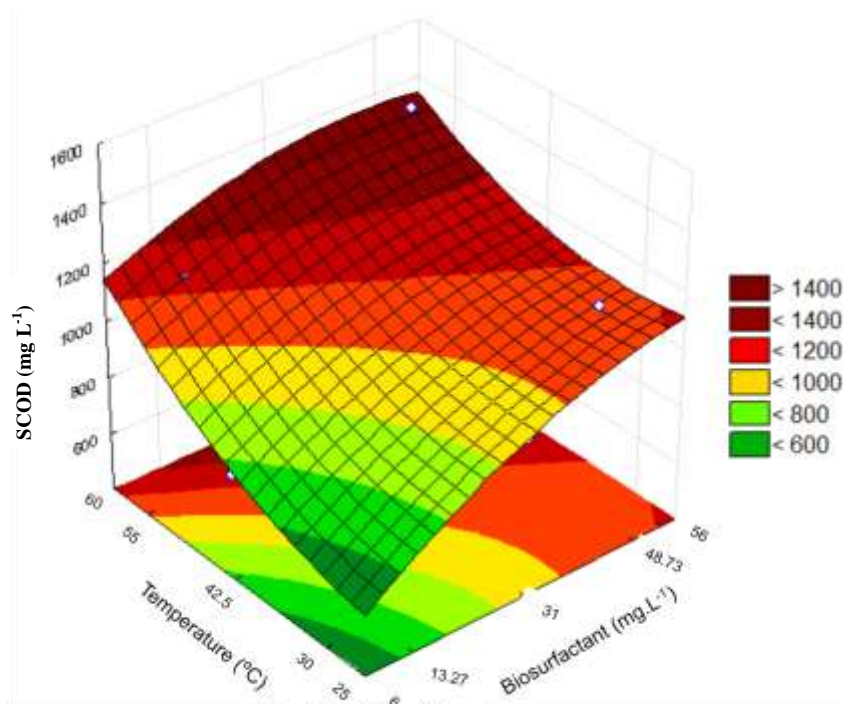
greases (OG), pH, total solid series (TS, FTS and VTS) and dissolved solid series (TDS, FDS and VDS) were obtained from the methods described in APHA (2005). The specific methanogenic activity (SMA) of anaerobic sludge and the specific production of methane (SPM) were determined according to the adapted methodologies of Chernicharo (1997), Rocha (2001) and Aquino et al. (2007). The emulsification index (EI%) of biosurfactant was determined according to Cooper and Goldenberg (1987). Surfactin concentration in crude extract was determined by HPLC according to Slivinski (2012). The surface tension of surfactin was obtained by Krüss (1994) cited by Slivinski (2012) and the critical Micelle concentration (CMC) followed the technique described by Sheppard and Mulligan (1987).

## RESULTS AND DISCUSSION

### Pretreatment of effluent with surfactin

Table 3 shows the values of increased SCOD based on the evaluated periods for each condition applied to the assays of effluent pretreated with surfactin as well as coded and real matrix of planning. It was observed that the initial COD (time 0 h) differed among treatments. This was due to the composition of each treatment, which there was application of different concentrations regarding surfactin.

Considering the eleven treatments applied in RCCD, three assays showed the maximum value of COD solubilization at 4.5-h incubation. Eight assays showed the maximum value at other incubation times, but the concentrations were very close to the ones obtained at 4.5 h. From the foregoing, it is possible some solubilizing increase up to 4.5 h incubation, except for the fourth assay, which showed reduction of solubilization at this time (RCCD). Laboratory analysis was repeated for this sample, but the result remained at the same trend (decrease of soluble COD in 4.5 h).



**Figure 2.** Response surface to increase soluble COD according to temperature and biosurfactant concentration.

Authors such as Valladão et al. (2007) and Leal et al. (2002) pointed out as the best hydrolysis time (action of enzymes on effluent), a total of 4 h to solubilize COD. For biosurfactants, Damasceno (2013) was a pioneer in evaluating the associated action of enzymatic pool and biosurfactant of rhamnolipid on COD solubilization that can be seen in wastewater from a poultry slaughterhouse. The author observed that 4.5 h was enough to promote such response.

Thereby, this trial applied 4.5 h as maximum time for the wastewater pretreatment, since the treatments in which there was an increased solubility of COD varied from 4.5 to 6 h, and such increase was little significant. It was also considered that, in some treatments, SCOD decreased during this time interval. After statistical analysis regarding the responses obtained in 4.5 h period, it was possible to determine variables that showed some effects on the increase of soluble COD.

The parameters considered significant were the ones with p-values lower than 10% ( $p < 0.1$ ), due to the large variability inherent to the processes. It was observed that variables such as biosurfactant and temperature, both in linear or quadratic ways, present a significant effect on the response variable. The effect of interaction between biosurfactant and temperature was excluded from the model because it was not significant. This answer differed from Damasceno (2013), who observed that when temperature was analyzed as a variable, there was a significant positive effect on the response variable, both

in linear and quadratic forms, but there was also a significant interaction between biosurfactant and temperature.

Silva et al. (2013) evaluated the use of an enzyme produced by fermentation in solid state using babassu oil residue as a culture medium by *P. simplicissimum* and by a rhamnolipid biosurfactant, produced by *P. aeruginosa* in pretreatment and anaerobic treatment of poultry effluent from a poultry slaughterhouse. The authors selected 8 h as the time of pretreatment with the enzyme and biosurfactant. They also observed that enzyme and biosurfactant concentrations as well as the interaction between biosurfactant and temperature have shown the most significant effect on hydrolysis of fat effluent.

The analysis of variance (ANOVA) was calculated based on SCOD average of time as pretreatment (4.5 h) that showed significant values for regression, that is, the biosurfactant concentration or temperature have affected organic matter solubilization, expressed as soluble COD. Thus, the following model equation was obtained:

$$\text{SCOD} = 1035.801 + 124.847 \cdot \text{BS} - 41.854 \cdot \text{BS}^2 + 135.059 \cdot \text{T} + 50.831 \cdot \text{T}^2$$

Where: BS = biosurfactant and T = temperature.

Based on the generated model to an increase of soluble COD, a response surface was built (Figure 2), according to temperature and biosurfactant concentration.

**Table 4.** Results from the anaerobic biodegradability assays of the pretreated effluent with surfactin.

Treatment	Initial VA/TA ratio	Final VA/TA ratio	Initial SCOD (mg L <sup>-1</sup> )	Final SCOD (mg L <sup>-1</sup> )	SCOD removal (%)	Initial OG (mg L <sup>-1</sup> )	Final OG (mg L <sup>-1</sup> )	OG removal (%)	Methane Volume (mL)	SPM (gCODCH <sub>4</sub> / gVSS d <sup>-1</sup> )
1 = Control	1.76	0.10	505.63	85.89 <sup>a</sup>	83.01	519.93	62.54	87.97	3500	0.78
2 = 6 mg L <sup>-1</sup> BS	1.06	0.07	529.69	104.07 <sup>bc</sup>	80.35	519.93	75.27	85.52	4822	0.98
3 = 13.27 mg L <sup>-1</sup> BS	0.86	0.07	492.92	85.04 <sup>a</sup>	82.74	519.93	97.26	81.29	4665	0.83
4 = 31 mg L <sup>-1</sup> BS	1.26	0.09	508.24	92.65 <sup>ab</sup>	81.77	519.93	0	100	5130	0.91
5 = 48.73 mg L <sup>-1</sup> BS	1.21	0.08	629.3	97.30 <sup>ab</sup>	84.53	519.93	141.62	72.76	5020	0.87
6 = 56 mg L <sup>-1</sup> BS	1.28	0.10	763	113.37 <sup>c</sup>	85.14	519.93	144.07	72.29	4115	0.87

\*The same letters represent equal averages. VA/TA = Volatile acidity ratio/total alkalinity; SCOD = soluble chemical oxygen demand; OG = oils and greases; BS = biosurfactant surfactin; SPM = specific production of methane.

Temperature and biosurfactant concentration conditions can be observed by analyzing the response surface generated by the model, which resulted in higher COD solubilization. The highest values of soluble COD concentration are recorded at temperatures above 60°C and at higher biosurfactant concentrations (56 mg L<sup>-1</sup>). This implies that despite the applied factor, there will be an increase in COD solubilization. Therefore, biosurfactant applicability can be extremely advantageous for effluent treatment, since a heating system for treating this kind of effluent demands financial resources for energy as well as a structure designed to implement and keep it during the treatment.

#### Anaerobic biodegradation of a pretreated effluent

During the anaerobic biodegradation assays, it was observed that pH samples have not changed much and their initial range stayed from 6.0 to 7.0 (initial and final) for all the treatments. The maintenance of a relatively neutral pH range

shows that the anaerobic process has been developed (Keefer and Urtes, 1962). This was appropriate for the survival of methane producing bacteria (Sosa et al., 2004; Chernicharo, 2007). The results obtained during the anaerobic biodegradability assays of the pretreated effluent with surfactin are shown in Table 4. The VA/TA ratio indicates stability of a reactor and it was observed that after the first week of digestion, VA/TA ratio was already in the reactor stability range, which according to Barana and Cereda (2000) varies from 0.1 to 0.3. Values of about 0.4 have shown some instability and when they were superior to 0.8, there could be a collapse of digestion.

The differences in the initial concentrations of SCOD among treatments may be due to the addition of surfactin or the presence of pieces of floating organic material in the effluent, which were broken during the pretreatment. The analysis of variance (ANOVA) was obtained based on the final results (Week 5) of SCOD and the test of multiple comparison of Tukey averages. Thus, it was observed that, statistically, treatments 1, 3, 4 and 5 presented the same

average of soluble COD removal, consequently, they differed from averages of 2 and 6 treatments. Regarding the tested level of significance, averages of 2, 4 and 5 treatments do not differ, as well as averages from 2 and 6 treatments.

Nakhla et al. (2003) studied the effect of biosurfactant addition from cactus for anaerobic wastewater treatment with a high content of oils and greases (OG = 38,800 mg L<sup>-1</sup>) from an animal diet industry. The authors concluded that after 16 days of anaerobic digestion, the addition of biosurfactant in raw effluent removed SCOD from 11,200 to 7,050 mg L<sup>-1</sup>.

Daverey and Pakshirajan (2011) evaluated the pretreatment of an effluent from a dairy industry using a sophorolipid biosurfactant produced by *Candida bombicola*. The results showed that COD removal efficiency was 93% after 96 h of operation. The synthetic surfactant Tween 80 has been used by Kumar et al. (2008) as a substrate during anaerobic treatment process of tannery residues. The addition of surfactant in this process significantly increased hydrolytic and fermentative activities of enzymes (proteases and deaminases). These results indicated that when

this surfactant is added to the treatment process, there is an increase in using residues by microorganisms, and in turn, there is an improvement on metabolic conversions.

