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Review

Toxicological and safety assessment of tartrazine as a synthetic food additive on health biomarkers: A review

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Recently, progressive use of synthetic food additives increase the attention paid on their benefit and toxicity in food, especially for the young. One of these additives is artificial azo dyes tartrazine. This study aimed to provide an outline of the existing evidence on the beneficial and side effect of food additive with special reference to tartrazine on different organ health. The methods include updated search for the relevant databases. The studies included a description of the types of food additives and products containing tartrazine and focused on the effect of tartrazine on liver, kidney function, lipid profile, oxidative stress biomarkers, nervous system, hyperactivity, behavior, cancer, reproductive and developmental toxicity and some bioelement levels of tartrazine. Several studies were identified and some investigated advantage and disadvantage of tartrazine. Summary of the study provides potentially harmful effects of tartrazine on liver, renal function, lipid profiles, behavior, carcinogenicity and forthcoming research recommendation are outlined. This review gives a broad evaluation of the safety and various toxicity effects of tartrazine. It can be concluded that there is a need for professional assistance for consumers regarding food safety issues. Cumulative indications have been increased, demonstrating the potential danger of tartrazine, and the possibility to avoid its consumption.

Key word: Food additives, tartrazine, liver, kidney, oxidative stress, cancer.

INTRODUCTION

Various kinds of food additives (more than 2,500 substances) have been employed to improve the taste, tint, constancy, quality and price of foods. These are the result of industrialization and advances in the technology of food processing and treatment (NRC, 1983).

Acute, subacute and chronic toxicity are investigated before food additives are ready for customer consumption. However, post-marketing investigations of

food additives properties needs to be reserved for an extensive period. Evidence regarding the security of the long-term usage of such substances, their pooled impact, and variability within the organism is uncommon. Several papers reviewed the effect of tartrazine including *in vivo* and experimental studies, while there is scarce literature on clinical aspect. The aims and concerns of this review are to assess the role of tartrazine as coloring food

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Table 1. Field report on food additives present in the hypermarket (frequently consumed by kids).

Product name	Company name	Label found
Tito wefer	Candy makers food industry	Flavors food grade
Beco-biski	EL-Jawhara for food industry	Edible flavors
Sposa Cake	Over seasas Co. for food products	Food flavors
Fruity, juice powder	Dream A.S.E.	Allowed artificial colorants (E102-E110)
Ice man, ice cream	Egyptian Co. for food industries	Healthy allowed flavors
Lika, Gum	Sima Food industry	Healthy allowed artificial color (E132).

additive in hepatic function, lipid profile and biomarkers of oxidative stress and bioelement contents in blood and different tissue. Its role in the nervous system, hyperactivity and behaviour is also discussed.

FOOD ADDITIVES

Importance and disadvantages of food additives

Many people consume various food additives every day, which have both advantages and disadvantages. Food additives have an important effect in currently abundant and nourishing food sources, and allow people to appreciate a diversity of nutritious, delicious and safe foods over the year. Food additives have different beneficial effects on foods. However, food additives may contain several metabolites, such as monosodium glutamate and nitrous compounds that are found to be carcinogens. Toxicity or benefit depends on the extent to which the food components interrupt absorption, elimination or metabolism. Description of the appropriate safety limits for human ingestion is further complicated due to the interaction between several substances.

In nutrition, the probability of toxicity of chemical compounds means that all new compounds should be regarded as toxic until their safety is confirmed. Food additives sometimes destroy vitamins in food (adding caramel to a food is found to cause a deficiency of vitamin B6), are used to make bad quality food look good and can cause allergy in many people like diarrhoea, skin irritation, stomach disorders, vomiting or an increase in the body heat. Also, it may destroy the nutritional value of food. Several food colourings have been banned due to their tendency to cause cancers and tissue injuries. Tartrazine as a food additive has been proven to cause many different side-effects and allergic responses in individuals. These may comprise migraines, nervousness, asthma attacks, hazy vision, eczema, other skin rashes and thyroid cancer.

Types of food additives

Food additives are classified into 6 main groups: preservatives, nutritive additives, flavoring, coloring,

texturizing and miscellaneous compounds. Examples of widely used food products containing target food additives and frequently consumed by children are shown in Table 1. The food additives include:

Preservatives: Preservatives are added to prolong the shelf life of foods. There are three types of preservatives. The first is antimicrobials that prevent microbial growth, which can cause life threatening illnesses such as salmonellosis or botulism, for example benzoic acid, ascorbic acid and propionic acid. This preservative can be used in cheeses, margarine and dressings, bakery products and dried fruit preparations.

The second preservative include antioxidants, which are added to oils containing unsaturated fats that are more susceptible to oxidation. Food oxidation is a damaging process, causing alterations in the chemical structure and biochemical properties result in loss of its dietary value. The antioxidant slow the degree of its oxidation, prevent them from becoming rancid and prolong food life. Natural and synthetic antioxidants provide comparable performance and they are frequently used in the mixture (Fiorentino et al., 2008). Some important antioxidants include vitamin C and E, citric acid, butylated hydroxytoluene (BHT) and butylated hydroxyanisole. The third preservative includes antibrowning which is added to fruits vegetables to prevent enzymatic browning, for example. alpha tocopherol.

Nutritional additives: They are added to increase the nutritional value of the food and comprise antioxidant vitamins, amino acids and bioelement.

Flavoring agents: Flavoring substances include the largest number of applied food additives. There are 3 main kinds of flavoring agents:

- 1) Sweeteners: These are substances that have a strong sweet taste but little or no caloric values. They are therefore useful for diabetics and include saccharin, sorbitol and aspartame.
- 2) Synthetic flavoring enhancers: They are used in general at very low level and they are synthetic, mainly esters, aldehydes and ketones.

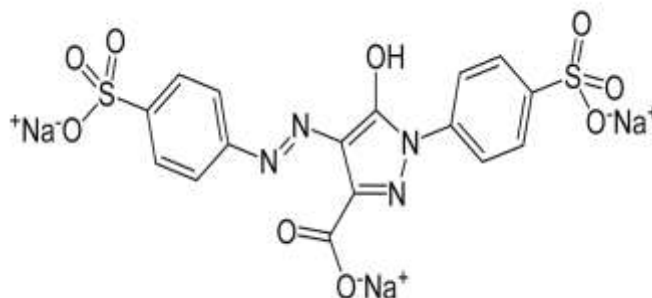


Figure 1. Structure of tartrazine.

3) Natural flavoring enhancers: They are used to modify the flavor of food without contributing any flavor on their own, for example, monosodium glutamate (MSG) and nucleotides such as disodium guanylate.

Texturizing agents: These are stabilizers and emulsifiers agents that are used to add or modify the overall texture or mouthful of food products. Emulsifiers include natural substances such as lecithin, mono-or diglycerides and several artificial byproducts. The major effect of the texturizing compound is to permit oils and flavors to be distributed through the food products. Stabilizers comprise many natural gums as carrageenan in addition to modified and natural starches. It has been used for many years in both dry and liquid form to provide the desired texture in products such as ice cream. Also, it is used to avoid deterioration and evaporation of volatile taste oils (Branen and Haggerty, 2001).

Coloring agents: These substances are added to enhance the visual appeal of food products. Some of these substances are derived from the natural colors, such as carotene and chlorophyll; others are synthetic such as indigotin, allura red, tartrazine and carmoisine.

Color additives released from authorization are intended for an extensive diversity of purposes in the foods, cosmetics and drugs (Van-Bever, 1989). Although, most color food additives are similar in relations to the FDA's monitoring description, they are controlled in 2 classes; natural (color additives exempted from certified) and the synthetic (certified) color additives. In studies from Asia and Africa, exposure to colors additives like tartrazine and sunset yellow FCF surpassed the acceptable daily intake (ADI) especially in festive and wedding times. The consumption of tartrazine and sunset yellow increased in high consumers, especially in children without control. The condition may be aggravated in an adult in developing countries (Rao and Sudershan, 2008). Tartrazine mainly affect young people because they cannot control their feeding, and are attracted to colour more easier than adult. Young people consume these additives several times daily in

chocolates, gum, chips, drinks and cared little about its health effect. Moreover nervous system, behaviour and other metabolic and organ function more easily affected in young than adult .

The use of tartrazine was focused on was because of its extensive usage in many beverages and sweets, to provide color to colorless foodstuff, make the food more attractive, appetizing and is a synthetic compound.

RESEARCH UPDATE ON TARTRAZINE

Tartrazine is an artificial lemon yellow azo dye, recognized as E102 or C.I. 19140 or FD&C Yellow 5 and used for the coloring of food. It is water soluble, derived from coal tar and its structure is shown in Figure 1.

Products containing tartrazine

Foods: Several foods have tartrazine in different quantities, relying on the industrialist or the cook administrator. Nowadays, the tendency is to prevent its addition or its replacement with a non-synthetic coloring material like annatto, malt color or beta carotene. Foodstuffs containing tartrazine include sweetmeat, soft drinks, cotton candy, cereals (corn flakes and muesli), flavored chips (Doritos and Nachos), cake combinations, soups, jam, sauces, ice cream, some rice, candy, chomping gum, marzipan, jelly, gelatins, mustard, marmalade, yogurt, noodles, fruit pleasant and product, chips and several expediency foods together with glycerin, lemon and honey products, soft drinks (Mountain Dew and Mirenda), energy drinks, prompt desserts, and some product containing tartrazine as shown in Figure 2.

Non-food products: Tartrazine may be found in non-food products like soaps, cosmetics, shampoos and other hair products, conditioners, pastels, crayons and stamp dyes.

Medications: Particular medicinal preparations comprise



Figure 2. Some products containing tartrazine.

tartrazine as antiacids, vitamins, certain prescription medications and medical capsules. Accruing research has been performed on tartrazine and its effect on the health

METABOLISM AND BIOLOGICAL EFFECTS OF TARTRAZINE

Tartrazine is reduced inside a organism to an aromatic amine which is greatly sensitized, since it is a nitrous derivatives (azo class). The chief metabolite recognized so far is sulfanilic acid. Tartrazine is identified to cause allergy such as urticaria and asthma, besides the emphasis of studies on its carcinogenesis and mutagenesis because of its metabolic conversion into aromatic amine (sulfanilic acid) via the gut microflora (Moutinho et al., 2007) and possibly by mammalian azo reductase in the hepatic or intestinal wall after consumption (Chequer et al., 2011). When these azo dyes are reduced totally into aromatic amines, they are oxidized to N-hydroxy derivatives by the enzymatic system of P450 (Demirkol et al., 2012). This mechanism of biotransformation takes place in many species including humans (Chequer et al., 2011), which is responsible for various disorders including anemia, pathological lesions in the brain, liver, kidney and spleen,

beside allergic reactions, tumor and cancer. However, tartrazine has no possibility to induce malignant or benign neoplasias. Moreover, Tanaka (2006) did not determine any adverse role of tartrazine in the development of neurobehavior, also, harmful impact on reproductive markers were not established at tartrazine dose of 1225 and 773 mg/kg BW/day for females and males, respectively. In earlier assessments, there were no suggestions of Tartrazine-linked contrary effects on reproduction. However, superoxide anion, hydroxyl radical and H_2O_2 reactive oxygen species (ROS) might be formed in the nitrosamines metabolism and raise oxidative stress (Bansal et al., 2005).

Role of tartrazine on sensitivity

A diversity of immunologic reactions has been recognized in tartrazine consumption, comprising general fatigue, nervousness, migraines, clinical depression, purple skin spots, and disruption in sleep. Either consumption or cutaneous contact with a material containing tartrazine can produce symptoms of sensitivity. Some claim involvement signs of tartrazine sensitivity even at minor dosages, and until 72 h following its exposure. In kids, asthma attack and rashes have been claimed, as well as possible links with chromosomal injury, thyroid cancer

and hyperactivity. Particular investigators have related tartrazine with infantile obsessive-compulsive disturbances and hyperactivity. Some common food additives including tartarazine, monosodium glutamate have been suggested as risk factors for exacerbations of asthma. Tartarazine is also used in many medications, and may increase asthma severity only in a few susceptible individuals, while MSG may exacerbate asthma severely (Romieu, 2005).