Damasceno et al. (2012) evaluated the combined use of rhamnolipid biosurfactant and an enzymatic preparation to treat wastewater from a poultry slaughterhouse with high fat concentration ( $2,403 \text{ mg L}^{-1}$ ) and  $8,692 \text{ COD mg L}^{-1}$ . The authors carried out the pretreatment of such effluent with this enzyme and biosurfactant, and, subsequently, the anaerobic treatment at the best condition was evaluated. It was found that enzyme concentrations above 0.5% (w/v) or biosurfactant concentrations below CMC ( $<205 \text{ mg L}^{-1}$ ) promoted inhibitory or toxic effects to anaerobic bacteria. At optimal concentration, both microbial metabolites showed simultaneous action on the availability/hydrolysis of fats, and the authors concluded that there is potential to treat wastewater from a poultry slaughterhouse and there will be no need for a flotation phase.

Jacobucci et al. (2009) observed, by COD reduction, the application of two bacterial species (*Pantoea agglomerans* and *Planococcus citreus*) that are biosurfactant producers and of a biosurfactant produced by them, in a greasy effluent that comes from a soap and margarine industry. The effluent presented  $4,400 \text{ mg L}^{-1}$  COD and 70 to 76% of removals that were obtained from COD with an application of bacterial strains and biosurfactants after 24-contact hours with the effluent.

The samples' results developed as replicas showed that there was higher conversion of soluble organic matter at the first three weeks of anaerobic biodegradation and the percentage of SCOD removal ranged from 80.35 to 85.14%. Based on these results, it can be inferred that anaerobic treatment of wastewater from a poultry slaughterhouse is a feasible option, since Chernicharo (2007) considers as efficient, the anaerobic system on wastewater treatment when the removal of organic matter exceeds 65%. It is noted that, in these samples, there is almost no difference on OG removal among the treatments, that is, either with or without biosurfactant application, there was an excellent OG removal from wastewater.

In this trial, an anaerobic sludge sample from each treatment was evaluated at the end of digestion to check some possible adsorption of fat to sludge; therefore, OG concentration in this material was not detected. This indicates that fat present in wastewater from a poultry slaughterhouse was degraded in such process. Accordingly, Gomes et al. (2011) reviewed the application of porcine pancreatin enzyme in the pretreatment of synthetic dairy wastewater with a subsequent anaerobic treatment in UASB reactor, and they recorded that lipids were completely removed from the anaerobic sludge digestion used as inoculum.

On the other hand, regarding methane production (mL), it was observed that in the early days of samples incubation, there was slight displacement of NaOH

solution from eudiometers. It lasted effectively almost until the 25<sup>th</sup> incubation day, consequently, after this term, the treatments tended to stabilize methane production. The assays ended at 36 days of incubation, when some stabilization was observed for three consecutive days of methane production. Treatment T1, which had no surfactin, showed the least and final volume of methane production. This may be an evidence that biosurfactant may have promoted some effect on microbial consortium.

Valladão et al. (2007) reported that the pretreatment of wastewater from a poultry slaughterhouse with a hydrolase pool obtained from fungi increased methane production, reduced time as well as reaction volume of reactors. When comparing the cumulative methane production with data of SCOD and OG removal for the studied period, there already had a removal superior to 80% SCOD as well as 90% OG of destructive samples, which are replicas of treatments applied to eudiometers. Therefore, there was no high concentration of organic material to be degraded or converted into biogas.

Huang et al. (2015) studied the improved volatile fatty acid production during waste activated sludge anaerobic fermentation by different bio-surfactants. They reported that volatile fatty acid production was increased to approximately 4-fold versus the blank using surfactin, rhamnolipid and saponin. Surfactin mainly increased the dissolution of organic matters to reach a high volatile fatty acid accumulation.

ANOVA analysis was carried out with maximum SPM values for each of the treatments' replicas. The p-value and calculated F showed that there is no statistical difference among the maximum SPM values from the applied treatments.

## Conclusions

With RCCD, it was observed that at higher temperatures and concentrations of surfactin (superior to their CMC), there are highest values of increased COD solubilization, but, effectively heating an effluent to carry out its pretreatment is not feasible.

The removal of soluble organic matter was statistically equal among the applied treatments as well as for SPM. The control treatment was the lowest one observed among the others concerning the cumulative volume of methane at the end of this trial, when surfactin was not added to the pretreatment.

These results demonstrated that the application of biosurfactant surfactin produced by *B. subtilis* LB5a above its CMC ( $> 28 \text{ mg L}^{-1}$ ) did not inhibit the activity of microbial consortium responsible for anaerobic biodegradation, and this process took place appropriately in all applied treatments.

## Conflict of interests

The authors have not declared any conflict of interests.



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## Full Length Research Paper

# Effect of activators and inhibitors on extracellular thermostable alkaline protease isolated from *Bacillus subtilis* obtained from eastern province of Saudi Arabia

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Alkaline proteases need some ions to achieve highest activity. These metallic ions help in, enzyme stability and activity through its thermal stability at high degrees. This protects the enzyme from being destroyed by heat. The results show that the ideal condition for enzyme activity was, using  $Mn^{2+}$  ions as the activator with 1112.5 U/ml activity, while  $MgSO_4 \cdot 7H_2O$  has lower effectiveness of an enzyme activity 247.5 U/ml. Benzene was the best solvent (867.5 U/ml), however ethylene glycol extract had lower solvents on enzyme activity (530 U/ml).  $KMnO_4$  has the best inhibitor with enzyme activity of 1325 U/ml, while ethylenediaminetetraacetate (EDTA) has the most effective inhibitor enzyme activity with 575 U/ml. Traditional methods in leather industries are based on using  $H_2S$  and other chemicals, which are considered as ways of pollution and threaten the environmental health. So, biological treatment using protease enzyme is environmentally safe and help to ease manipulation, less byproducts and wastes. Alkaline protease is used in soaking leather, incubate at  $50^\circ C$  for different periods then wash and hair remove by distaining processes. The enzyme control remove hair for 30 min, its activity decreased for a period of 180 min making the enzyme potentially suitable for biotechnological applications.

**Key words:** Alkaline protease, *Bacillus subtilis*, de-hairing, leather, enzyme stability.

## INTRODUCTION

Protease enzyme is one of the most important commercially produced enzymes. Genus *Bacillus* is considered as a rich source of alkaline protease (Jisha et al., 2013). Its product represents about 35% of the products of that field (Bhunja et al., 2012). Akcan and Uyar (2011) proved that adding  $MgSO_4 \cdot 7H_2O$  and  $FeSO_4 \cdot 7H_2O$  support protease production by *Bacillus*

*subtilis* RSKK 96. Kumar (2002) reported that  $Ca^{+2}$ ,  $Mg^{+2}$  and  $Mn^{+2}$  ions play an important role in enzyme stability, activity and thermal tolerance. Rattray et al. (1995) mention that  $CO^{+2}$ ,  $Mg^{+2}$ ,  $Ba^{+2}$ ,  $Zn^{+2}$  and  $Fe^{+3}$  ions are used for enzyme stability and to protect the enzyme against destruction after increased reaction temperature.  $Ca^{+2}$  ions are known as active enzyme production

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through thermal stability (Kotlova et al., 2007).  $\text{Hg}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Cu}^{+2}$ ,  $\text{Ag}^{+2}$ ,  $\text{Fe}^{+2}$ , on the contrary, found to inhibit pretense activity (Pena-Montes et al., 2008). Bhunia et al. (2012), proved that some ions are important for enzyme production like  $\text{Ca}^{+2}$ ,  $\text{CO}^{+2}$ ,  $\text{Cu}^{+2}$ , boron,  $\text{Fe}^{+2}$ ,  $\text{Mn}^{+2}$ ,  $\text{mg}^{+2}$  and molybdenum and the source of the enzyme detect the important ions heeded. Besides, salts as NaCl and phosphate ions are used to stimulate protease production. Growth of *Bacillus* sp. was found to be controlled by gradients of sodium ions, these ions interfere with balancing of pH and construction of ATP (Bhunia et al., 2012 and Lakshmi et al., 2014).

Rao et al. (2009) reported that proteinases are industrially important products used in detergents, leather and meat processing industries. The enzyme performs 35% of the microbial products of enzyme (Bhumia et al., 2012). The alkaline enzyme is important in leather industry; in soaking processes and hair removed from skin to get rid of undesired stains soda lime. Sodium sulfate were previously used due to their efficiency and environmentally friendship (Macedo et al., 2005). The enzyme was used at pH 9 to 10 during soaking process to facilitate water absorption from leather, which is also used with softeners and keratin for hair removal during leather processing in a short time (Mukherjee et al., 2008). Proteinases from *B. subtilis* shows an activity to hydrolyze keratin instead of sodium sulfate during hair removing (Arunachalam and Saritha, 2009), while Verma et al. (2011) used proteases from *Thermoactinomyces* sp. RM4 to remove goat hair.

The aim of this work was the study of the effect of some additions of activators and inhibitors on our isolate and its alkaline protease production and determination of alkaline protease efficiency in application during animal hair removal processes.

## MATERIALS AND METHODS

### The bacterial isolate

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

### Cultural conditions and production of enzyme

Isolate was grown for enzyme production in an ideal cultural conditions in shake flask fermentation through incubation at 37°C for 5 days in media containing fructose (10 g), potassium nitrate (5 g), NaCl (150 g), dipotassium mono hydrogen phosphate (5 g), magnesium sulfate 0.4 (g),  $\text{CaCl}_2$  (0.2 g) and Tween 80 (10 g) in 1 L (Al-Khaldi, 2014). The fermentation conditions were maintained at 50°C, 50 rpm agitation for 5 days. The enzyme was separated through centrifugation at 10,000 rpm. Substrate was 1 g azocasein in 100 ml buffer phosphate at pH 8.

### Effect of different ions on enzyme activity

The effect of different metal ions on protease activity was determined by, the addition of the corresponding ion at a final concentration of 1.0 mM to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of  $\text{CaCl}_2$  -  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  -  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  -  $\text{MnSO}_4$  - NaCl -  $\text{K}_2\text{HPO}_4$ . Reactions were incubated at 50°C in water bath for 30 min and assayed under standard condition then, enzyme protease activity was measured. The control sample was prepared for calibration and reset for spectrophotometer (Usharni and Muthuraj, 2010).