Food additives examinations revealed that tartrazine increased sulphido-leukotriene released by peripheral leucocyte in patients with confirmed intolerance to food additives (atopic dermatitis). The mechanism of these changes may be due to a pathophysiological involvement of food additive that facilitated exaggeration of atopic dermatitis (Worm et al., 2001).

A number of studies in humans recorded adverse reactions such as vasculitis and urticaria following tartrazine consumption. EFSA Panel (2009) concluded that Tartrazine seems to be capable of producing intolerance responses in few exposed people and noted that sensitive persons may respond to the level of ADI dose. JECFA in 2016 and European Commission SCF (1984) evaluated tartrazine. In 2008, the EFSA Scientific Board of Food Additives, flavourings, evaluated tartrazine, against claims that it cause hyperactivity in children (EFSA, 2008c). In 2009, the EFSA ANS Panel accepted a finding on the reevaluation of tartrazine (E-102) as food additives (EFSA ANS Panel, 2009).

However, the Brazilian Sanitary Surveillance Agency (ANVISA) issued a consultation on the opportunity of distributing a ticket warning against rise of urticaria, asthma and allergic rhinitis in atopic patient consuming food and drugs containing tartrazine. While, Pestana et al. (2010) reported that a group of atopic subjects with asthma, nasal allergy, pseudo-allergic or urticaria responses to non-steroidal anti-inflammatory (NSAID) drugs, 35 mg of tartrazine dye, did not produce any kind of significant respiratory, cutaneous or cardiovascular responses when compared with the placebo and there were no statistical changes among the groups.

Effect of tartrazine on albumin and hemoglobin binding

Tartrazine could result in conformational and some microenvironmental alteration of both human and bovine serum albumin, which might disturb the biological functions of serum albumins. This provides a significant understanding on the mechanism of tartrazine toxicity *in vivo* (Pan et al., 2011). The synchronous fluorescence investigation indicated that tartrazine binds with the hemoglobin central cavity, which was confirmed by a molecular demonstrating study (Li et al., 2014). Recently, the interaction of the food colorant tartrazine with hemoglobin was considered by Basu and Suresh Kumar

(2016a). They found that tartrazine slaked the intrinsic fluorescence of hemoglobin by inducing conformational alterations and substantial damage in the helicity of Hb.

Effect of food azo dyes on liver enzymes and hepatotoxicity

Previous studies were conducted until 2015. The findings in Table 2 are as follows: the activities of hepatic serum enzymes (AST and ALT) increased in rats administrated food colorants particularly at high doses, suggesting elevated permeability, injuries and impairment of the hepatic cells. Also, elevation in both ALT (located in the cytoplasm) and AST (located mainly in organelles such as mitochondria) activities indicated the injury of both the hepatic cellular and mitochondrial membranes in food azo dyes administered rats (Senthil et al., 2003).

Moreover, the enzymatic activities of ALT, AST and ALP showed significant increases with consumption of an extraordinary dose of tartrazine (500 mg/kg BW) for 30 days or a high dose of carmoisine (100 mg/kg BW) when compared with control rats (Table 2). While the low dose of both tartrazine (15 mg/kg BW) and carmoisine (8 mg/kg BW) displayed a significant rise in the ALT and alkaline phosphatase activities, respectively as compared to the control rats (Amin et al., 2010). In addition, Saxena and Sharma (2015) reported that consumption of food color including tartrazine induces hepatic tissue damages in Swiss Albino Rats. These effects assessed through significant increased serum total protein, albumin, ALP and hepatic MDA level and significant lowered levels of SOD, reduced GSH and CAT in the hepatic tissue. The alteration in the liver includes necrosis of hepatocytes, infiltration, vacuolation and drastic alteration in the antioxidant defense system.

The findings of Amin et al. (2010) is in agreement with that of Mekkawy et al. (1998) who specified that two low or high doses of artificial dyes contain both carmoisine and tartrazine (ponceau, carmoisine, erythrosine, sunset yellow, tartrazine, fast green, indigotine, brilliant blue and brilliant black) which revealed a significant elevation of serum ALT, AST and alkaline phosphates activities; they credited these changes to hepatocellular injury produced by the toxic properties of these artificial dyes that is associated with swelling, pyknosis, vacuolation and necrosis of the hepatic cells. The elevated activities of aminotransferases with the histopathological changes suggested that the tissue impairment of mainly liver, heart and kidney is associated with synthetic dyes.

An alternative mechanism of the significant rises in aminotransferases may be due to the biochemical and pathological state of the hepatic lobules and failure to perform vital functions, that trigger disturbance or imbalance in intermediary metabolism. Some enzymes such as ALT, AST, LDH and ALP leak out from the cells into the serum and so their serum activities determine the

Table 2. Effect of food colorants, tartrazine both low and high doses, on liver, renal function, lipid profile and oxidative stress.

Parameter	Biomarkers	Control Groups	Low and high tartrazine	References
Liver function	ALT, AST and ALP (U/l)	Normal	↑	Amin et al. (2010); Himri et al. (2011).
Renal function	Urea and Creatinine (mg/dl)	Normal	Significant ↑	Helal et al. (2000); Ashour and Abdelaziz (2009); Amin et al. (2010); Himri et al. (2011)
Lipid profile	TC and TG (mg/dl)	Normal	↑	Ashour and Abdelaziz (2009); Amin et al. (2010)
Oxidative stress/antioxidant markers	SOD (U/g)	Normal	↑	Amin et al. (2010)
	Catalase	Normal	↑	Mohamed et al. (2015)
	GSH(nmol/100 mg)	Normal	↑	Mohamed et al. (2015)
	Malondialdehyde (nmol/g/h)	Normal	Decrease	Demirkol et al. (2012); Mohamed et al., (2015)

↑ Indicates increased value of the biomarkers.

type and degree of destruction.

Histopathological examination of groups that ingested 10 mg/kg BW of tartrazine showed severe hepatic changes, swollen hepatocytes, a single large vacuole surmounting the whole cytoplasm and wide trabeculae from degenerated hepatic cells compressing and constricting the sinusoids lumen, besides, deposition of brown pigment inside the K pffer cells and hepatic fatty degeneration with Tartrazine treatment at dose of 7.5 and 10 mg/kg BW. Also, a significant rise in the mean liver weight and congested blood vessels and areas of hemorrhage in the liver were not confirmed (Himri et al., 2011).

Meyer et al. (2017) found that initial systemic administration of tartrazine in mice resulting in a periportal recruitment of inflammatory cells, raised serum alkaline phosphatase activity and mild periportal fibrosis. Moreover, Tartrazine alone induced the colon and hepatic NF-  B activities but there was no periportal recruitment of inflammatory cells or fibrosis. Tartrazine, its sulphonated metabolites and the contaminant inhibited sulphotransferase activities in murine hepatic S9 extracts. Systemic tartrazine exposure is potentially associated with an inhibition of bile acid sulphation and excretion and not oestrogen receptor-mediated transcriptional function.

Effect of tartrazine on kidney function

Everyday consumption for 30 days, of low or high doses of tartrazine revealed a significant rise in renal function tests of urea and creatinine level when compared with control group, and the high dose indicated higher significance in serum creatinine level (Amin et al., 2010). These results are parallel to those reported by Helal et al. (2000) on synthetic or natural food colorants. Additionally, these results are in accordance with that recorded by

Ashour and Abdelaziz (2009) on organic azo dye fast green for 35 days. Also, Tartrazine presented a significant elevation in serum creatinine level in a dose response manner (Himri et al., 2011).

Impairment of renal function is closely associated with higher levels of urea and creatinine (Varely, 1987). The renal injuries occur in all forms of renal diseases such as hydronephrosis congenital cystic, kidney renal tuberculosis, a condition in which there is calcium deposition (hypervitaminosis D). Increases in plasma creatinine in renal diseases provide a predictive importance than those of other nitrogenous substances. Concerning renal histopathological examination, Himri et al. (2011) showed tubular dilatation with thickened basement membrane, tubular degeneration and dilatation of the glomerular capillaries, and intercapillary sclerosis, atrophy of glomerulus in the group treated with 5, 7.5 and 10 mg/kg BW of Tartrazine, respectively.

For both liver and kidney phenomena represented by hepatic impairment, edema, congestion, and kidney apoptosis, with atrophy of renal corpuscles were observed. Degree and severity of histopathological aspects observed were directly proportional to the concentration of the administered dyes (Rus et al., 2009).

Effect of food azo dyes (tartrazine and carmoisin) on lipid profile

The study of Amin et al. (2010) and Ashour and Abdelaziz (2009) indicated the reduction in serum cholesterol and triglycerides levels (Table 2) when food color azo dye (fast green) was given orally to male albino rats for 35 days.

Approximately, 50% of the intestinal cholesterol pool is reabsorbed and recirculated via the enterohepatic flow, while the rest is eliminated in the feces. The abnormality

of serum cholesterol level is considered as indicator of hepatic diseases and so, the diminished cholesterol level may suggest liver injuries.

Recently, Elbanna et al. (2017) found that rats treated with food azo dyes (sunset yellow (E110) and carmoisine (E122)) produced highly significant changes in the hematological index. Also, liver function (ALT, AST, amylase and total bilirubin), renal function (BUN and creatinine), glucose and globulins were significantly elevated. Besides, noticeable histopathological changes several body organs, and these changes and inflammation were improved by treatment with lactic acid bacteria.

Role of food colorants, azo dyes on oxidative/antioxidant biomarkers

Hepatic GSH level and catalase activity decreased significantly in rat that ingested low and high carmoisine dose and a high dose of tartrazine (Amin et al., 2010). Also, hepatic super oxide dismutase (SOD) decreased significantly in high and low doses of tartrazine, while hepatic MDA as oxidative stress biomarker indicated significant increases with a high dose of tartrazine. Increased production of free radicals or ROS may induce autooxidation and lipid peroxidation of the hepatocytes, causing obvious hepatic injuries and subsequent release of hepatic function enzymes ALT and AST.

Tartrazine could be regarded as toxic due to its possible oxidative impairment induced by depletion of GSH, the main antioxidant for the cell, and a significant increase in MDA levels, where the researchers strongly believe that the usage of these possibly toxic colors in food needs to be re-evaluated (Demirkol et al., 2012). In a recent study, tartrazine, a widely used synthetic azo dye, induced a sharp deficiency in the biomarkers of antioxidant (SOD, catalase and GSH) and a marked rise in MDA concentration in the brain cortex in comparison with the other groups of male rat pups (Mohamed et al., 2015; Saxena and Sharma, 2015).

A possible effect of frequently consuming beverages on stimulation of the risk of pathophysiology associated with ROS and peroxy radical-facilitated events is suggested. Therefore, a healthy food consists of real food, without any artificial additives and high-quality food has no need for Tartrazine or any artificial color to maintain good health.

Effect of tartrazine on the nervous system, hyperactivity and behavior

The dose levels of 125 to 500 mg/kg of tartrazine given for 30 days induced a rare adverse effects on memory and learning in animals model, this is might be because of its promotion of lipid peroxidation metabolites and ROS, preventing endogenous enzymes of antioxidant

protection and the brain tissue injury (Gao et al., 2011). Taken together, because of the current evidence presented, the daily consumption of Tartrazine as agreed by the ADI rate seems to be reasonably harmless; however, exposure is unlikely to be reached after ingestion of food.

Tartrazine induced hyperactivity, antisocial behavior and anxiety in male Wistar rats at 0, 1 and 2.5% doses in drinking water as recorded for different animal models of raised plus-maze, open ground and the dark-light transition experiments (Kamel and El-Iethy, 2011). Moreover, Tanaka et al. (2008) found that 0.05, 0.15 and 0.45% tartrazine doses induced a few antagonistic effects on neurobehavioral markers all over generations in mice. The dose level of tartrazine induced altered neurobehavioral parameters during the lactation period in mice (Tanaka, 2006). In a clinical study, the effect of a mixture of sunset yellow, carmoisine and tartrazine on 3 to 9 years old children behavior was assessed, and it was found that synthetic colors in the diet result in exaggeration of the hyperactive behaviors (overactivities, inattentiveness, and impulsivity) in children at least up to middle infantile. Raised hyperactivity is accompanied by the development of problems in education, particularly those linked to reading, which could affect the kid's skill in school (McGee et al., 2002). These results show that adverse properties are not only seen in children with great hyperactivity but also seen in the overall population with a range of hyperactivity severities (McCann et al., 2007).