### Effect of solvents on protease activity

Different solvents were tested as methanol, 1-propanol, 2-propanol, ethylene glycol, ethyl acetate, acetone, xylene, toluene, benzene by adding 20 ml of one of each solvent to 100 ml substrate (1 g casein in 100 ml buffer phosphate at pH 8) (Najafi et al., 2005), tubes were incubated at 50°C in water bath for 30 min, and assayed under standard condition. The control sample was prepared for calibration and reset for spectrophotometer, then protease activity was applied.

### Effect of inhibitors on protease activity

One ml of the following inhibitors ethylenediaminetetraacetate (EDTA) 0.01 M -  $\text{H}_2\text{O}_2$  -  $\text{KMnO}_4$  -  $\text{KIO}_3$  -  $\text{AgNO}_3$  were added separately to 100 ml substrate using the method of Kezia et al. (2011). Reactions were incubated in water bath at 50°C for 30 min and assayed under standard condition. The control sample was prepared for calibration and reset for spectrophotometer, then enzyme activity was measured.

### Measuring the alkaline protease activity in hair removing

Cow skin was washed to get rid of impurities, left to dry at 50°C for 30 min, then cut to small piece (4x4 cm), followed by incubation with an extracted enzyme (crud protease) (2 U/ml in (50 ml) M Tris- HCL-at pH= 8) at 50°C for different incubation time (30, 60, 90, 120, 150, and 180 min). The skin pieces were washed and examined for hair removal, by hand or using forceps (Najafi et al., 2005).

## RESULTS

Results in Table 1 proved that  $\text{MnSO}_4$  was the best to activate protease activity (1112.5 U/ml), followed by  $\text{K}_2\text{HPO}_4$  with 977.5 U/ml, NaCl with 891.5 U/ml,  $\text{CaCl}_2$  with 866.5 U/ml,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  with 543 U/ml and the least of which was  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  which where the effectiveness of the enzyme reached 247.5 U/ml.

Also results in Table 2 show that benzene was the best solvent in stimulating interaction of enzyme with high percentage of enzyme activity of 867.5 U/ml, while ethylene glycol recorded low enzyme activity 530 U/ml followed by 1-propanol with 610 U/ml, acetone with 620 U/ml, 2-propanol with 625 U/ml, toluene with 630 U/ml, methanol with 640 U/ml, xylene with 725 U/ml then ethyl acetate with lower percentage of enzyme activity of 530 U/ml.

**Table 1.** Effect of various metal ions on alkaline protease activity.

Metal ions (0.4 g/100 ml)	Optical density (OD)	Protease activity ± (SE) (U/ml)
K <sub>2</sub> HPO <sub>4</sub>	1.2244	977.5±49.81
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.7619	543±27.22
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.4574	247.5±37.50
MnSO <sub>4</sub>	1.3635	1112.5±71.98
NaCl	1.1243	891.5±73.06
CaCl <sub>2</sub>	1.1221	866.5±54.13

**Table 2.** Effect of various solvents on alkaline protease activity.

Solvents (20 g/100 ml)	Optical density (OD)	Protease activity ± (SE) (U/ml)
Methanol	0.8666	640±25.00
1-Propanol	0.8344	610±72.11
2-Propanol	0.8484	625±26.46
Ethylene glycol	0.7511	530±34.73
Ethyl acetate	0.9560	727.5±82.27
Acetone	0.8440	620±33.07
Xylene	0.9531	725±44.23
Toluene	0.8563	630±41.76
Benzene	1.1032	867.5±97.82

**Table 3.** Effect of various inhibitors on alkaline protease activity.

Inhibitors (1 g/100 ml)	Optical density (OD)	Protease activity ± (SE) (U/ml)
EDTA	0.7937	575±17.50
H <sub>2</sub> O <sub>2</sub>	0.9568	727.5±30.00
KMnO <sub>4</sub>	1.5837	1325±17.50
KIO <sub>3</sub>	1.1512	912.5±23.85
AgNO <sub>3</sub>	0.8905	660±0.00

Results in Table 3 show that EDTA showed the highest inhibition which recorded high percentage of enzyme activity inhibition, with low enzyme activity of 575 U/ml followed by silver nitrate AgNO<sub>3</sub> which inhibited enzyme at 660 U/ml, H<sub>2</sub>O<sub>2</sub> with 727.5 U/ml activity, KIO<sub>3</sub> with 912.5 U/ml and KMnO<sub>4</sub> was the weakest inhibitor, having enzyme activity of 1325 U/ml.

Alkaline protease produced by *B. subtilis* was used in this study. The protease activity of the culture filtrate was used in the ideal condition (for enzyme activity). The alkaline enzyme was active in skin hair removal process during the first 30 min up to 180 min. This activity decreased but was still functional. At the end of the treatment, the hair was completely removed leaving the skin clean (both of which can be reused) (Figures 1 to 7).

**Figure 1.** A piece of cow's skin untreated with enzyme extract.**Figure 2.** The piece of cow's skin after immersion in crude alkaline enzyme extract for 30 min.**Figure 3.** The piece of cow's skin after immersion in crude alkaline enzyme extract for 60 min.

## DISCUSSION

Na, Ca<sup>+2</sup>, Mn<sup>+2</sup>, Mg<sup>+2</sup>, K, Fe<sup>+3</sup>, Cu<sup>+2</sup> and cobalt ions play



**Figure 4.** The piece of cow's skin after immersion in crude alkaline enzyme extract for 90 min.



**Figure 7.** The pieces of cow's skin immersion by crude alkaline enzyme after 180 min.



**Figure 5.** The piece of cow's skin after immersion in crude alkaline enzyme extract for 120 min.



**Figure 6.** The piece of cow's skin after immersion in crude alkaline enzyme extract for 150 min.

an important role in industrial production of alkaline enzyme which sometime catalyze the reaction between enzyme and substrate. This ions can also act as election carriers in oxidation- reduction reactions.

Results proved that magnesium ions ( $Mn^{+2}$ ) was the ideal ions which activated enzyme reaction; these findings agreed with that found by Sharaf and Al-Fadel (2013) while the result contradict those of Sharaf and Al-Fadel (2013) and Vijayaraghavan et al. (2014b) who found that calcium ions were the best. Sathyavathan and Krithika (2014) found that  $Cu^{+2}$  ions was the best for this reaction. Jadhav et al. (2013) recommended potassium ions for this reaction. Muthulakshmi et al. (2011) recommended  $Zn^{+2}$  ions as the best conditions for the reaction, while barium ions ( $Ba^{2+}$ ) were the best as mentioned by Shahbazi and Heidari (2012).

Sandhya and Tambekar (2013) found that, adding calcium ions was the best for this reaction. Ferric ions were chosen by Saraçoğlu et al.(2013), cobalt and aluminum ions were using organic solvent stimulate protease analysis of peptide bonds. New peptide bonds were formed by specificity and stability of the enzyme in the presence of organic solvents (El-Hadj-Ali et al., 2007). Benzene was the best solvent as mentioned by (Najafi et al., 2005; Olajuyigbe and Ehiosun, 2013). Acetone was recommended by Bahobil (2011), while toluene was the best mentioned by (Padmapriya et al., 2012).

Vijayaraghavan et al. (2012c) found out that butanol was the best but n-hexadecane was found by Abusham (2009) to be the best solvent. Pathak and Deshmukh (2012) found that n-hexane was the best solvent. Vijayaraghavan et al. (2014a) recommended methanol, but dimethyl sulfoxide (DMSO) was the best solvent, as mentioned by Bhunia and Dey (2012).

Inhibitors are organic compounds or ions which stop or decrease enzyme activity. Natural structure of enzyme protein facilitate precipitation, which may block receptive sites on the enzyme structure. EDTA was found to act on metallic ions forming the structure of the enzyme. This recorded the highest percentage of inhibition of an enzyme activity, as agreed with Sharaf and Al-Fadel (2013). Bahobil (2011) found that the highest percentage



of inhibition was recorded after using cadmium chloride, while Annapurna et al. (2012) proved that maruric chloride was the highest inhibitor. Mrudula and Shyam (2012), found that H<sub>2</sub>O<sub>2</sub> potentially inhibit the enzyme activity. AgNO<sub>3</sub> and N-ethyl maleimide (NEM) were recommended as inhibitors by Najafi et al. (2005). Others proved that Phenylmethylsulfonylfluoride (PMSF) was the best for enzyme reaction inhibition (Saraçoğlu et al., 2013.)

In this study, alkaline enzyme protease was effective in removing hair during the first 30 min and then declined a little in the effectiveness of an enzyme, with an increase in the period of incubation. The reason for this is to expose an enzyme to a temperature of 50°C which is an optimum temperature for different time periods. Factor time is important for the stability of this temperature, which has effect on the nature of protein synthesis (break some secondary links or weak) and thus loses its activity. This result is consistent with those of Bholay et al. (2012).

Shivasharana and Naik (2012) proved that, enzyme extracted from *Bacillus* sp. JB-99 can help in hair removing by 100% rate, and the enzyme destruct the basal cells of hair follicle and cells surrounding the hair which subsequently facilitate its removal. Also, Krishnaveni et al. (2012) indicated that, alkaline protease hydrolyze hair roots in skin layer and facilitate the removing of hair. Najafi et al. (2005) and Mukesh et al. (2012) explained that, alkaline enzyme hydrolyses collagen during incubation for 3 h and help in hair removing. Macedo et al. (2005) could completely remove skin hair by using keratinase extracted from *B. subtilis* S14 after 9 h incubation, but Bholay et al. (2012) proved that at 12 and 18 h, if incubated at 37°C skin hair can also be removed and this was also mentioned by Vijayaraghavan et al. (2014b). Verma et al. (2011) mentioned that, alkaline protease could remove the epidermal layer with hair by skin treatment after 24 h, while Prabhavathy et al. (2013) stated that the enzyme could remove skin hair after 24 h, as the enzyme destroy the collagen and facilitate hair remove without any skin damage. Sharaf and Al-Fadel (2013) proved that alkaline enzyme could eradicate sheep wool and also used in hair removing.

### Recommendation

Our study proved the activity of alkaline protease from microbial origin in hair removal during leather processing. It is recommended to prepare enzyme of mutant strains which produce alkaline protease with high quality in hair removal by increasing studies about the products of *Bacillus* spp. especially *B. subtilis*.

### Conflicts of Interests

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Optimization of biomass and glucoamylase production by *Candida famata* using response surface methodology

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**Glucoamylase is among the most important enzymes in biotechnology. The present study aims to determine better conditions for growth and glucoamylase productivity by *Candida famata* and to reduce the overall cost of the medium using central composite design (CCD) with one central point and response surface methodology. A three-level central composite design (CCD) factorial design based was employed to obtain optimal medium combination of four independent variables such as soluble starch,  $(\text{NH}_4)_2\text{HPO}_4$ , yeast extract, and  $\text{MgSO}_4$ . 25 randomized mediums were incubated in flask on a rotary shaker at 105 rpm for 72 h at 30°C. The production of biomass was found to be starch,  $(\text{NH}_4)_2\text{HPO}_4$  and yeast extract dependent; maximum production was obtained when the starch concentration was 5 g/L, yeast extract, 5 g/L and  $(\text{NH}_4)_2\text{HPO}_4$  2 g/L. Positive interaction was observed between  $(\text{NH}_4)_2\text{HPO}_4$  and both starch and yeast extract. All the variables were highly significant for glucoamylase production according to their *p* values; maximum production was found at 5 g/L of yeast extract, 7 g/L of starch and 3 g/L of  $(\text{NH}_4)_2\text{HPO}_4$ ; furthermore, yeast extract and  $(\text{NH}_4)_2\text{HPO}_4$  interacted positively. Central composite design used for the analysis of treatment combinations gave a second-order polynomial regression model with  $R^2 = 0.99$  for biomass and  $R^2 = 0.98$  for glucoamylase. The final biomass and glucoamylase activity obtained was very close to the calculated parameters; the predicted optimal parameters were confirmed and provide a basis for further studies in the valuation of starch waste products.**

**Key words:** *Candida famata*, glucoamylase, fermentation, central composite design, RSM correlation coefficient, analysis of variance (ANOVA).

## INTRODUCTION

The microbial amylases are the most used enzymes, due to their reproductivity (Burhan et al., 2003). Natural fermented media (foods, soils and wastes) are sources for isolation of microorganism strains producing

amylases. Amylases have been used as industrial enzymes since decades, these include hydrolases which cause starch hydrolysis and are of great importance in biotechnology with broad range of applications in

fermentation, food, textile, brewing, bakery and paper industries (Mushtaq et al., 2016). Amylases can be easily obtained from several sources including plants and animals but microbial enzymes are most preferred (Adrio and Demain, 2014).

Generally, two main methods (classical and statistical) are used for the process of optimization. The classical method is based on the 'one-factor-at-a-time' method, in which one independent variable is observed, whereas the other factors are kept at a fixed level. However, this method cannot guarantee the determination of optimal conditions and is unable to study the interactions between the factors, thus probably leads to unreliable results and inaccurate conclusions (Hu et al., 2016). The statistical optimization method (response surface methodology) uses the data from a few sets of experiments to determine equations; this method can overcome the limitations of the classical method, it has been proved to be a powerful tool for designing experiments, building models, evaluating the effects of factors and analyzing optimal conditions of factors for desirable responses (Wu et al., 2014).

Such statistical design had already been used in many research works such as in the improvement of biomass production and glucoamylase activity by *Candida famata* (Mosbah et al., 2015), in the optimization of  $\alpha$ -amylase production by *Aspergillus niger* (Oberoi et al., 2014), *Aspergillus oryzae* (Naili et al., 2016) and *Bacillus* sp. (Murat et al., 2015). These designs were also used for the optimization of the culture medium for the production of inulinase by *Kluyveromyces* S120 (Xiong et al., 2007) in the production of glucoamylase from food waste (Kiran et al., 2014), and in the optimization study of  $\alpha$ -amylase activity (Keharom et al., 2016).

As a continuation of a previous screening work (Lagzouli et al., 2007), the present study aims to determine better conditions for growth and glucoamylase productivity by *C. famata*, response surface methodology using central composite design with one central point to optimize media composition to reduce the overall cost of the medium to provide a basis for further studies in the baking additives.

## MATERIALS AND METHODS

### Microorganism and culture medium used

*C. famata* was isolated from traditional Moroccan sourdough (Lagzouli et al., 2007) using medium containing soluble starch (5 g/L),  $\text{KH}_2\text{PO}_4$  (3g/L),  $(\text{NH}_4)_2\text{SO}_4$  (1 g/L),  $\text{MgSO}_4$  (0.5 g/L) and yeast extract (4 g/L). Initial pH was adjusted to 5 with HCl 0.1 M. The medium was solidified by the addition of 1.5% agar, and autoclaved at 121°C for 15 min. Liquid medium was incubated in flask on a rotary shaker set at 105 rpm for 72 h at 30°C.

### Cultivation and production of glucoamylase by *C. famata*

Growth rate was determined after 72 h of incubation by measuring the absorbance of the suspension at 600 nm and then free substrate supernatant was obtained by centrifugation at 7000 rpm for 10 min and used for estimation of enzyme activity. Glucoamylase activity was determined by measuring the reducing sugar formed by the enzymatic hydrolysis of starch using the method of Nelson (1944); 0.25 ml soluble starch (1%), 0.15 ml phosphate buffer (0.1 M) and 0.1ml enzyme solution were mixed and then incubated at 40°C in water bath for 30 min. The reaction was stopped by 2 ml of Somogyi reactive, and 1.5 ml of distilled water, followed by boiling for 15 min to develop blue color. The absorbance was measured at 540 nm with a spectrophotometer against the control in which no enzyme was added. A calibration curve of absorbance and concentration of glucose was established with known amount of glucose.

One unit ( $\mu\text{mol/L/min}$ ) of amylase activity was defined as the amount of  $\mu\text{mole}$  of reducing sugar per liter of enzymes per min, measured as glucose under the conditions of assay.

### Optimization studies

A 3-level 4-factor central composite design was adopted to evaluate the effects of soluble starch ( $X_1$ ), yeast extract ( $X_2$ ),  $(\text{NH}_4)_2\text{HPO}_4$  ( $X_3$ ) and  $\text{MgSO}_4$  ( $X_4$ ) on the biomass and glucoamylase production by *C. famata*. In this study, the independent variables were studied at three different levels, namely low (-1), medium (0) and high (+1), providing 25 trials (mediums). The minimum and maximum ranges of independent variables were investigated with respect to their coded values given that the response variable was fitted by a second order model in order to correlate the response variables to the independent variables, the second order polynomial coefficients were calculated and analyzed using the adequate statistical software. The general form of the second-degree polynomial equation is:

$$Y = b_0 + \sum_{i=1}^K b_i X_i + \sum_{i=1}^K b_{ii} X_{ii}^2 + \sum_{i=1, i < j}^K \sum_{j=2}^K b_{ij} X_i X_j + \varepsilon$$

Where, Y is the predicted response;  $b_0$  is the intercept,  $b_i$  the linear coefficient,  $b_{ij}$  is the quadratic coefficient,  $b_{ii}$  is the linear-by-linear interaction between  $X_i$  and  $X_j$  regression coefficients, and  $X_i X_j$  are input variables that influence the response variable Y.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA), this analysis included Fisher's F test (overall model significance), its associated probability  $p(F)$ , correlation coefficient R, determination coefficient  $R^2$  which measure the goodness of fit of regression model. The quadratic models were represented as contour plots (2D) and response surface curves (3D) for each variable. The 25 randomized experiments with the coded and real values of the experimental variables are given in Tables 1 and 2.

The response variable was fitted by a second order model in order to correlate the response variables to the independent variables, the second order polynomial coefficients were calculated and analyzed using the adequate statistical software. The general form of the second-degree polynomial equation is:

$$Y = b_0 + \sum_{i=1}^K b_i X_i + \sum_{i=1}^K b_{ii} X_{ii}^2 + \sum_{i=1, i < j}^K \sum_{j=2}^K b_{ij} X_i X_j + \varepsilon$$

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**Table 1.** Levels of variables chosen for the central composite design optimization experiment.

	Factors	Number of levels	Levels		
X1	Starch	g/l	3	5.0	7
X2	Yeast extract	g/l	3	4.0	5
X3	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	g/l	1	2	3
X4	MgSO <sub>4</sub>	g/l	0.25	0.50	0.75

**Table 2.** Coded levels (in parentheses) and real values of experimental variables.

No. Exp.	Rand	Starch (X1)	Yeast extract (X2)	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	MgSO <sub>3</sub>
		g/L (level)	g/L (level)	g/L (level)	g/L (level)
1	16	3.0 (-1)	3.0 (-1)	1.0 (-1)	0.25 (-1)
2	22	7.0 (1)	3.0 (-1)	1.0 (-1)	0.25 (-1)
3	8	3.0 (-1)	5.0 (1)	1.0 (-1)	0.25 (-1)
4	3	7.0 (1)	5.0 (1)	1.0 (-1)	0.25 (-1)
5	12	3.0 (-1)	3.0 (-1)	3.0 (1)	0.25 (-1)
6	10	7.0 (1)	3.0 (-1)	3.0 (1)	0.25 (-1)
7	13	3.0 (-1)	5.0 (1)	3.0 (1)	0.25 (-1)
8	23	7.0 (1)	5.0 (1)	3.0 (1)	0.25 (-1)
9	15	3.0 (-1)	3.0 (-1)	1.0 (-1)	0.75 (1)
10	19	7.0 (1)	3.0 (-1)	1.0 (-1)	0.75 (1)
11	7	3.0 (-1)	5.0 (1)	1.0 (-1)	0.75 (1)
12	4	7.0 (1)	5.0 (1)	1.0 (-1)	0.75 (1)
13	2	3.0 (-1)	3.0 (-1)	3.0 (1)	0.75 (1)
14	14	7.0 (1)	3.0 (-1)	3.0 (1)	0.75 (1)
15	24	3.0 (-1)	5.0 (1)	3.0 (1)	0.75 (1)
16	11	7.0 (1)	5.0 (1)	3.0 (1)	0.75 (1)
17	5	3.0 (-1)	4.0 (0)	2.0 (0)	0.50 (0)
18	20	7.0 (1)	4.0 (0)	2.0 (0)	0.50 (0)
19	6	5.0 (0)	3.0 (-1)	2.0 (0)	0.50 (0)
20	1	5.0 (0)	5.0 (1)	2.0 (0)	0.50 (0)
21	9	5.0 (0)	4.0 (0)	1.0 (-1)	0.50 (0)
22	25	5.0 (0)	4.0 (0)	3.0 (1)	0.50 (0)
23	18	5.0 (0)	4.0 (0)	2.0 (0)	0.25 (-1)
24	21	5.0 (0)	4.0 (0)	2.0 (0)	0.75 (1)
25	17	5.0 (0)	4.0 (0)	2.0 (0)	0.50 (0)

No. Exp. = Number of experiment.