In a more recent experimental study, tartrazine was evaluated for potential neurotoxic effect, where it showed a significant decrease in gamma amino butyric acid, dopamine and serotonin levels as neurotransmitters in the brain and numerous apoptotic cells in the brain cortex were reported using an immunohistochemical staining with the anti-ssDNA antibody as apoptotic cell marker as compared to other groups (Mohamed et al., 2015).

Concerning the beneficial effect of Tartrazine, it had an important inhibitory role in fibrillogenesis and showed the potential anti-amyloidogenic property of food colorants (Basu and Kumar 2017).

Effect of tartrazine on DNA and as carcinogen

Various dye applications (0.25 to 64.0 mM) revealed that tartrazine had no cytotoxic properties. Nevertheless, at all examined levels, this dye had a significant genotoxic effect. While most of the injuries were responsive to repair, some damages persisted more than +ve control following 24 h of repair. These results show that tartrazine could be harmful to health and its prolonged consumption might generate carcinogenesis (Soares et al., 2015).

Investigations using spectroscopic titration for the interaction of food additives, tartrazine with DNA, showed that these dyes bind to DNA of calf thymus and different

isosbestic points clearly, indicating binding of DNA with the dyes. Tartrazine as food colorants had a possible toxic effect on human lymphocytes *in vitro* and it seems that they bind directly to DNA (Mpountoukas et al., 2010). In a novel study, the interaction of tartrazine and endogenous compound as bovine hemoglobin was defined for the dye (Li et al., 2014).

Sasaki et al. (2002) observed an extensive DNA damage in glandular stomach and the colon at doses higher than 10 mg/kg b.w. This effect may be due to the acute dye cytotoxicity or insufficient repair of DNA at the 3 h sampling time. Poul et al. (2009) verified the non-mutagenicity of tartrazine when given orally up to doses of 2000 mg/kg b.w. and reported that the dye does not increase the quantity of micronucleated colonic cells at any of the examined doses as compared to control groups. The spectroscopic and calorimetric study indicated that tartrazine induces hypochromism in DNA without any bathochromic effects. However, tartrazine improved the thermal stability of DNA by 7.53 K under saturation circumstances (Basu and Kumar, 2016b).

The literature on the cytotoxic, mutagenic and genotoxic effect of tartrazine are controversial and unsatisfactory in some cases. In this concern, the work of Soares et al. (2015) demonstrated that tartrazine has no cytotoxic effects and concluded that tartrazine may be unsafe to health and its extended usage could generate carcinogenesis. On the other hand, infrequent studies indicated that tartrazine, erythrosine and indigo carmine are strong inhibitors of skin tumor promotion in mice treated with TPA and DMBA (Kapadia et al., 1998). Khayyat et al. (2017) found that rats administered tartrazine exhibited an obvious increased hepatic and renal function, and also increased oxidative markers and decreased total antioxidants markers. Alternatively, giving tartrazine was linked to severe histopathological and cellular changes in hepatic and renal tissues, moreover, tartrazine initiates leukocyte DNA damage as identified by comet assay.

Recently, the study of Sekeroglu et al. (2017) indicated that both tartrazine and its metabolites have possible genotoxic effect on human lymphocyte cultures with and without a metabolic activator (S9 mix) while, tartrazine can induce cytotoxicity at the highest level in culture without S9 mix under the experimental situations. These evident indicated that tartrazine had an adverse effect on health.

Concerning long-term carcinogenicity of tartrazine, some studies are now available on the chronic effect of tartrazine. Himri et al. (2011) found that a 90 daily oral dosing of 5 to 10 mg/kg b.w in Wistar rats, revealed significant dose-related high blood biochemical markers of glucose, triglycerides, total cholesterol, blood urea nitrogen, creatinine, AST and total protein, as compared to the control.

When tartrazine was given at 0, 1 and 2% in drinking water to 50 female and male rats for 2 years,

carcinogenicity was not seen (Maekawa et al., 1987). Nontoxic injuries were reported at all the dye doses in the treated groups. The tumor detected, in the control and treated groups, was spontaneous in the strain of rats and the author determined that the tumors that were found in 1% of the treated group were not associated with the administration of the dye.

Collectively, these records revealed that Tartrazine might generate carcinogenesis at an extraordinary dose or accumulative exposure, however, this is improbable to occur.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY OF TARTRAZINE

Tartrazine did not play a significant teratogenic toxic role at a dose of 0, 60, 100, 400 and 600 mg/kg BW in pregnant Osborne-Mendel during the 1st 19 days of pregnancy (Collins et al., 1990, 1992). Meanwhile, Mehedi et al. (2009) reported that tartrazine has toxic effects on the reproductive organs comprising, decrease in reproductive performance, reduced sperm count and increased rate of sperm anomalies in mice with doses of 0, 0.1, 1.0 and 2.5% for 13 weeks.

There are particular clinical studies on assessment of the effects of various colorant mixtures, as they may be consumed in ordinary life (McCann et al., 2007). Regrettably, these works have many limitations; hence it is difficult to determine a clear conclusion on the matter (Amchova et al., 2015). The selection of a definite technique for assessing a food additive compounds can be based on expected mechanism of action and its chemistry. Moreover, there is a need for continuous estimation of novel chemicals and existing ones too, due to the contradicting data and inadequate results to conclusively classify several regularly used materials as safe or carcinogenicity.

Effect of tartrazine on tissues bioelement contents

In a few manuscripts on bioelement and tartrazine, there were reports on significant changes in levels of bioelements in rats' liver, kidney and brain tissues exposed to tartrazine (Cemek et al., 2014). The changes include increased Cu and iron level in renal tissue, which is important because copper accumulation in the tissues leads to Wilson's disease and hepatic cirrhosis (Shazia et al., 2012); this effect may be due to binding of copper and iron to the artificial food colorants, resulting in its tissues accumulation (Stevens et al., 2013).

The levels of the trace elements, aluminium and barium, reduced by consuming high and low doses of tartrazine in the brain. Moreover, low dose tartrazine induced reduced liver zinc content and high dose tartrazine has the same result in kidney, this may be due

unsaturated fatty acids peroxidation in cell membranes by ROS produced during tartrazine administration, resulting in a decrease of membrane flexibility and disturbance in cell function and integrity which affected the pumping and selection of activities of membranes and the level of bioelements may be altered in tissues (Cemek et al., 2014).

The safety effect of tartrazine as food additive

A number of subchronic and chronic feeding investigations on the role of tartrazine in mice and rats for periods of over one year without any given or significant opposing role, has been formally defined and evaluated (EFSA ANS Panel, 2009). Insignificant discoloration of fur, fecal and urinary output had been observed in doses from 10 g/kg of feed upwards (Borzelleca and Hallagan, 1988), which is greater than ADI of tartrazine. In the authors' opinion, the use of tartrazine in children's food and the presence of discoloration in body fluid demonstrated its incomplete metabolism. The authorities that confirmed its safety are now somewhat dated. On the other hand, several recent publications have been provided in this review. This is the first paper that covers most of the available literature including the relationship between tartrazine, oxidative stress biomarkers, hyperactivity, behavior, carcinogenicity, reproductive and developmental toxicity and some bioelement levels. Also, it provides some important recommendation on food additives that are vital to the health of the consumer. Various aspects of tartrazine and health, however, still demand supporting evidence.

CONCLUSION AND RECOMMENDATIONS

Existing literature and accumulated evidence indicate the various harmful effects of tartrazine on several organs and health systems. It can be firstly concluded that food additives, including the colorant tartrazine, adversely affect and modify the biochemical biomarkers in important organs such as the kidney and liver, even when used in low doses. The risk increases when a higher dosage is taken and when consumed daily for 30 days, given the hepatic oxidative stress caused by the formation of ROS. Children consume these additives several times daily in chocolates, gum, chips, drinks and many other products and are susceptible to the adverse effects of tartrazine.

Secondly, tartrazine can be converted by intestinal flora into aromatic amines that may be changed to nitrosamine. This releases ROS. It is therefore, essential to make consumers awareness of the side effects of these food azo dyes.

Thirdly, these food additives can affect body weight and the growth of children, as normal food consumption is reduced. Furthermore, the azo dye group including tartrazine, induce hypersensitivity and allergic reactions.

Consumption of tartrazine as a food additive should be limited; particularly in children.

Fourthly, continuous updating of the safety evaluations of the effect of tartrazine on health is recommended using modern methodological approaches and by making available all current results that include: data from studies on the nervous system, behavior, injuries to body organs, and results concerning issues of genotoxicity, reproductive toxicity and chronic carcinogenicity/toxicity.

Fifthly, many companies that produce products containing these food additives have never revealed the type or level of food additives added to their products. The public cannot determine the type of food additives or the dosage that they have consumed. Therefore, the food industry is obliged to mention the name and concentration of food additives found in their products, with reference to those foods mainly consumed by young children and, further, they should focus more when labelling products, to offer clear and detailed information; particularly to persons who are intolerant to such products.

Finally, all currently available evidence highlights the potentially harmful effects of tartrazine and how it is ineffective as a nutritive additive. It is recommended that its consumption should be avoided.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Development and improved selected markers to biosurfactant and bioemulsifier production by *Rhizopus* strains isolated from Caatinga soil

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This study screened four *Rhizopus* species as biosurfactant producers using different markers. First of all, *Rhizopus* spp. UCP 1607 was identified as *Rhizopus arrhizus* by morphological and molecular methods. The production of biosurfactant/bioemulsifier was investigated by submerged fermentation using soybean post-frying oil (5% v/v) and sodium glutamate (1% w/v) medium. The primary markers' hemolysis and parafilm M tests showed that *R. arrhizus* UCP1607 strain exhibited higher hemolytic activity (49 mm of clear zone) on sheep blood agar and a larger drop diameter (12 mm) on parafilm hydrophobic surface. The experimental results showed the most promising biosurfactant production by *R. arrhizus* UCP 1607 strain led to a reduction of surface tension (31.8 mN/m) and the diameter of the oil-spreading covered an area of 66.4 cm². The strains *Rhizopus microsporus* var. *chinensis* UCP1296, *R. microsporus* var. *microsporus* UCP1304, and *R. arrhizus* UCP1607 were capable of forming stable emulsions corresponding to 91.7, 94.8, and 82.6%, respectively in crude oil.

Key words: Tensio-active agent, bioemulsifier, screening of *Rhizopus* strains, submerged fermentation.

INTRODUCTION

Caatinga is a biome that comprises an extensive semi-arid area of 969.589,4 km², located in the Northeast of Brazil. A prominent feature of the semi-arid region of

Caatinga is the climate markedly characterized by severe environmental conditions where high temperatures with the minimum above 15°C and the maximum around

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40°C, intense insolation, scanty water resources, and the annual rainfall in the area is estimated to be lower than 1000 mm, leading to prolonged periods of serious drought. Moreover, the predominantly shallow soils present low natural fertility (Menezes et al., 2012; Silva et al., 2015). These environmental conditions of the semi-arid region of Caatinga biome have a direct influence on soil microbial life. Thus, microorganisms that survive in these stressful conditions do develop adaptive mechanisms of response, synthesizing appropriate metabolites (Gorlach-Lira and Coutinho, 2007; Silva et al., 2015).

Studies aiming to explore the biotechnological potential of genus *Rhizopus* have demonstrated that species of this genus are able to produce different types of compounds of an enormous industrial importance, namely enzymes (Freitas et al., 2014), organic acids (Abe et al., 2007), chitin, and chitosan (Berger et al., 2014) including biosurfactant (Freitas Silva et al., 2012).

Biosurfactants are products of the metabolism of living cells especially of bacteria, yeasts, and filamentous fungi that may be produced extracellularly or as part of cell membranes (Mulligan, 2005).

Structurally, biosurfactants are amphipathic molecules possessing hydrophobic and hydrophilic domains as basic information according to Desai and Banat (1997). Their complex structural organization gives them important physico-chemical properties such as lowering surface and interfacial tensions between immiscible phase systems, promoting the formation of micelles through which hydrophobic compounds can be solubilized in water or vice-versa (Fracchia et al., 2015). In addition, these compounds are known to be efficient dispersing and emulsifying agents, exhibit high foaming and wetting abilities, and display low critical micelle concentration (Mnif and Ghribi, 2015). These properties make biosurfactants molecules with a wide range of practical applications in the bioremediation of contaminated environments, enhanced oil recovery, as ingredients in the food processing industry, cosmetics and pharmaceutical industry (Makkar et al., 2011; Mnif and Ghribi, 2015).

The natural origin of these molecules turn them more interesting compounds, along with non/low toxicity, high biodegradability, effectiveness at extreme conditions (pH, temperature, and salinity), biocompatibility, and specificity in their function (Makkar et al., 2011).

Due to their advantages and numerous possible uses in different areas, microbial surfactants have been the central point of diverse studies aiming to identify potential microorganism producers of these molecules (Walter et al., 2010). However, the majority of screening studies have been carried out using bacteria (Kebbouche-Gana et al., 2009; Nwaguma et al., 2016; Joy et al., 2017; Batool et al., 2017), and outgrows by far those evaluating the fungi producing potential (Sari et al., 2014; Lodha et al., 2016). Till date however, the need for discovery of

biosurfactant producing microorganisms capable of inhabiting environments featured by adverse typical conditions such as extreme salinity, higher temperatures, and scanty humidity is still enormous (Techoei et al., 2007; Kebbouche-Gana et al., 2009; Kiran et al., 2010). Hence, the objective of the current study was to evaluate the potential of *Rhizopus* strains in the production of biosurfactants using different screening methods (Kiran et al., 2010; Sari et al., 2014).

MATERIALS AND METHODS

Micro-organisms

Rhizopus strains from the Caatinga soil used were: *Rhizopus arrhizus* var. *arrhizus* UCP 1295 and *Rhizopus microsporus* var. *chinensis* UCP 1296 and var. *microsporus*, UCP 1304 and were kindly provided by the culture collection, UCP (Catholic University of Pernambuco), Recife-PE, Brazil which is registered in the World Federation for Culture Collections (WFCC).

Fungus isolation

The new *Rhizopus* species strains were isolated from Caatinga soil sample collected in Natal, Rio Grande do Norte state, Northeast of Brazil and were used in the following media (g/L): wheat germ agar medium (wheat germ 15, glucose 5, and agar 15, supplemented with chloramphenicol 0.1), malt extract agar (MEA; malt extract 20 and agar 20), Sabouraud dextrose agar (SDA; peptone 10, glucose 40, and agar 20). The isolation of the fungus was carried out by soil sprinkling technique according to Benny (2008). Briefly, 5 mg of soil sample was weighed using a precision balance and then were spread onto wheat germ agar medium plates and incubated at 28°C until sporulation. Afterwards, using a sterile syringe mature sporangiospores, they were transferred directly from the colonies to MEA plates and then incubated at 28°C for 7 days. Pure culture of the isolate was maintained on SDA slants and stored at 4°C in a refrigerator. Transfers were done to fresh SDA slants, each three months to maintain the isolate viable.

Morphological and molecular identification

The macroscopic and microscopic identification was conducted according to Zheng et al. (2007). The macroscopic morphology (colony size, aspect and color) was attained by naked eye examination of 5 to 7 days old culture grown on potato dextrose agar (PDA) medium (g/L peeled potato 200, dextrose 20, and agar 15). The microscopic morphology was observed by light microscopy using Lactophenol Cotton Blue staining and distilled water.

The genomic DNA was extracted from mycelium grown on PDA for 72 h, at 28°C by the cetyltrimethylammonium bromide (CTAB) DNA Extraction Protocol method, adapted from Doyle and Doyle (1991). The ribosomal DNA ITS1-5.8S-ITS2 and LSU regions were amplified by polymerase chain reaction (PCR) on a Peltier PTC-100® thermocycler (MJ Research, Inc., USA) in a total volume of 25 µL of sample. The rDNA ITS regions were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (3'-GCTGCGTTCTTCATGAGC-5') (White et al., 1990). The D1/D2 LSU region of the rDNA was sequenced using primers NL1 (5' GCATATCAATAAGCGGAGGA-3') and NL4 (5'-GGTCCGTGTTTCAAGACGGGTCG-3') (O'Donnell, 1992). The amplicons were purified with PureLink-PCR Purification Kit C/50rxn

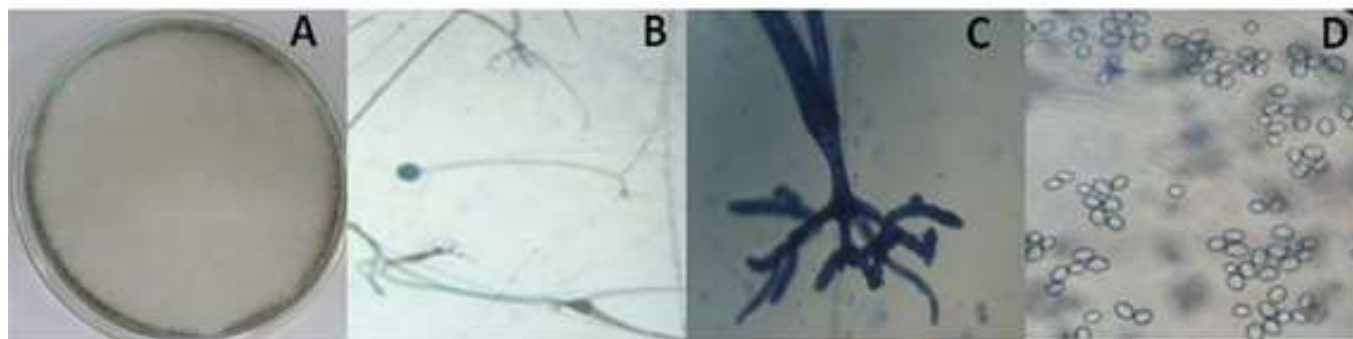


Figure 1. Macroscopic characteristics of *Rhizopus* spp. UCP 1607 grown on potato dextrose agar (A), under light microscope straight and opposite sporangiophores arising from rhizoids were observed (B). Rhizoids finger-like branched (C). Sporangiospores (D)

Columns, Invitrogen and sequenced by ACT Gene Molecular Analyses, Alvorada-RS.

Screening for biosurfactants production

Primary screening: Hemolysis and parafilm M tests

Preliminary identification of the potentially biosurfactant-producing *Rhizopus* strains was performed by the hemolytic activity test (Satpute et al., 2010). Spores of *Rhizopus* strains were inoculated on the central part of the agar plate containing 5% (v/v) of defibrinated sheep blood and incubated at 28°C for four days. The experiments were monitored for observation of hemolytic activity which was detected by appearance of clear zone on blood agar plate.

Parafilm M assay is a rapid and simple test that does not require specialized equipment and can be carried out with small sample volumes. The test consisted of placing 25 µL of mycelia-free metabolic liquid on hydrophobic surface of the parafilm M strip. The shape of the drops was examined after 1 min and its diameters were measured using a caliper. The presence of the surface active compounds in the mycelia-free metabolic liquid was detected by the flat shape of the drop, while the semispherical shape indicates the absence of biosurfactant/bioemulsifier. The medium without microorganism was used as control (Sari et al., 2014).

Biosurfactant/Bioemulsifier production

The strains *R. arrhizus* var. *arrhizus* UCP 1295, *R. microsporus* var. *chinensis* UCP 1296, *R. microsporus* var. *microsporus* UCP 1304, and *Rhizopus* spp. UCP 1607 were grown on PDA for 96 h at 28°C. Spore suspensions were prepared in the sterile water and adjusted to 10⁷ spores/mL, and 5% of suspensions were inoculated in Erlenmeyer flasks containing 100 mL of the medium constituted by soybean post-frying oil (5% v/v), sodium glutamate (1% w/v), and salt solution (g/L): (NH₄NO₃ 1.0, KH₂PO₄ 0.2, and MgSO₄·7H₂O 0.2), and the pH was adjusted to 5.5. The flasks were incubated in orbital shaker at 150 rpm, at 28°C during 96 h. The net metabolic liquid containing biosurfactant was obtained by filtration followed by centrifugation (10.000 ×g for 15 min), and was used for secondary screening.

Secondary screening

Surface tension determination

The measurement of surface tension in the mycelia-free broth was performed using an automatic Tensiometer (model Sigma 70 KSV

Ltd, Finland) utilizing the Du Nouy ring method as described by Kuyukina et al. (2001). The results were reported as the average of measurements in triplicate.

Oil spreading assay

In order to determine the biosurfactant dispersing ability, oil spreading test was applied (Andrade Silva et al., 2014). Distilled water (40 mL) was inserted in a Petri dish (15 cm of diameter), and this was followed by addition of 1.0 mL of burnt motor oil onto water layer surface. After that, 0.5 mL of metabolic liquid (A), 0.5 mL of commercial detergent (B), 0.5 mL of chemical surfactant SDS (C), and 0.5 mL of distilled water (D) were placed in the center of the oil film. The presence of the biosurfactant/bioemulsifier in the mycelia-free broths was detected by the spreading of oil resulting in the formation of oil displacement areas. The clear zone diameters were measured and the respective oil displacement areas (ODA) were determined and expressed in cm² using the equation that follows. The experiments were performed in triplicate.

$$\text{ODA} = 3.14 \times r^2$$

Emulsification index (E₂₄)

The emulsifier properties of the biosurfactant in crude extracts produced by *Rhizopus* strains were evaluated by emulsification index assay. For determination of emulsification index, 1.0 mL of mycelia-free metabolic liquid containing biosurfactant and 1.0 mL of burnt motor oil were mixed together in a test tube, and then homogenized vigorously for 2 min at room temperature (25°C). After 24 h, measurements were performed through the equation:

$$E_{24} (\%) = \frac{He}{Ht} \times 100$$

where He = emulsion height; Ht = mixture total height (Liu et al., 2013).

RESULTS AND DISCUSSION

Isolation, phenotypic and molecular identification

The isolation of *Rhizopus* spp. UCP 1607 from the Caatinga semi-arid region soil sample was accomplished on basis of colony morphology (Figure 1). The isolate

Table 1. Evaluation of tensio-active produced by *Rhizopus* strains using parafilm M test and surface tension determination.

Strain	Parafilm M test (mm)	Surface tension (mN/m)
<i>Rhizopus arrhizus</i> var. <i>arrhizus</i> UCP 1295	8	35.0
<i>Rhizopus microsporus</i> var. <i>chinensis</i> UCP 1296	10	33.3
<i>Rhizopus microsporus</i> var. <i>microsporus</i> UCP 1304	9	34.7
<i>Rhizopus</i> spp. UCP 1607	12	31.8
Fresh medium (control)	6	-

showed rapid growth on PDA plates at a temperature of 28°C, covering the entire Petri plate of 9 cm in diameters with abundant mycelia, and the colonies were cottony, initially white and later turned gray blackish (A). Under light microscope straight and opposite sporangiophores arising from rhizoids were observed (B). Rhizoids finger-like branched (C). Straight to substraight, 2 to 3 in groups and opposite sporangiophores arising from rhizoids, 285 to 840 to 1000 µm long, 8 to 17 µm diameters. Sporangia black, globose to slightly depressed globose, 72 to 168 µm diameter. Columellae subglobose, 50 to 114 × 78 to 149 µm, light grayish-brown. Sporangiospores ovoid, the mostly regular in shape and size, 5 to 7 × 4 to 6.5 µm, light gray the solitary (D). The fungus was identified as belonging to *R. arrhizus* (Zheng et al., 2007).