Where, Y is the predicted response;  $b_0$  is the intercept,  $b_i$  is the linear coefficient,  $b_{ij}$  is the quadratic coefficient,  $b_{ii}$  is the linear-by-linear interaction between  $X_i$  and  $X_j$  regression coefficients, and  $X_i X_j$  are input variables that influence the response variable Y.

## RESULTS

In earlier studies, various nutrients were screened for the biomass and glucoamylase production using the traditional one-factor-at-a-time technique (Lagzouli et al., 2007). It

was found that starch, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, yeast extract and magnesium sulfate were the most effective in promoting both biomass and extracellular glucoamylase production.

The experimental and calculated results of experiments carried out with the central composite design are given in Table 3. The analysis of variance (ANOVA) was calculated for each response to determine significant parameters then was carried out by Fisher's statistical test for the analysis of variance.

The F value is a measure of variation of the data on the mean. Generally, the calculated F value should be

**Table 3.** Central composite design for the production of biomass and glucoamylase production by *Candida famata*.

No. Exp.	Biomass		Glucoamylase	
	(Do 6000)		( $\mu\text{mol}/\text{min}/\text{L}$ )	
	Yexp.	Ycalc.	Yexp.	Ycalc.
1	5.52	5.50	1815.50	1918.16
2	7.34	7.49	3154.70	3098.55
3	5.55	5.58	2265.47	2310.06
4	7.56	7.48	3888.15	3761.29
5	5.61	5.54	2405.35	2413.01
6	8.00	8.01	3330.28	3280.32
7	6.15	6.17	3460.68	3361.31
8	8.52	8.55	4406.19	4499.45
9	5.15	5.18	2275.22	2257.05
10	7.21	7.14	2673.33	2693.76
11	5.35	5.29	3204.27	3175.29
12	7.05	7.17	3815.40	3882.83
13	5.48	5.51	2956.96	3004.88
14	7.91	7.94	3098.00	3128.50
15	6.26	6.17	4348.26	4479.50
16	8.55	8.52	5055.56	4873.96
17	5.36	5.51	3005.81	2818.26
18	7.82	7.68	3402.75	3605.69
19	7.10	7.04	3823.30	3738.41
20	7.30	7.37	4706.81	4807.09
21	7.29	7.21	3288.56	3283.60
22	7.82	7.90	4006.24	4026.59
23	7.12	7.07	4104.84	4189.01
24	6.83	6.89	4614.49	4545.71
25	7.24	7.21	4129.01	4082.85

No. Exp. = Number of experiment.

**Table 4.** ANOVA for central composite model results.

		Sum of squares	Degrees of freedom	Mean square	Ratio	Prob (P) > F
<b>Biomass</b>	Regression	26.5257	14	1.8947	131.9645	< 0.01 ***
	Residues	0.1436	10	0.0144		
	Total	26.6692	24			
<b>Glucoamylase activity</b>	Regression	1.65434E+0007	14	1.18167E+0006	53.5982	< 0.01 ***
	Residues	2.20468E+0005	10	2.20468E+0004		
	Total	1.67638E+0007	24			

\*\*\*Very high signification degree.

several times greater than the tabulated value if the model is a good prediction of the experimental results and the estimated factor effects are real. The corresponding analysis of variance (ANOVA) is presented in Table 4.

The value  $R^2$  of Biomass (Y1) was 0.99 and of

glucoamylase (Y2) was 0.98, which indicated good correlation between observed and experimental values of amylase activity. The determination coefficient (R) indicated the fitness of model. The high value (close to 1) of R indicates good correlation between predicted and observed values.

**Table 5.** Significance of regression coefficients of biomass and extracellular glucoamylase production model.

Nom	Biomass (Do 6000)		Glucoamylase ( $\mu\text{mol}/\text{min}/\text{L}$ )	
	Coefficient	Signif. %	Coefficient	Signif. %
b0	7.212	< 0.01 ***	4082.851	< 0.01 ***
b1	1.085	< 0.01 ***	393.713	< 0.01 ***
b2	0.165	0.0166 ***	534.342	< 0.01 ***
b3	0.349	< 0.01 ***	371.496	< 0.01 ***
b4	-0.088	1.12 *	178.352	0.0467 ***
b11	-0.615	< 0.01 ***	-870.877	< 0.01 ***
b22	-0.008	91.8	189.898	6.9
b33	0.344	0.102 **	-427.757	0.0984 ***
b44	-0.231	1.16 *	284.508	1.21 *
b12	-0.021	50.3	67.709	9.8
b13	0.118	0.273 **	-78.271	6.1
b23	0.137	0.102 **	139.098	0.380 **
b14	-0.008	79.6	-185.921	0.0531 ***
b24	0.010	75.3	131.582	0.531 **
b34	0.071	3.86 *	63.242	11.9

\*Signification degree; \*\* High signification degree; \*\*\* very high signification degree.

Values of Probability  $P>F$  indicated that model terms were significant. The ANOVA of quadratic regression model demonstrates that the model is highly significant, as is evident with the two results from the Fisher's test with a very high probability value ( $P$  model >  $F$ ) <0.001\*\*\*) (Table 5).

Among the variables tested, soluble starch (b1), yeast extract (b2) and  $(\text{NH}_4)_2\text{HPO}_4$  (b3) were highly significant for biomass production. However,  $\text{MgSO}_4$  indicated very low effect on the biomass production according to the  $p$  value ( $p=1.12$ ), a positive interaction was observed between  $(\text{NH}_4)_2\text{HPO}_4$  and both soluble starch (b13) and yeast extract (b23).

For all the variables tested, soluble starch, yeast extract,  $(\text{NH}_4)_2\text{HPO}_4$ , and  $\text{MgSO}_4$  were very highly significant for glucoamylase production conforming to  $p$  values indicating that the model was extremely affected by these variables. The  $p$  value of the linear effect of  $\text{MgSO}_4$  (b4) was very significant ( $p=0.0467$ ), but interact negatively with soluble starch on the glucoamylase production model. Furthermore, interaction between soluble  $(\text{NH}_4)_2\text{HPO}_4$  and both yeast extract (b23) and  $\text{MgSO}_4$  (b24) were positively significant, considering  $p$  values.

The contour of the effect of soluble starch (b1) and yeast extract (b2) on the biomass production (Figure 1) are not elliptical; maximum biomass production was represented corresponding to the maximum level of soluble starch (7 g/L), which indicated that maximum biomass production would be reached above (7 g/L). Yeast extract showed a linear plot, which means that it effected slightly the biomass production.

Figure 2 shows that interaction between  $(\text{NH}_4)_2\text{HPO}_4$  and  $\text{MgSO}_4$  on the biomass production indicated a hyperbolic shape with positive effect, biomass activity decreased gradually with an increment of  $(\text{NH}_4)_2\text{HPO}_4$  to reach to a minimum value at 2 g/L, higher than this value, biomass production increased. In the same plot, maximum biomass production was attained at 0.5 g/L of  $\text{MgSO}_4$ , beyond this value, biomass production decreased.

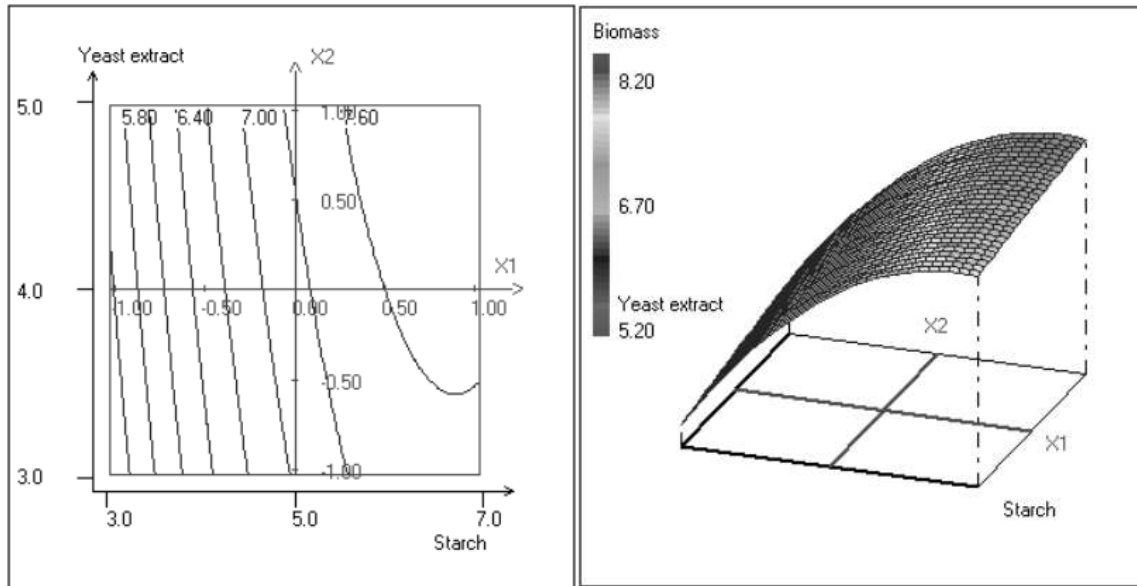
Graphical representation of starch and yeast extract on the glucoamylase production reported in Figure 3 shows a net elliptical peak at 5.0 g/L of yeast extract, the shape of the effect of soluble starch indicates that maximum glucoamylase activity could be reached at 5.5 g/L.

It is evident in Figure 4 that the hyperbolic plot indicates the contour plot is not helicoids; maximum glucoamylase activity shown with 2 g/L of  $(\text{NH}_4)_2\text{HPO}_4$  and 0.75 g/L of  $\text{MgSO}_4$  show a higher effect on glucoamylase.