Species of *Rhizopus* are worldwide distributed, inhabiting different environments (Ribes et al., 2000), so they may be isolated from soil, dung, decaying organic material and mature fruits (Santiago et al., 2013), and a variety of food products (Abdel-Hafez, 1984). Some species of this genus live as pathogens causing diseases in humans, animals and plants (Santiago et al., 2013).

There are various studies on isolation and assessment of diversity of tropical areas filamentous fungi including Brazil (Siqueira and Brussaard, 2006; Cavalcanti et al., 2006). However, only few reports referred to isolates from semi-arid environments (El-Said and Saleem, 2008; Grishkan and Nevo, 2010). In this context, little is known about filamentous soil mycota of the Caatinga biome.

Considering this fact, Cavalcanti et al. (2006) studied the diversity of soil filamentous fungi in Xingó, state of Bahia, a region with typical Caatinga ecosystem. Among Zygomycota, two *Rhizopus* spp. were isolated and identified as *R. microsporus* var. *chinensis* and *R. microsporus* var. *microsporus*. Santiago et al. (2013) worked from soil samples of three different semi-arid areas of the state of Pernambuco to evaluate the distribution of Mucorales order. These authors reported the *R. microsporus* var. *microsporus* (10.19%) and *R. arrhizus* var. *arrhizus* (7.41%) as one of the most frequent genus in the three areas. In this study, *Rhizopus stolonifer* and *R. microsporus* var. *chinensis* were isolated as well. Oliveira et al. (2013) assessed the diversity of filamentous fungi from soil in the same state and identified same species of *Rhizopus* (*R. microsporus* var. *microsporus* and *R. arrhizus*).

It was observed that molecular identification of the isolate, using the nucleotide sequence found was compared to those deposited in National Center for Biotechnology Information (NCBI) website using the BLAST program. The results identified homology of 95% of similarity to *R. arrhizus*. In the current study, the phenotypic characteristics of the *R. arrhizus* matched with the molecular analysis for the definitive identification of the fungus. Different rDNA regions have been frequently used for the identification of Mucorales. Moreover, the 18S regions of the small ribosomal subunit, the region of the internal transcribed spacer (ITS), and the large ribosomal subunit (LSU) region are the most commonly used as markers (Kasai et al., 2008; Bellemain et al., 2010; Yang et al., 2016; Ziaee et al., 2016).

Detection of biosurfactant-producing *Rhizopus* strains

According to Thavasi et al. (2011), microorganisms with positive hemolytic activity for production of biosurfactants show a clear zone in the blood agar plates. In this context, only the strain *Rhizopus* spp. UCP 1607 produced extracellular compound during the radial growth on the blood agar and formed a higher clear zone diameter (40 mm), after 72 h of incubation. The hemolytic activity demonstrated by the strain was superior to that presented by *Aspergillus* species MSF1 that caused the appearance of a clear zone with a diameter of 7 mm in blood agar medium as described by Kiran et al. (2010). The hemolytic activity was employed by several authors as initial criterion for selection of biosurfactant producers (Batool et al., 2017; Nwaguma et al., 2016). However, Satpute et al. (2010), recommend the use of more than one screening method for detection of biosurfactant-producing microorganisms.

Parafilm test and surface tension determination are both physical methods widely applied for identification of biosurfactant-producing microorganisms (Sari et al., 2014; Korayem et al., 2015). The results from parafilm M assay showed that *Rhizopus* strains tested in this work were able to produce biosurfactants with different surface-active properties, since the droplet diameter of the metabolic liquid of each strain was larger, compared with fresh culture medium (Table 1). However, the best



Figure 2. Tensioactivity of crude biosurfactants of the *Rhizopus* strains on parafilm M hydrophobic surface.

Table 2. Oil displacement area by biosurfactants/bioemulsifiers produced by different *Rhizopus* strains compared with chemical surfactant and commercial detergent.

Strain	Oil displacement area, ODA (cm ²)
<i>Rhizopus arrhizus</i> var. <i>arrhizus</i> UCP 1295	22.0
<i>R. microsporus</i> var. <i>chinensis</i> UCP 1296	56.7
<i>R. microsporus</i> var. <i>microsporus</i> UCP 1304	39.6
<i>Rhizopus</i> sp. UCP 1607	66.4
Commercial detergent	44.2
Chemical surfactant (SDS)	75.4
Distilled water	Negative

results were exhibited by the new strain of *R. arrhizus* UCP 1607 forming largest drop (12 mm diameter) on the hydrophobic surface (parafilm M strip) (Figure 2).

The surface tension of the cell free broth from *R. arrhizus* UCP1607 reached the lowest value (31.8 mN/m). Correlations between the drop diameters and the reduction of surface tension and the drop spreading value from *Rhizopus* strains were observed (Table 1).

Sari et al. (2014) evaluated the capability of isolates of *Pseudozyma* strains for biosurfactants production, and found the surface tensions varying from 35.8 to 44.3 mN/m. Therefore, it was concluded that the biosurfactants produced by all *Rhizopus* strains were their primary metabolite, due to the production of growth-associated biosurfactant.

According to Sharma et al. (2016), a microorganism is considered a good surface-active compounds producer if its net metabolic liquid is able to reduce the surface tension of water from 72 to 35 mN/m or below this value. Similar criterion for biosurfactants-producing microorganism detection was applied by Ariech and Guchi (2015), considering surface-active potential biomolecule reduced the surface tension below 40 mN/m.

Dispersing capacity and emulsification activity of the *Rhizopus* strains

The oil displacement assay requires no specialized equipment, and also the method is rapid and simple

which can be undertaken with small volumes of sample (Walter et al., 2010). Table 2 shows the results for dispersing ability of the crude biosurfactant extracts produced by *Rhizopus* strains. Thus, significant oil displacement activities were demonstrated by biosurfactants produced by *R. microsporus* var. *microsporus* UCP1304 and *R. microsporus* var. *chinensis* UCP1296 corresponding to 39.6 and 56.7 cm² of oil displacement areas, respectively. However, the biosurfactant produced by *R. arrhizus* UCP1607 exhibited excellent potential in the dispersion of burnt motor oil on water surface that resulted in 66.4 cm² of oil displacement area (ODA). The results showed that the oil displacement area of the biosurfactant produced by *R. arrhizus* UCP1607 (Figure 3A) was superior to dispersion induced by commercial detergent (44.2 cm²) (Figure 3B). Synthetic surfactant dodecyl sulphate (SDS) showed the best dispersion (75.4 cm²) (Figure 3C) as positive control, as well as the negative control in which the burnt motor oil with distilled water was used (Figure 3D).

The data obtained in this study using *Rhizopus* strains were superior to dispersion capacity of the biosurfactant produced by *Pleurotus ostreatus* (12.56 cm²) of oil displacement area (Velioglu and Öztürk Ürek, 2015), and the bioemulsifier production by *Cunninghamella echinulata* (37.36 cm²) (Andrade Silva et al., 2014).

According to Walter et al. (2010), the emulsification index is a reliable method for detection of bioemulsifier producers. *Rhizopus* strains showed emulsifying capacity of biosurfactants produced (Figure 4).

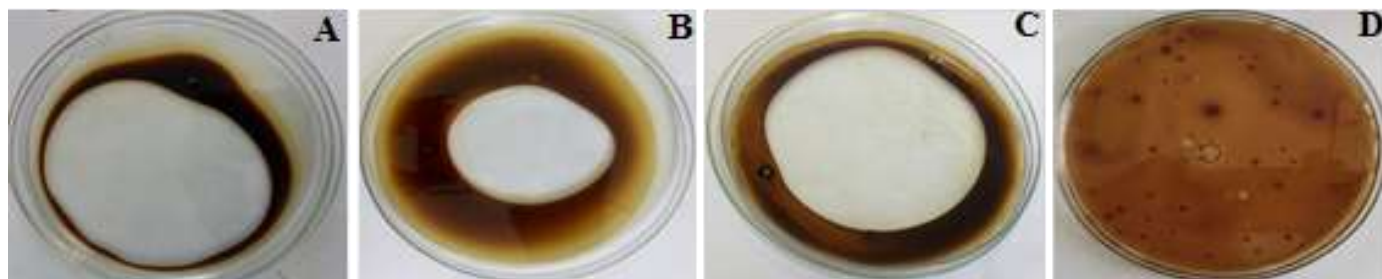


Figure 3. Oil displacement area (ODA) for: (A) Biosurfactant produced by *Rhizopus arrhizus* UCP1607, (B) Commercial detergent, (C) Chemical surfactant (SDS), and (D) Distilled water (negative control).

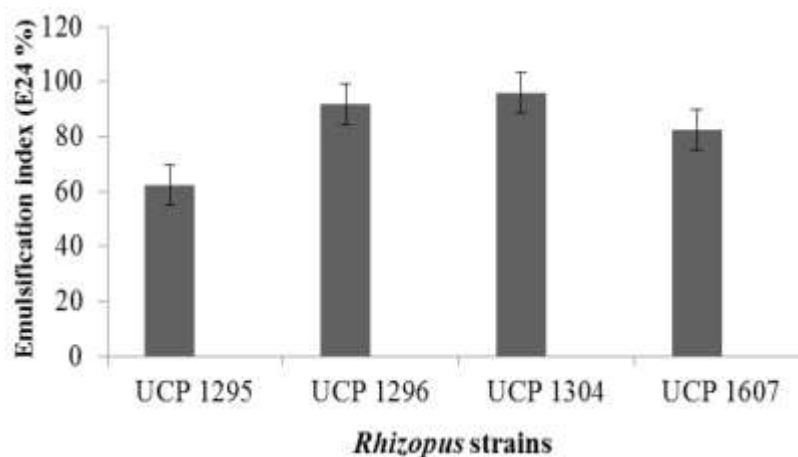


Figure 4. Emulsification index (E₂₄) of burnt motor oil with biosurfactants produced by *Rhizopus* strains.

The significant positive values were considered to be above 50% after 24 hours of emulsion according to Kebbouche-Gana et al. (2009); thus, the best emulsification property against burnt motor oil were observed by *R. microsporus* var. *microsporus* UCP 1304 (94.8%), followed by *R. microsporus* var. *chinensis* UCP 1296 (91.7%) and *R. arrhizus* UCP 1607 (82.6%) of emulsification.

All results obtained in this study were higher than the biosurfactants produced by *Aspergillus niger* CF12 (18.5%) and *Rhizopus nigricans* CF3 (21.66%), as described by Lodha et al. (2016).

In addition, the current study suggests that the importance of the screening methods mainly based on primary and secondary assays led to isolation of biosurfactant and bioemulsifying agents using mycelia-free broths, in particular from filamentous fungi. Those assays considered the important properties of higher dispersion oil activity, lower surface tension of the tension active agent, and bioemulsification. According to Uzoigwe et al. (2015), emulsification index test is a suitable screening method for detection of bioemulsifier producing microorganisms.

Most of the surfactants compounds are chemically synthesized. However, the main drawback for the biosurfactants commercialization is associated with non-economical production and is not yet competitive with the synthetics products. The renewable substrates used, especially from industrial wastes as soybean post-frying oil, supplemented with sodium glutamate showed excellent results to biosurfactant and bioemulsifier production at an experimental scale. The agro-industrial waste soybean post-frying oil is considered as the promising substrate for reduction of the cost of production to tenso-active and emulsifier molecules (Andrade Silva et al., 2014; Freitas et al., 2014).