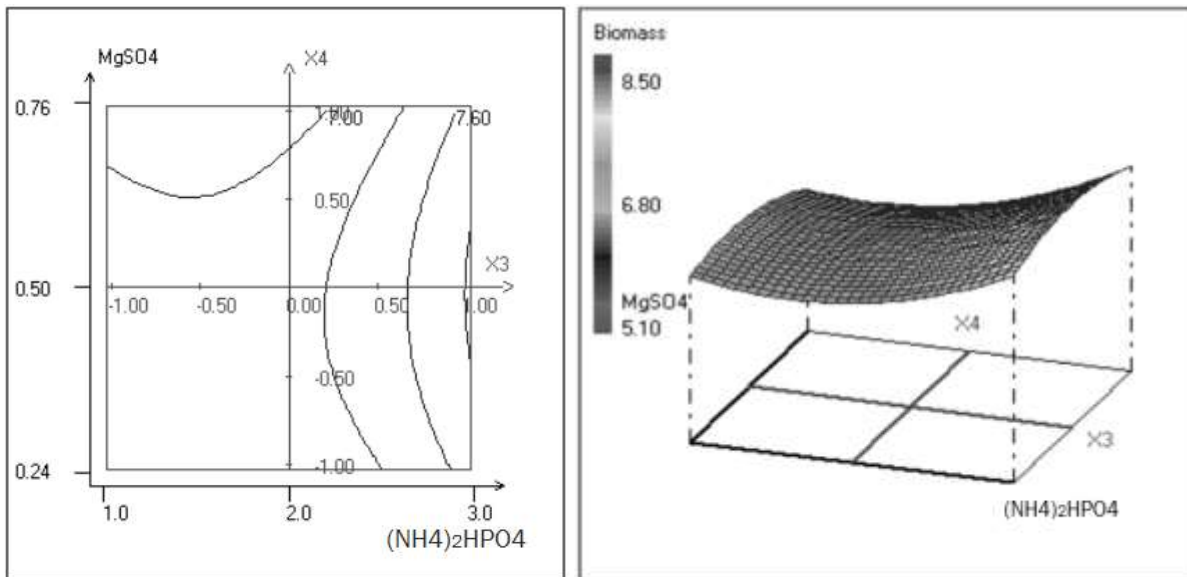
The mathematical model describing the production of glucoamylase with the independent process variables, b1, b2, b3 and b4 as a function of the coded independent variables is given by the following equation:

$$Y = 4082.851 + 393.713 b_1 + 534.342 b_2 + 371.496 b_3 + 178.352 b_4 + -870.877 b_{11} + 189.898 b_{22} - 427.757 b_{33} + 284.508 b_{44} + 67.709 b_{12} - 78.271 b_{13} + 139.098 b_{23} - 185.921 b_{14} + 131.582 b_{24} + 63.242 b_{34}$$

Where  $Y$  is the response variable, glucoamylase activity ( $\mu\text{mol}/\text{min}/\text{l}$ ) and  $b_1$ ,  $b_2$ ,  $b_3$  and  $b_4$  are the values of independent variables, soluble starch, yeast extract,  $(\text{NH}_4)_2\text{HPO}_4$  and  $\text{MgSO}_4$ .



**Figure 1.** Contour and 3D plot of effects of soluble starch and yeast extract on biomass production.



**Figure 2.** Contour and 3D plot of effects of  $\text{MgSO}_4$  and  $(\text{NH}_4)_2\text{HPO}_4$  on biomass production.

## DISCUSSION

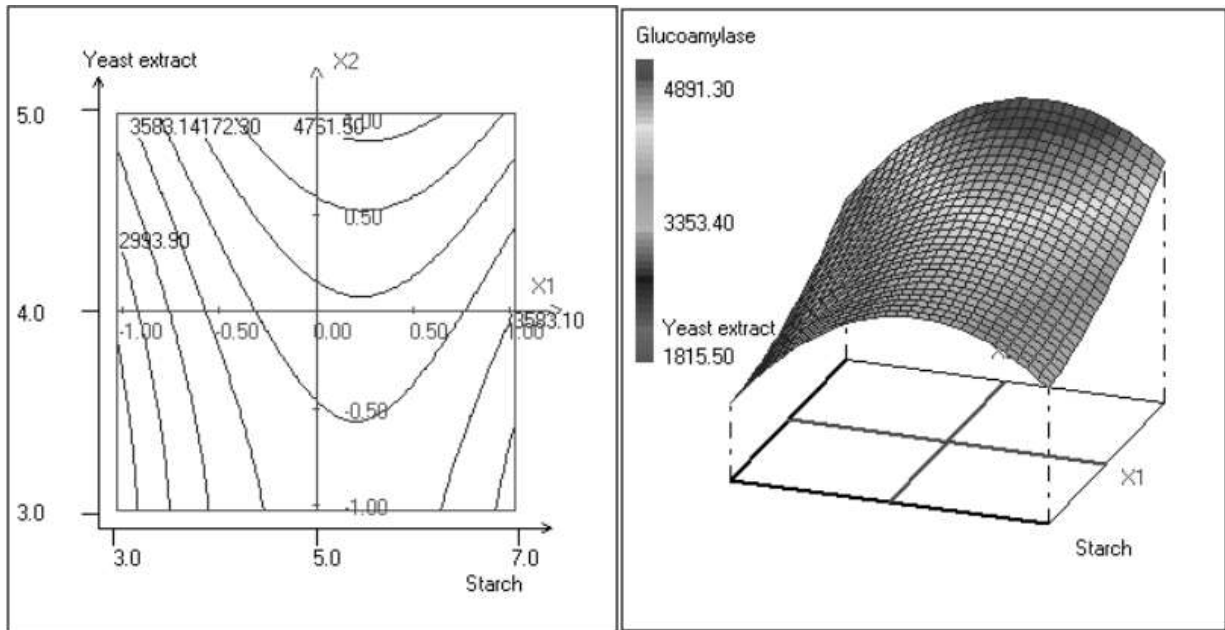
The main goal of response surface is hunt efficiency for the optimum values of the variables such that the response is maximized. Each contour curve represents an infinitive number of combinations of two test variables. The maximum predicted value was indicated by the surface confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained whenever there

is a perfect interaction between the independent variables (Guo et al., 2010).

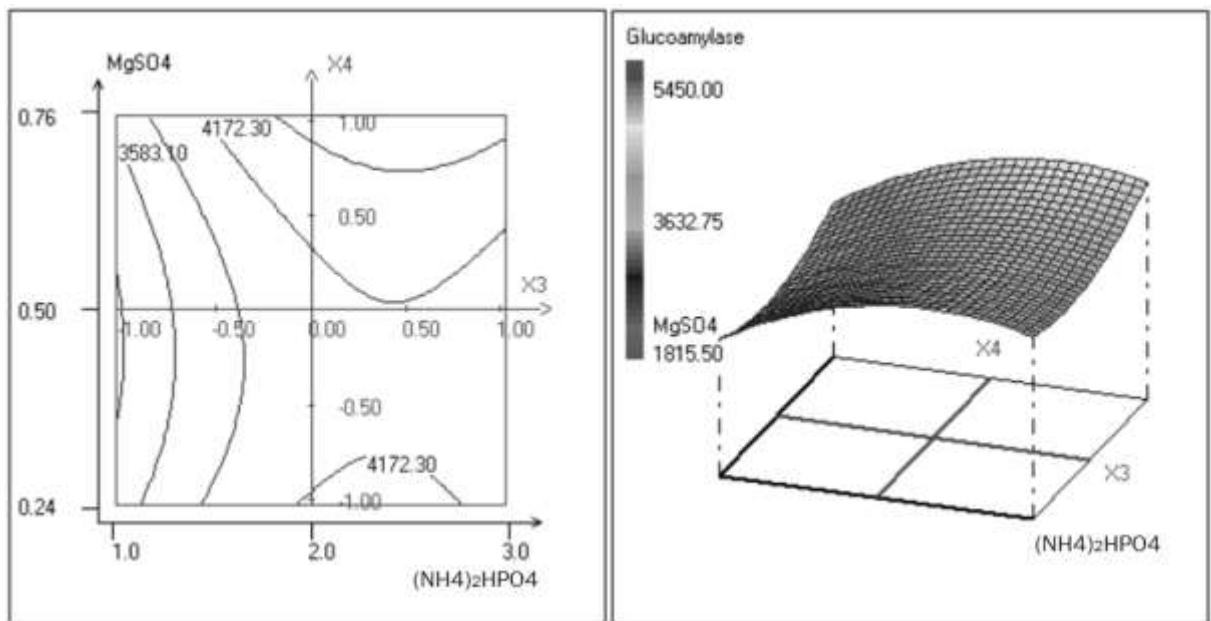
### Effect of starch

Biomass became more active in relation to the increase in starch concentration from 2 to 7 g/L, plots are note elliptical which indicated that higher biomass production





**Figure 3.** Contour and 3D plot of effects of soluble starch and yeast extract on glucoamylase production.



**Figure 4.** Contour and 3D plot of effects of  $\text{MgSO}_4$  and  $(\text{NH}_4)_2\text{HPO}_4$  on glucoamylase production.

would be reached at above 7g/L (Figure 1); whereas, maximum glucoamylase production was found at 6.5 g/L (Figure 3); above this concentration, the production decreases.

Literature abounds on instances, which show the prominent role played by starch as a carbon source for high biomass and glucoamylase production. In contrast,

maximum  $\alpha$ -amylase production by *Candida guilliermondii* was at 5 g/L of starch (Acourene et al., 2013); whereas, other studies showed maximum glucoamylase activity by thermophilic fungus, *Humicola grisea* MTCC 352 at 28.41 g/L (Ramesh and Ramachandra, 2014); starch seems to have an “inductive effect” and portrays a significant role in glucoamylase

production (Prajapati et al., 2013).

### Effect of yeast extract

Yeast extract exhibited the high biomass production at 4 g/L (Figure 1), in addition, maximum glucoamylase activity was attained at 5 g/L of yeast extract (Figure 3); the contour graphics of the effect of yeast extract on glucoamylase production is not elliptical, which suggest that the maximum glucoamylase could be given by higher concentrations.

As in many other studies, yeast extract helps in the development of mycelial structures with a corresponding higher yield of enzymes (Ramesh and Ramachandra, 2014; Kumar and Satyanarayana, 2007; Arnthong et al., 2015). However, some studies showed that the peptone was the best nitrogen source with *Thermomyces lanuginosus*, *Penicillium fellutanum* and *Bacillus licheniformis* (Kunamneni et al., 2005; Kathiresan and Manivannan, 2006).

McTigue et al. (1994) reported that the results of enzyme yield are probably due to the excessive amount of the yeast extract, which may inhibit the production of enzyme when concentration of yeast exceeds a critical value (Alam et al., 1989; Pedersen and Nielsen, 2000).

### Effect of $(\text{NH}_4)_2\text{HPO}_4$

The glucoamylase production was affected by  $(\text{NH}_4)_2\text{HPO}_4$  as nitrogen source, which confirms the results found with *C. famata* in a previous work (Lagzouli et al., 2007). Also, the beneficial effects of  $(\text{NH}_4)_2\text{HPO}_4$  on the production of amylase was reported by many researchers on *Aspergillus fumigatus* (Akhter et al., 2013) and by *Bacillus subtilis* (Demirkan, 2011; Aiyer, 2005).

### Effect of $\text{MgSO}_4$

Biomass production highly occurred at 0.5 g/L of  $\text{MgSO}_4$ , which are comparable to previously reported results (Lagzouli et al., 2007), in same way, effect of  $\text{MgSO}_4$  on the production of biomass and glucoamylase by *Humicola grisea* MTCC 352 (Ramesh and Ramachandra, 2014) and with *Thermomucor indicaeseudaticae* by Kumar and Satyanarayana (2007).