Conclusion

In this work, four biosurfactant/bioemulsifier-producing strains were isolated from Caatinga soil of Brazil, namely *R. arrhizus* var. *arrhizus* UCP 1295, *R. microsporus* var. *chinensis* UCP 1296, *R. microsporus* var. *microsporus* UCP 1304 and *Rhizopus* spp. UCP1607, collectively identified as *R. arrhizus*. The experimental result showed

that among the four strains, the best biosurfactant activity was achieved in *R. arrhizus* UCP 1607 strain. The biosurfactant produced by *R. arrhizus* UCP 1607 strain had a large hemolytic activity, parafilm drop, oil-spreading diameter, and low surface tension, while the best emulsifying activity was observed using *R. microsporus* var. *microsporus* UCP1304. The screening methods mainly based on surface tension determination have led in many cases to the elimination of bioemulsifying agents. The promising physico-chemical results showed that evaluation of emulsifying activities from mycelia-free broths demonstrated great possibility for production of bioemulsifiers with powerful potential to induce stable emulsion. The better surface active properties with its great effectiveness confirmed the lowering of surface tension and oil dispersion in water by the new strain of *R. arrhizus* UCP 1607. Further studies are under way to scale up growth conditions and to optimize biosurfactant and bioemulsifier productions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

A comparative study on bacterial conversion of selected agricultural biomass to ethanol

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The aim of the study was to investigate the bioethanol production potentials by indigenous bacterial isolates screened for amylolytic and cellulolytic activities. Cassava peels were obtained from local 'garri' producers, while corn grains and corn cobs were gotten from retailers within the Erinfun farm settlement along Afe Babalola University Road, Ado-Ekiti, Ekiti State, Nigeria on June 4, 2015. The substrates (corn flour, corn cob and cassava peels) were sundried for 5 days and ground into fine powder using a hammer mill and analyzed for proximate nutrient composition and reconstituted to a 5% concentration in water before fermentation. The fermentation was set up for 20 days using single cultures of bacteria and co-cultures of bacteria and yeast selectively isolated and screened for amylolytic and cellulolytic activities. Parameters such as pH, total titratable acidity, reducing sugar and optical density were taken at intervals of 5 days and ethanol production was analyzed at the end of fermentation. Three bacterial isolates (*Bacillus macerans*, *Bacillus subtilis* and *Micrococcus varians*) yielded ethanol in all the feed stocks. The highest percentage ethanol yield was observed in the fermentation of corn flour and corn cobs with *B. macerans* which were 3.6 ± 0.009 and 3.5 ± 0.009 , respectively. There was reduction in the pH and total titratable acidity and increase in the reducing sugar content and optical density. *B. macerans* exhibited the highest average ethanol production ($3.5 \pm 0.07\%$). A proximate analysis of the feed stocks showed presence of nitrogen, protein, fat, carbohydrate and ash. This study investigated the potentials of wastes (cassava peels and corn cob) used as feed stocks for bio-ethanol production, in substantially replacing major food crops (corn) as a case study.

Key words: Fermentation, amylolytic activity, cellulolytic activity, corn flour, corn cob, cassava peels, feed stocks.

INTRODUCTION

Bioethanol as an alternative source of energy has received special attention worldwide due to the depletion of fossil fuels (Sanat et al., 2014). The world production

of bioethanol increased from 50 million m³ in 2007 to over 100 million m³ in 2012. The United States and Brazil are known to produce approximately 80% of the total world

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supply derived from corn and sugarcane. In developing economies, food related feedstocks are preferably substituted with non-food raw materials. This is being achieved with farm products that are considered to be less edible (Qian et al., 2014). Interest in the production of biofuel from agricultural wastes is driven by several reasons such as global search for alternative source of energy and transporting fuel to replace the depleting fossil fuel. It is also as a result of several benefits derivable from the use of ethanol such as its use as solvents, germicides, antifreeze and intermediates for other organic chemicals (Kingsley, 2012).

Cassava production in Africa accounts for over 54% of the total world production, with Nigeria taking the global lead with a production of about 54.8 million metric tons in 2014. Most of the cassava harvested in Nigeria is processed into food to obtain 'garri', 'fufu' and 'lafun'. There is little processing of cassava into products such as ethanol, chips, syrups and starch (Sahel, 2016). The value of corn cobs as a feedstock for ethanol production could be attributed to the large amount of carbohydrates, specifically starch, present in corn and can be rather easily processed to break it down into simple sugars, which can then be used to biologically produce ethanol (Nathan, 2006). There is an emerging trend in the utilization of biomass conversion technologies on rice husk and sugarcane bagasse, and gasification of other agricultural residues. Biomass is still largely underutilized and left to decompose in the fields; this is a common phenomenon in developing countries that do not have strong regulatory instruments to control such polluting practice (UNEP, 2007). Ethanol producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of the yeasts presently used for the practical production of fuel alcohol and with the recent advances in biotechnology (Zuber and Anjani, 2013). The inability of yeast fermentation to utilize xyloses also hampers the yield of ethanol from lignocellulosic biomass (Qian et al., 2014). As a result, this study aimed to investigate bioethanol production potentials by indigenous bacterial isolates screened for amyolytic and cellulolytic activities.

MATERIALS AND METHODS

Sample collection and preparation

Cassava peels were obtained from local garri producers, while corn grains and corn cobs were gotten from retailers within the Erinfun farm settlement along Afe Babalola University Road, Ado-Ekiti, Ekiti State, Nigeria on June 4, 2015. They were sundried for 5 days and ground into fine powder using a hammer mill. The ground samples were then collected in clean polyethylene bags and stored in the laboratory at room temperature until use. The waste water from the fermentation of cassava was also obtained in sterile container and transported to the laboratory for storage at refrigerating conditions.

Isolation of bacteria

The bacteria were isolated from soil contaminated with the effluent

discharged during cassava fermentation for 'garri' production. The soil was obtained using a hand trowel to collect some surface and sub-surface soil materials. Bacterial isolates were also obtained from the other samples (cassava peels, corn grains and corn cobs).

Identification of bacterial isolates

The bacterial isolates were identified according to the biochemical tests described by Bergey and John (2000) and Barrow and Feltham (1993).

Screening of isolates for starch hydrolysis

Ten grams each of the powdered samples (cassava peel flour, corn flour and corn cob) were weighed into separate 1000 ml conical flasks containing 500 ml of water and boiled at 100°C for 30 min. The preparation was allowed to cool at room temperature and the starchy liquid sieved with a muslin cloth. Two percent of the starch extract from the samples were used to enrich the nutrient agar and malt extract agar for the bacteria and yeast respectively. The preparation was sterilized at 121°C for 15 min and dispensed into 15 Petri dishes for 15 bacterial isolates and the sterilized starch enriched malt extract agar dispensed into 5 Petri dishes for the 5 yeast isolates. The media were allowed to solidify and the isolates streaked on the solidified media. The plates were incubated at 37°C for 24 h. Drops of Lugol's iodine were added to the bacterial growth observed on the plates after 24 h of incubation. Clear zones around the cultures indicated the ability of the isolate to hydrolyze starch while the absence of clear zones indicated the inability of the isolate to hydrolyze starch (Barrow and Feltham, 1993).

Inoculum preparation

Sterile 10 ml nutrient broth in test tubes was inoculated with 24 h cultures of bacterial isolates.

Preparation of sample for fermentation

Twenty-five grams each of the samples (cassava peel flour, corn flour and corn cob) were weighed in a 1000 ml conical flask each. Five hundred milligrams of distilled water was added and the preparation were sterilized at 121°C for 15 min. The samples were allowed to cool and dispensed into 1 L sterile flasks. 10 ml of the inoculum was introduced into the prepared samples for a 20-day fermentation trial.

Analysis of fermentation medium

Physiochemical parameters such as pH, total titratable acidity, optical density, determination of reducing sugar and analysis of ethanol concentration were carried out with the fermenting media according to AOAC (2000).

Enzyme assays

Cellulase activity was determined by the method of Camassola and Dillon (2012) and the amylase activity by the method of Naguib (1964).

Proximate analysis

The proximate analysis of the samples such as moisture content,

Table 1. Biochemical characteristics and identification of bacterial isolates.

Code/Isolate	Spore staining	Starch hydrolysis	Catalase	Voges/Proskauer test	Methyl Red test	Swollen test	6.5% NaCl	Acid from Arabinose	Gas from Glucose	Acid fast test	Citrate test	Probable organism
CFWI	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	ND	ND	<i>Bacillus macquariensis</i>
CFW2	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	ND	ND	<i>Bacillus subtilis</i>
CFW3	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	ND	ND	<i>Bacillus circulans</i>
CF1	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	ND	ND	<i>Bacillus macerans</i>
CP1	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	ND	ND	<i>Bacillus macquariensis</i>
CP2	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	ND	ND	<i>Bacillus circulans</i>
CC1	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	ND	ND	<i>Micrococcus varians</i>
CC2	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	ND	ND	<i>Bacillus circulans</i>
FCS.A	-ve	-ve	+ve	ND	ND	ND	ND	ND	ND	-ve	ND	<i>Corynebacterium xerosis</i>
FCS.B	-ve	-ve	+ve	ND	ND	ND	ND	ND	ND	ND	ND	<i>Corynebacterium xerosis</i>
FCS.C	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	<i>Bacillus azotoformans</i>
FCS.D	-ve	-ve	+ve	ND	ND	-ve	ND	ND	ND	-ve	+ve	<i>Bacillus insolitus</i>
FCS.E	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	<i>Bacillus azotoformans</i>
FCS.F	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	<i>Bacillus azotoformans</i>
FCS.G	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	<i>Bacillus azotoformans</i>

ND, Not determined; +ve, positive; -ve, negative; CFW, cassava fermentation water; CC, corn cob; CP, cassava peels; CF, corn flour; FCS, soil containing fermented cassava effluent.

crude protein, crude fibre, crude fat, ash content and the determination of the carbohydrate content were carried out as described by AOAC (2000).

Analysis of data

The results are presented as the mean standard values of three replicates. A one-way analysis of variance (ANOVA) was carried out using SPSS 16.0. Significant difference was at $P \leq 0.05$.

RESULTS

Morphological and biochemical characteristics of the bacterial isolates

The bacterial isolates were mostly Gram positive rods. The biochemical tests carried out on the

bacterial isolates showed the following organisms: *Bacillus subtilis*, *Bacillus macerans*, *Bacillus macquariensis*, *Micrococcus varians*, *Corynebacterium xerosis*, *Bacillus azotoformans* and *Bacillus insolitus*. Three isolates (*B. macquariensis*, *B. subtilis* and *B. circulans*) were isolated from effluent obtained from cassava fermentation, two isolates (*M. varians* and *B. circulans*) were got from corn cob, *B. macquariensis* from cassava peels while three other isolates (*C. xerosis*, *B. azotoformans* and *B. insolitus*) were obtained from the soil sample (Table 1).

Sample starch hydrolysis test

Six of the bacterial isolates (*B. macquariensis*, *B.*

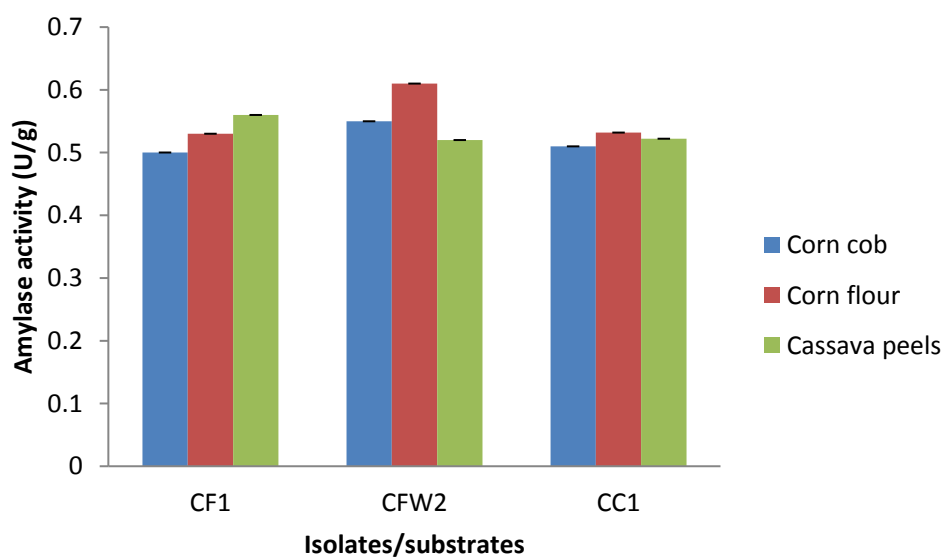
subtilis, *B. circulans*, *M. varians*, *B. macerans* and *B. azotoformans*) from the soil samples were observed to be capable of starch hydrolysis (Table 2).

Amylase and cellulase activity

The amylase activity of the bacterial isolates was examined in all samples on the 20th day of fermentation. The highest activity of 0.61 U/mg was observed in corn flour by *B. subtilis*, while the lowest (0.50 U/mg) was observed in corn cob (C.C) by *B. macerans*. The cellulase activity of the bacterial isolates was examined in all samples on the 20th day of fermentation. The highest activity was observed in corn flour by *B. macerans* (CF1) in the cassava peels (C.P) to be 0.0470 FPU

Table 2. Result of starch hydrolysis test.

Isolates/code	Reaction
<i>Bacillus macquariensis</i>	+ve
<i>Bacillus subtilis</i>	+ve
<i>Bacillus circulans</i>	+ve
<i>Micrococcus varians</i>	+ve
<i>Bacillus circulans</i>	+ve
<i>Bacillus macerans</i>	+ve
<i>Bacillus macquariensis</i>	+ve
<i>Bacillus circulans</i>	+ve
<i>Corynebacterium xerosis</i>	-ve
<i>Corynebacterium xerosis</i>	-ve
<i>Bacillus azotoformans</i>	-ve
<i>Bacillus insolitus</i>	-ve
<i>Bacillus azotoformans</i>	-ve
<i>Bacillus azotoformans</i>	-ve
<i>Bacillus azotoformans</i>	-ve

**Figure 1.** Amylase activity of the bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis*.

while the lowest was observed in corn flour (C.F) by the *M. varians* to be 0.0301 FPU (Figures 1 and 2).

Ethanol yield of the different isolates using corn cob, corn flour and cassava peels

Most of the isolates and substrates used for fermentation gave ethanol yield of between 3.0 and 3.6%. The lowest ethanol yield was recorded for cassava peels ($2.0 \pm 0.07\%$) by *B. subtilis* while the highest percentage ethanol yield was observed in corn flour ($3.6 \pm 0.07\%$) by

B. macerans (Figure 3). At $P \leq 0.05$, there was statistical significant difference in the percentage ethanol yield of the samples after 20 days of fermentation.

pH of samples inoculated with bacterial isolates

There was a decrease in the pH of the samples inoculated with the bacterial isolates as the fermentation period increased. *B. macerans* with corn cob had an initial pH on day 0 of 6.80 and decreased to 4.10 on day 20. The lowest pH on day 20 was recorded for *B.*

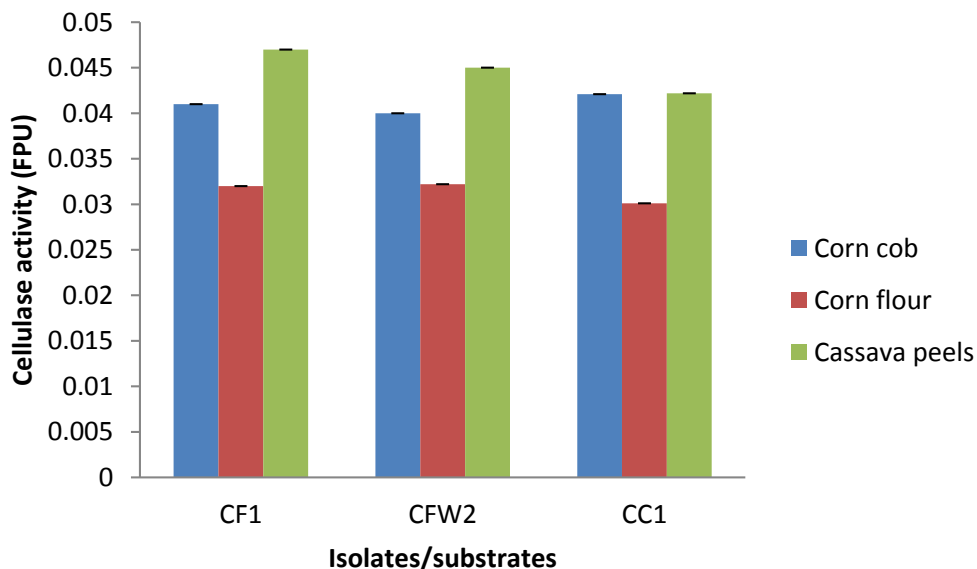


Figure 2. Cellulase activity of the bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis*.

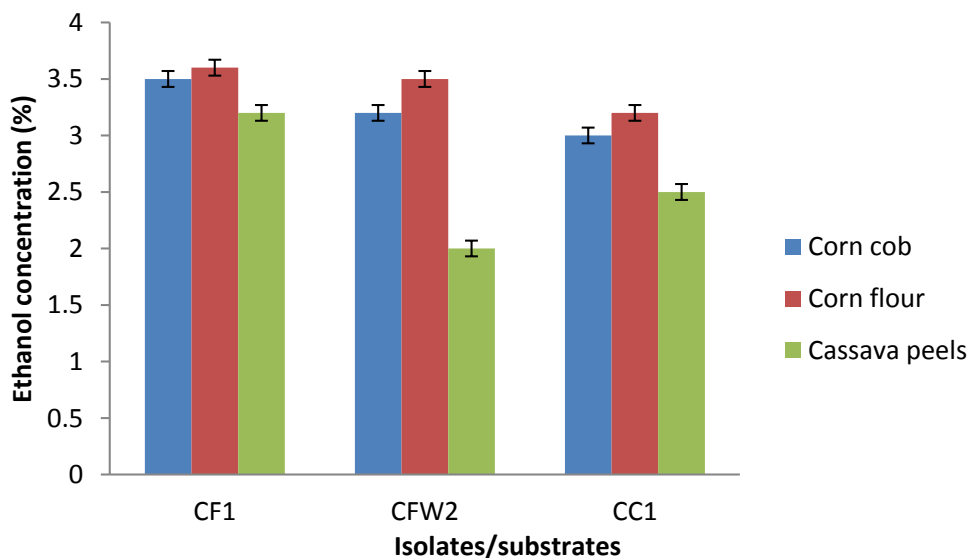


Figure 3. The ethanol yield of isolates using different substrates after 20 days of fermentation. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis*.

macerans with cassava peels which was 3.85 from the initial value of 7.04 (Table 3).

Total titratable acidity of samples inoculated with bacterial isolates

The total titratable acidity of the samples during fermentation by selected bacteria are presented in Table 4. There was a progressive increase with time. The

highest Total titratable acidity (TTA) at day 20 (0.180 moles) was observed for fermentation with *B. subtilis* on the corn cob, while the least value of 0.120 moles was obtained for *B. macerans* with corn cob.

Reducing sugar values of samples inoculated with ethanol yielding bacterial isolates

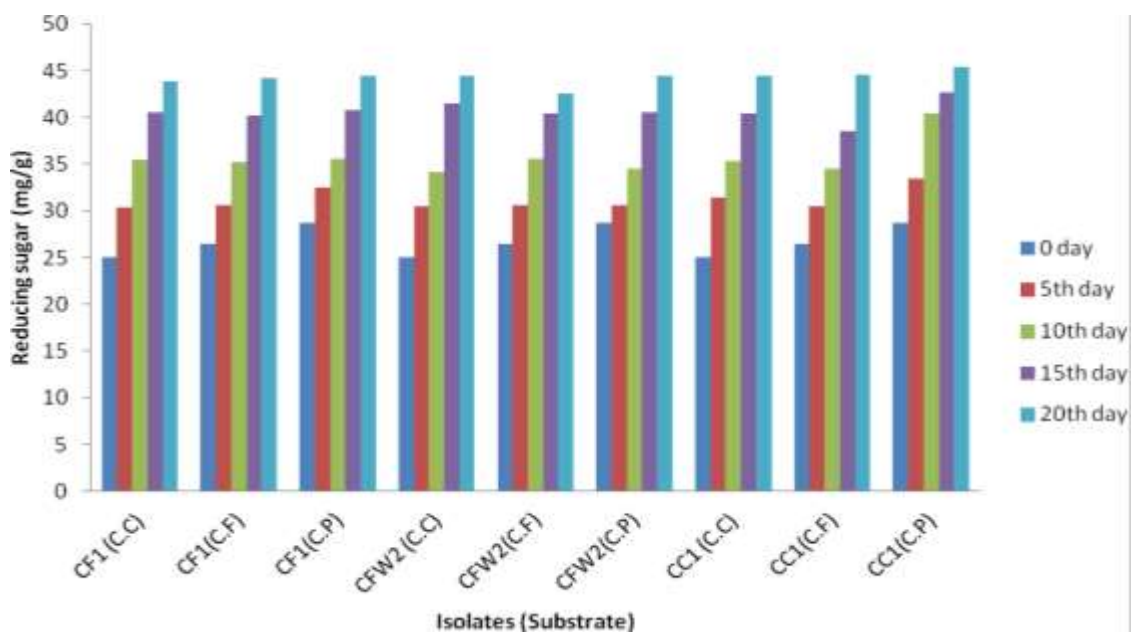
The reducing sugar of the samples during fermentation by bacterial isolates is shown in Figure 4. There was a

Table 3. pH of samples inoculated with bacterial isolates during fermentation.

Isolate/substrate	Number of days				
	0	5	10	15	20
CF1 (C.C)	6.80	5.52	4.75	4.41	4.10
CF1(C.F)	7.10	5.67	4.62	4.32	4.11
CF1(C.P)	7.04	5.41	4.45	3.97	3.85
CFW2 (C.C)	6.80	5.61	4.71	4.25	4.12
CFW2(C.F)	7.10	5.76	4.62	4.31	4.20
CFW2(C.P)	7.04	5.31	4.35	4.22	4.12
CC1 (C.C)	6.80	5.41	4.68	4.31	4.20
CC1(C.F)	7.10	5.71	4.65	4.22	4.10
CC1(C.P)	7.04	5.52	4.85	4.35	4.20

Table 4. Total titratable acidity of samples inoculated with bacterial isolates

Isolate/substrate	Number of days				
	0	5	10	15	20
CF1 (C.C)	0.00	0.050	0.055	0.081	0.120
CF1(C.F)	0.00	0.053	0.056	0.083	0.122
CF1(C.P)	0.00	0.056	0.058	0.086	0.142
CFW2 (C.C)	0.00	0.055	0.066	0.091	0.180
CFW2(C.F)	0.00	0.061	0.065	0.088	0.151
CFW2(C.P)	0.00	0.052	0.062	0.082	0.146
CC1 (C.C)	0.00	0.053	0.062	0.082	0.152
CC1(C.F)	0.00	0.052	0.063	0.084	0.144
CC1(C.P)	0.00	0.055	0.064	0.088	0.130

**Figure 4.** Reducing sugar values of samples inoculated with ethanol yielding bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis*.

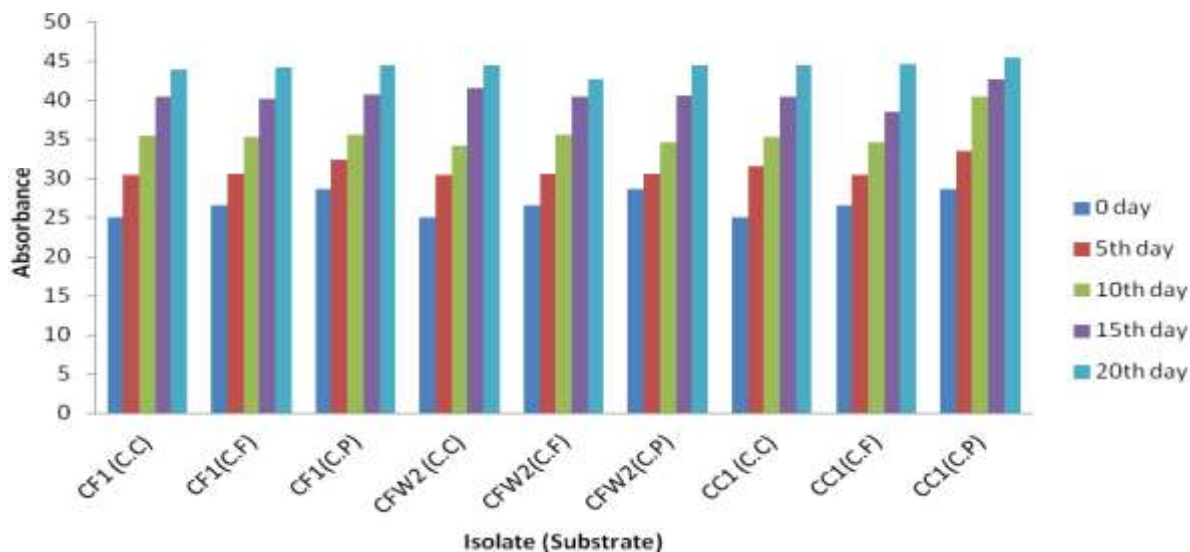


Figure 5. Optical density of samples inoculated with ethanol yielding bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis*.