### Conclusion

In the present study, the authors demonstrated the use of statistical design for the rapid identification and optimization of significant media components for the production of biomass and glucoamylase by *C. famata*.

Biomass production was found to be starch  $(\text{NH}_4)_2\text{HPO}_4$  and yeast extract dependent, the maximum

was obtained when the starch concentration was 5 g/L, yeast extract was 5 g/L and  $(\text{NH}_4)_2\text{HPO}_4$  was 2g/L; Positive interaction was observed between  $(\text{NH}_4)_2\text{HPO}_4$  and both starch and yeast extract.

All variables were highly significant for glucoamylase production according to their  $p$  values, highest production was found at 5 g/L of yeast extract, 7 g/L of starch and 3 g/L of  $(\text{NH}_4)_2\text{HPO}_4$ ; furthermore, yeast extract and  $(\text{NH}_4)_2\text{HPO}_4$  interacted positively.

The predicted values were verified experimentally and gave a second-order polynomial regression model, which was in good agreement with experimental results, with  $R^2 = 0.99$  for biomass production and  $R^2 = 0.98$  for glucoamylase, according to the  $p$  values ( $p < 0.01$ ). These predicted optimal parameters were confirmed in the laboratory and the final biomass and glucoamylase activity obtained was very close to the calculated parameters.

The optimized media composition found in the present investigation might reduce the overall cost of the medium, and provide a basis for further studies, as a potential candidate for application in baking, detergent industry, or in the valuation of starch waste products.

### Conflict of Interests

The authors have not declared any conflict of interests.

### ACKNOWLEDGEMENT

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## Full Length Research Paper

## Selection of filamentous fungi producing lipases from residual waters of slaughterhouses

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The exploration and use of lipases produced by microorganisms are in line with societal concerns since they provide clean development options within the standards of environmental sustainability. The objective of this work was therefore to isolate microorganisms with the potential of producing lipases, which could be used in the waste water treatment systems of slaughterhouse units. Waste water samples were collected from the treatment lagoons of a poultry slaughterhouse. The PCA culture medium was used for the isolation of fungi. The pH was adjusted to 6.5 and autoclaved at 1 atm of pressure, for a time of 30 min. The isolated and purified colonies were evaluated in order to obtain lipase producing strains. The results obtained in this study enabled the conclusion that the waste water produced in slaughterhouse has a high microbial load, highlighting bacteria with  $6.8 \times 10^8$ , filamentous fungi with  $3.4 \times 10^4$  and yeasts with  $2.2 \times 10^2$  CFU.ml<sup>-1</sup>. Out of this microbial population, six species of lipase producing fungi were isolated, with the fungi *Aspergillus flavus* and *Aspergillus terreus* being those that obtained the highest enzymatic indices.

**Key words:** Lipase, fungi, effluent, lipids, enzymes.

### INTRODUCTION

Industrial processes are one of the major factors responsible for water pollution and contamination through the untreated release of effluents into natural waterways,

causing significant damage to the environment and the population. One of the main sources of agro-industrial waste that needs special attention in order to avoid the

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pollution of the waters, are the effluents from slaughterhouses (Cammarota and Freire, 2006). The undue release of slaughterhouse effluents causes changes in the characteristics of the water and soil, and can pollute or contaminate the environment (Mees, 2004; Chaubey et al., 2013; Miranda and Souza, 2015).

Slaughterhouses represent an important sector in the food industry from the economic point of view. However, considering the large number of companies that still dispose of their effluent without any kind of treatment into the waterways, the contribution of these industries to water pollution is quite significant. Most of these industries are not qualified to deal with the changes required for the implementation of clean technologies, nor for the operation of effluent treatment systems (Vazoller et al., 1991; Machado, 1999; Klibanov, 2001; Kopec et al., 2016). The waste water from slaughterhouses has a high flow rate and high load of suspended solids, organic nitrogen and a BOD<sub>5</sub> of 4,200 mg/L (Aguilar, 2002), depending on the reuse or treatment of the effluent. Because of its composition, this waste is highly putrescible and starts to decompose in a few hours, producing gases that produce bad odors that make it difficult to breathe in the establishment's surroundings, causing nuisance to the local population. These effluents are therefore responsible for the bad image that the public has of these establishments.

Slaughterhouses generally release their effluents, treated or not, into bodies of water. Resolution no. 357 of the National Environmental Council (CONAMA) of March 17, 2005, established the classification of water bodies and environmental guidelines for this framework, in addition to the conditions and standards of effluent discharge. Article 34 of this resolution states the conditions and standards for the release of effluents, including a pH between 5 and 9, up to 1 mL/L of sedimentary material, in a test of 1 h in a Imhoff cone, up to 20 mg/L of mineral oils, and up to 50 mg/L of vegetable oils and animal fats (Conama, 2006).

The lipids present in these effluents cause flotation of the biomass and poor formation of sludge granules in anaerobic reactors with upward flow (Hanaki, 1981; Gavala, 1999); toxicity to acetogenic and methanogenic microorganisms (Hanaki, 1981); foaming due to the accumulation of non-biodegraded fatty acids (Vazollér et al., 1991) and a decrease in the concentration of adenosine triphosphate (ATP) (Hanaki, 1981; Perle, 1995; Manoel et al., 2015; Melais et al., 2016; Pecha et al., 2016). The anaerobic digestion of these residual waters rich in fat of animal origin is therefore a slow process, with the release of fatty acids by specific microorganisms with lipolytic activity being a limiting factor (Perle, 1995; Pecha et al., 2016).

The use of lipases promotes an increased reaction speed in the sequential hydrolysis of lipids, reducing the biodegradation time of these compounds (Mendes and Castro, 2004). Lipases are enzymes of animal, plant or

microbial origin classified as glycerol ester hydrolases (E. C. 3.1.1.3). They act in the organic/aqueous interface, catalyzing the hydrolysis of the ester-carboxylic bonds present in acylglycerols with the release of fatty acids and glycerol (Yahya, 1998; Smaniotto et al., 2012; Melais et al., 2016; Pecha et al., 2016). The use of microbial lipases is more appropriate for the pre-treatment of industrial waste water rich in animal fats. These lipases are more efficient in the hydrolysis of triacylglycerols containing fatty acids with more than 12 carbons (Masse et al., 2001; Wu et al., 2014; Pecha et al., 2016).

A great diversity of microorganisms are involved in the anaerobic degradation of complex substrates, such as those found in effluents of animal husbandry activities, such as slaughterhouses, poultry slaughter and dairy farming, among others (Novaes, 1986). The microbial lipases can be produced by yeasts of the genus *Candida* sp. and *Torulopsis* sp. (Benzoana and Esposito, 1971; Sakiyama et al., 2001; Lee et al., 2001), by the filamentous fungi *Penicillium* sp., *Aspergillus* sp., *Rhizopus* sp. and *Geotrichum* sp. (Iwai and Tsujisaka, 1974; Rodriguez et al., 2006; Diaz et al., 2006), *Humicola* sp. (Ibrahim et al., 1987) and by the bacteria of the genus *Pseudomonas* (Bucky et al., 1987; Sugiura et al., 2003; Suzuki et al., 2003); *Staphylococcus* sp. (Alford et al., 1964); *Streptococcus* sp. (Tripathi et al., 2004); *Burkholderia* sp. (Fernandes et al., 2006); *Sporidiobolus pararoseus* (Smaniotto et al., 2012) and *Aspergillus* sp. (Colla et al., 2015; Melais et al., 2016).

The microorganisms hydrolyze the triacylglycerides in the extracellular medium through the action of lipases, producing fatty acids and glycerol. The lipases (triacylglycerol acyl hydrolases, E.C.3.1.1.3) comprise a group of hydrolytic enzymes that act on the organic-aqueous interface, catalyzing the hydrolysis of ester-carboxylic bonds, present in glycerides. These enzymes cleave specific triglycerides, but may not be completely specific and catalyze the hydrolysis reactions of triacylglycerides that contain different fatty acids in their composition, which may be of great interest for the treatment of effluents with high fat content (Akersson et al., 1983; Quilles et al., 2015; Pecha et al., 2016).

Within this context, alternative processes are being used for the reduction of the concentration of lipids in these effluents through the action of enzymes and microbial lipases, in particular. These enzymes are of particular importance because they specifically hydrolyze fats and oils, which can be of great interest for the treatment of effluents with high fat content (Iwai and Tsujisaka, 1974). Their use reduces the levels of suspended solids and lipids, enabling better operating conditions in the anaerobic treatment and freeing up piping from oily films, increasing the useful life of equipment (Brockerhoff and Jensen, 1974; Arnold et al., 1975; Hasan et al., 2006; Gupta et al., 2012; Chen et al., 2012; Nascimento et al., 2013; Silalahi et al., 2014). The objective of this work was therefore to isolate





**Figure 1.** Waste water collection location from the slaughterhouse.

microorganisms with the potential of producing lipases, which could be used in waste water treatment systems.

## MATERIALS AND METHODS

### Collection of biological material

Waste water samples were collected from the treatment lagoons of a slaughterhouse located in the municipality of Francisco Beltrão in the State of Parana, Brazil (Figure 1), at the geographical coordinates with Latitude: 26° 02' 54" S and Longitude: 53 03' 74" W. The waste water was collected in cleaned and sterilized plastic containers in order to conserve its characteristics. After collection, the material was transported to the laboratory and stored in a refrigerator at 0°C. All the activities were performed in the Laboratory of Microbiology of the União de Ensino do Sudoeste do Paraná - Unisep - Francisco Beltrão Campus – Paraná, Brazil.

### Total microorganism count

For the isolation and total count of microorganisms (filamentous fungi, yeasts and bacteria), a PCA (Plate Count Agar) culture medium was used containing 1% of casein peptone; 2.5% of yeast extract, 2.5% of glucose, 2% of agar. The pH was adjusted to 6.5 and autoclaved at 1 atm of pressure, for a time of 30 min. The effluent samples collected from the lagoons were submitted to successive dilutions in test tubes. The material of each tube was homogenized and inoculated with the *pour plate* method by adding 1.0 mL of each tube to the bottom of the Petri dish. The plates were subsequently submitted to slow agitation, kept until cooling,

incubated upside down at a temperature of 28°C, and monitored every 24 h for the total count.

Based on the microbial growth, each colony characteristic of filamentous fungi was transferred to the culture medium containing 5% fat of animal origin, in order to isolate microorganisms with the ability to grow in a fat rich medium, and incubated at 28°C and monitored every 24 h. After the growth, the isolated and purified colonies were subcultured in tubes containing medium, tilted for keeping, at a temperature of  $\pm$  -4°C for later identification and assessment of their capacity to produce lipases. The fungal colonies that were distinct from one another, according to macroscopic observations (color and growth features in the culture medium) were purified in PDA medium, preserved by subculture and stored at 4°C. To identify the genera, microcultures from the selected isolates were made using agar block, and literature was consulted (Booth, 1971; Barnett and Hunter, 1972; Larone, 1993; Menezes and Oliveira, 1993).

### Assessment of enzyme activity

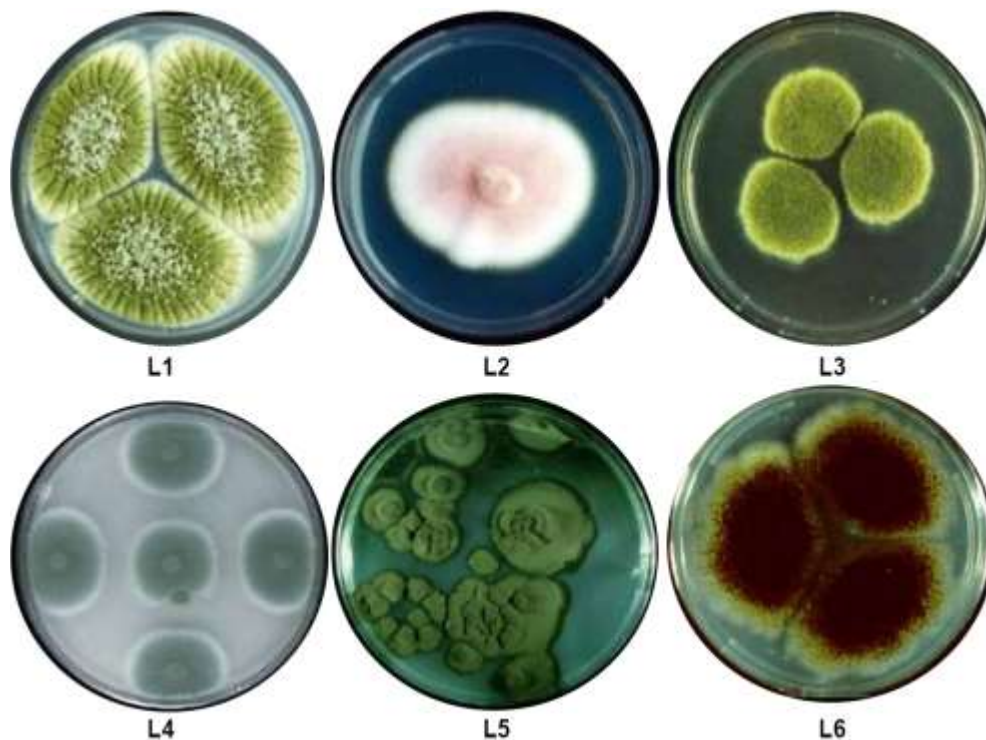
The isolated and purified colonies of filamentous fungi were evaluated in order to obtain lipase producing strains. The methodology described by Hankin and Anagnostakis (1975) was followed to perform this activity. The culture medium used for this activity was composed of: peptone (1%), sodium chloride (0.5%), calcium chloride (0.01%) and agar (2%). To this medium, 1% sorbitan monolaurate was added (Tween-80®). After 72 h of incubation at 28°C, the Petri dishes were kept in the refrigerator for 7 days. As such, lipase activity was observed through the presence of calcium salt crystals of the lauric acid, released by the enzyme around the formed colonies. Each colony was measured with a caliper and the enzymatic index was determined using the following



**Table 1.** Total count of microbial flora present in the slaughterhouse effluent.

Micro-organism	Count in CFU mL <sup>-1</sup>
Bacteria	$6.8 \times 10^{8a}$
Filamentous fungi	$3.4 \times 10^{4b}$
Yeasts	$2.2 \times 10^{2c}$

\*Values followed by the same letter vertically, do not differ significantly at the level of 5% by the Tukey Test.



**Figure 2.** Morphological characteristics of the isolated and identified fungal species. *Aspergillus flavus* (L<sub>1</sub>), *Paecilomyces* sp. (L<sub>2</sub>), *Aspergillus terreus* (L<sub>3</sub>), *Penicillium chrysogenum* (L<sub>4</sub>) and *Cladosporium herbarum* (L<sub>5</sub>) and *Aspergillus niger* (L<sub>6</sub>).

ratio: EI = diameter of the colony + the halo / diameter of the colony.

#### Statistical analysis

The statistical analysis was performed using the software Statistica, version 5.0. The analyses of variance were carried out according to standards of ANOVA. The significant differences between the means were determined by Tukey's test. All activities were performed in triplicate.

## RESULTS AND DISCUSSION

After the development of the work, the microbial flora of the effluent collected in the treatment lagoons of the evaluated slaughterhouses could be quantified. The

results are summarized in Table 1. By analyzing Table 1, one can see that bacteria are the predominant microorganisms in the slaughterhouse effluent, with  $6.8 \times 10^8$  CFU.mL<sup>-1</sup>, followed by filamentous fungi with  $3.4 \times 10^4$  CFU.mL<sup>-1</sup> and yeasts with  $2.2 \times 10^2$  CFU.mL<sup>-1</sup>. This diversity can be seen as beneficial for the treatment process of these effluents because a greater diversity increases the biodegradation capacity of the effluents (Pereira, 2007; Bhuwal et al., 2013; Motiwalla et al., 2013).

The isolated, purified and identified fungi were: *Aspergillus flavus* (L<sub>1</sub>), *Paecilomyces* sp. (L<sub>2</sub>), *Aspergillus terreus* (L<sub>3</sub>), *Penicillium chrysogenum* (L<sub>4</sub>) and *Cladosporium herbarum* (L<sub>5</sub>) and *Aspergillus niger* (L<sub>6</sub>) (Figure 2). After isolation and identification, the fungi were evaluated for their ability to degrade lipids. The

**Table 2.** Lipolytic activity of the filamentous fungi strains isolated from the slaughterhouse effluent.

Micro-organism	Diameter of the colony	Diameter of the colony + halo (mm)	Enzymatic index*
L <sub>1</sub>	17.33	35.12	2.02 <sup>a#</sup>
L <sub>2</sub>	45.32	67.82	1.49 <sup>b</sup>
L <sub>3</sub>	15.41	32.41	2.10 <sup>a</sup>
L <sub>4</sub>	22.12	36.78	1.66 <sup>b</sup>
L <sub>5</sub>	18.62	28.29	1.51 <sup>b</sup>
L <sub>6</sub>	52.34	71.26	1.36 <sup>c</sup>

\*Enzymatic Index obtained from the ratio diameter of the colony / diameter of the colony + halo × 100. Average of three repetitions.

<sup>#</sup>Indices followed by the same letter in the column do not differ by Tukey's Test at the level of significance of 5%.



**Figure 3.** Halo of degradation caused by the lipolytic activity revealing the presence of calcium salt crystals of lauric acid.

results obtained are summarized in Table 2 and in Figures 3. The lipase activity was observed through the presence of calcium salt crystals from lauric acid, released by the enzyme around the formed colonies, revealing the degradation of the sorbitan monolaurate (Tween-80<sup>®</sup>) (Figure 3). This behavior is in line with the work by Sharma et al. (2001), Maia et al. (2001), Dominguez et al. (2003), Rodríguez-Contreras et al. (2012a), Rodríguez-Contreras et al., (2012b), Sandhya et al., (2013) and Vinish et al. (2015), who assessed the production of lipases using the same methodology of this work and noted that two of the tested strains showed the same precipitation pattern of calcium laurate forming a thin layer of precipitation around the colonies, which may also be a coarse grain precipitation.

After analyzing the lipolytic activity, no differences were found with respect to the production of lipases between

the strains L<sub>1</sub> of *A. flavus* and L<sub>3</sub> of *A. terreus*, with enzymatic indices of 2.02 and 2.10, respectively. Another group of strains that did not show any differences between themselves regarding the enzymatic indices, were the strains L<sub>2</sub> of *Paecilomyces* sp, L<sub>4</sub> of *P. chrysogenum* and L<sub>5</sub> of *C. herbarum*, presenting rates of 1.49, 1.66 and 1.51, respectively. The strain with the lowest enzymatic index, and which stood out from the other strains, was the L<sub>6</sub> strain of *A. niger*, with an enzymatic index of 1.36. After evaluation of the obtained indices, it was found that the fungi that produce the most enzymes were the fungi of the genus *Aspergillus*, with the species *A. flavus* and *A. terreus*.

The results obtained are in agreement with the results found in Soza-Gomes and Alves (1983), who found enzymatic indices between 1.45 and 1.86 evaluating strains of fungi with potential to produce extracellular enzymes. They also found enzymatic indices equal to 1 (one), indicating that these strains showed no lipolytic activity, at least not extracellularly. The fact that the fungal strains L<sub>2</sub>, L<sub>4</sub>, L<sub>5</sub> and L<sub>6</sub> have shown low enzymatic indices for the production of lipases may be related to the phenotypic responses to enzyme induction mechanisms (Fargues and Robert, 1983; Amirita et al., 2012; Lutz et al., 2013; Senanayake et al., 2016) or to the culture medium used. However, with respect to the type of medium used for the observation of lipolytic activity, it is important to emphasize the fact that fungi may have different responses to the composition of fatty acids used as source of lipids.

Gabriel (1968) and Silva et al. (2015), observed that *Metarhizium anisopliae* and *Beauveria bassiana* continually degrade lipids in a specific medium to detect lipase, but they do not produce enzymes capable of degrading olive oil or coconut oil, suggesting that this selective reaction could be related to the presence of different fatty acids, such as oleic acid in olive oil, and capric and caprylic acid in coconut oil. The lipolytic activity in different fungi may therefore be related to the species-specificity of the fungus to the substrate. As such, one can see that the medium containing Tween-80 may not be the most appropriate for the evaluation of lipolytic activity, since this activity may be related to other

types of fatty acids.

## Conclusion

The results obtained in this study enabled the conclusion that the waste water produced in slaughterhouses has a high microbial load, highlighting bacteria with  $6.8 \times 10^8$ , filamentous fungi with  $3.4 \times 10^4$  and yeasts with  $2.2 \times 10^2$  CFU.ml<sup>-1</sup>. Out of this microbial population, six species of lipase producing fungi were isolated, with the fungi *A. flavus* and *A. terreus* being those that obtained the highest enzymatic indices.

## Conflicts of Interests

The authors have not declared any conflict of interests.

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