Table 5. Proximate analysis of selected samples for fermentation.

Parameters (%)	Samples		
	Cassava peel	Corn cob	Corn flour
Moisture	28.06±0.007 ^c	27.69±0.007 ^b	31.00±0.007 ^a
Ash	1.94±0.007 ^c	1.99±0.007 ^b	2.1±0.007 ^a
Nitrogen	0.021±0.0007 ^c	0.028±0.0007 ^b	0.063±0.0007 ^a
Protein	0.13±0.0014 ^c	0.18±0.0014 ^b	0.39±0.0014 ^a
Crude Fibre	0.80±0.007 ^c	0.77±0.007 ^b	1.30±0.0007 ^a
Crude fat	0.58±0.007 ^c	0.65±0.007 ^b	1.80±0.0007 ^a
Carbohydrate	68.47±0.0014 ^b	68.70±0.007 ^a	63.34±0.0007 ^c

a, b and c, Means on the same row with different superscripts are statistically significant ($P \leq 0.05$).

progressive increase with time. *M. varians* had the highest value of 45.46 ± 0.00 mg/g when used on cassava peels. At $P \leq 0.05$, there was statistical significant difference between the reducing sugar yields in all the samples.

Optical density of samples inoculated with ethanol yielding bacterial isolates

The optical density of the samples is shown in Figure 5. The highest optical density of 4.04 was observed in cassava peels with *B. macerans* after fermentation for 20 days.

Proximate composition of selected sample for fermentation

The proximate analysis of the sample showed the

percentage composition of moisture, ash, nitrogen, protein, crude fibre, crude fat and carbohydrate of the samples (cassava peels, corn cob and corn flour) (Table 5). The highest value of moisture content was recorded for corn flour (31.00 ± 0.007^a) and was significantly different from the others. The corn cob had the highest carbohydrate value of 68.70 ± 0.007^a , while the highest value for crude fibre (1.80 ± 0.0007^a) was recorded for corn flour. At $P \leq 0.05$, all the analyses carried out on the samples were statistically significant.

DISCUSSION

The aim of this study was to investigate the potential of using indigenous bacterial isolates for the sole hydrolysis and conversion of cellulosic and starch containing biomass to ethanol. The characterization of the indigenous microorganisms isolated from the samples (corn cob and corn flour) and water from fermented

cassava showed the morphological and biochemical characteristics of the isolates. Bacterial isolates were identified as: *B. subtilis*, *B. macquariensis*, *M. varians*, *B. macerans*, *B. circulans*, *C. xerosis*, *B. azotoformans* and *B. insolitus*. Some bacteria identified in this study were similar to those obtained from study carried out by Olusola (2010) who isolated *B. subtilis* in a submerged fermentation of cassava. *B. macquariensis*, *B. macerans* and *B. subtilis* have also been shown to be present in fermenting media (Sarkar et al., 2002). This research established the isolation of *M. varians* and its ability to produce ethanol from the biomass used.

In addition, starch hydrolysis was carried out compounding the substrates as sources of starch and some of the bacterial isolates were able to hydrolyze starch. The isolates capable of hydrolyzing the starch compounded from the samples were used for the fermentation trials. This according to Mohammed (2009) is due to the inability of the organisms to produce the enzyme amylase which is a starch digesting enzyme. Such organisms lacking starch hydrolyzing enzymes were therefore screened out.

The result of this study on amylase activity was similar to that of Harihrishna et al. (2012) and Adeyemi (1990), who reported amylase production by *B. subtilis*, *B. macerans* and *M. varians* in the fermentation of cassava peels and corn. Production of cellulase by some microorganisms in this study was supported by studies carried out by Nisha et al. (2014) who reported cellulase activity in *M. varians* and *B. subtilis*.

At $P \geq 0.05$, there was no statistical significant difference between the ethanol yields of all bacterial isolates. It was also observed that at $P \leq 0.05$, there was statistical significant difference between the ethanol yield of the bacterial isolates in the corn cob, corn flour and cassava peels. Orji et al. (2016) reported 9% yield in ethanol using the corn cob as feed stock at a 10% substrate concentration. This study is comparable with that of Orji et al. (2016). The study recorded an average of 3.0% ethanol yield from corn cob at a 5% substrate concentration. Mohammed (2009) reported higher ethanol yields using corn starch with different organisms. The highest ethanol yield ($3.20 \pm 0.00\%$) obtained from the cassava peels fermentation was lower than 17.6% reported in the study of Oyeleke et al. (2012).

In this study, physicochemical parameters such as pH, total acidity, reducing sugar concentration, absorbance (optical density) and ethanol concentration were evaluated during and after the fermentation processes. A steady reduction was observed in the pH of the samples as fermentation proceeded for 20 days. This was attributed to fermentation by-products from the incomplete oxidation of glucose residues to organic acids such as acetic and formic acids according to Michelle (2011). Noe et al. (2009) and Michelle (2011) reported pH 4.0 as the optimum pH for ethanol production. The report of these two authors is in conformity with that obtained in

this study as the fermentation proceeded for 20 days after which, the ethanol concentration was determined and the optimum yield was less than 4.0.

No titratable acidity was reported on day zero before fermentation proceeded. The lowest titratable acidity observed in the cassava peels was between 0.05 and 0.061 moles in the bacterial fermentations on the 5th day of fermentation. It reached 0.13 moles on the 20th day of fermentation with the corn cob as substrate. At $P \leq 0.05$, there was statistical significant difference between the reducing sugar yields in all the samples using the bacterial isolates. Continuous increase in reducing sugar yield when the organisms were used for hydrolysis was reported by Mohammed (2009) to be due to substrate specificity of the enzymes. An increasing trend was observed in the optical density of the media while the bacteria fermented the media. This is similar to the report of Clark et al. (2013) who described this in a study on ethanol production as due to increase in reducing sugars and cell mass.

The samples (corn cob, corn flour and cassava peels) which served as substrates were analyzed for percentage proximate nutrient composition and at $p \leq 0.05$, the differences between the average values of moisture, ash, fat, protein, crude fibre and total carbohydrate of the samples were statistically significant. The highest percentage moisture content was observed in the corn cob while the lowest was found in the cassava peels. The percentage ash, nitrogen and protein content also showed similar trend. The highest crude fibre was found in the corn cob and the lowest in the cassava peels. The highest crude fat was found in the corn flour with the lowest in the cassava peels. The report on crude fat is similar to that of Ikram et al. (2012), while the reports on moisture, ash, fat, protein, crude fibre and total carbohydrate are similar to that of Ikram et al. (2012), Pointner et al. (2014), Ikeen et al. (2002), Christopher et al. (2016) and Ogbonna and Adewale (1993). The percentage ash and nitrogen, reported in the work of Pointner et al. (2014) are higher in the cassava peels when compared with the corn cob, while the percentage moisture, protein and carbohydrate were higher in the corn flour than in the other substrates. However, this study established that the percentage protein and ash content of the corn cob was higher than that of cassava peels.

Conclusion

This study investigated the potentials of wastes (cassava peels and corn cob) used as feed stocks for bio-ethanol production, in substantially replacing major food crops (corn) as a case study. The bio-conversion process of wastes shows that it is useful to mankind. This study also demonstrated the isolation of indigenous microflora as a successful approach to spontaneously carrying out fermentation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Morphophysiological diversity of rhizobia nodulating pigeon pea (*Cajanus cajan* L. Millsp.) growing in Ethiopia

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Pigeon pea (*Cajanus cajan* (L.) Millsp.) is an important protein source grown in several tropical and sub-tropical countries, and is considered a multi-purpose plant that is resistant to the conditions where drought and salinity is a common phenomenon. The aim of this study was to evaluate the diversity of rhizobial isolates obtained from root nodules of pigeon pea plants grown in central and southern Ethiopia. A total of 116 nitrogen-fixing rhizobial strains were isolated. The bacterial isolates were characterized by 91 phenotypic traits including cultural characteristics, intrinsic antibiotic and heavy metal resistance, salt, pH and incubation temperature tolerance, and carbon and nitrogen sources utilization ability. Preliminary symbiotic properties of the isolates were also evaluated. The isolates were compared with seven reference strains of rhizobia by application of the unweighted pair group method with arithmetic means (UPGMA) using NTSYSpc Version 2.1 software program. The dendrogram constructed from cluster analysis of 91 phenotypic traits, grouped them into six clusters and eight un-clustered positions at 80% relative similarity. Cluster I contained 83% of the test isolates that were grouped together with the reference strains *Bradyrhizobium japonicum* (HAMBI 2314^T) and *Bradyrhizobium elkanii* (LMG 6164), suggesting that pigeon pea is commonly nodulated by bradyrhizobia. Results from symbiotic effectiveness test revealed that majority of the isolates were found to be effective. Generally, this investigation demonstrated that rhizobial population nodulating *C. cajan* on the study area were phenotypically diverse and symbiotically effective. Furthermore, the result indicates the existence of strains in the collection, which can tolerate environmental stresses, thus can be developed into inoculant for pigeon pea inoculation and production in Ethiopia and beyond.

Key words: *Bradyrhizobium*, *Cajanus cajan*, phenotypic characteristics, *rhizobium*.

INTRODUCTION

Pigeon pea (*Cajanus cajan* L. Millsp.) is the only crop member of the Cajaninae tribe (Hancock, 2012), which

grows vigorously in soils with low fertility, mainly in marginal lands (Beltrame and Rodrigues, 2007). Indeed,

Table 1. Sites and associated environmental variables of the locations from where root nodules of pigeon pea was obtained.

Site of nodule	Annual T°C ranges	Annual rainfall ranges (mm)	Elevation in m.a.s.l.	Latitude °N	Longitude °E
Humbo	17.6-25	801-1600	1001-2500	6.51-6.79	37.59-38.05
Bodity	17.6-22.5	100-1400	1501-2500	6.88-7.12	37.75-38.00
Gofa	17-29	1401-1600	501-3000	6.12-6.72	36.68-37.20
Hawassa	12-27	801-1600	1501-3000	6.11-6.52	37.58-37.98
Badwacho	17.6-22.5	801-1400	1501-2500	7.07-7.30	37.73-38.10
Ziway	16-27	450-850	1640	7.56	38.43

Source: EARO (1998); AARC (2004).

pigeon pea is able to associate with a large diversity of indigenous rhizobia in soil, reaching more than 150 kg of fixed N per hectare per year (Peoples et al., 1995). To exploit the biological nitrogen fixation (BNF) potential of this crop, the selection and evaluation of new rhizobial strains from different areas where pigeon pea is cropped must be carried out.

The slow and fast growing pigeon pea rhizobia present great genetic and metabolic diversity and are likely to have new species among the culture collections worldwide (Fernandes et al., 2012; Ramsubhag et al., 2002). In addition to being efficient in fixing nitrogen in field conditions, pigeon pea rhizobia also present other biotechnological applications, such as biopolymer production and enzymatic activity (Fernandes et al., 2012; Júnior et al., 2011). Nevertheless, it should be noted that for Ethiopia, information is scarce about indigenous rhizobia that nodulate grain legumes such as pigeon pea. Few studies on Ethiopian collections of rhizobia from different legume species showed, however, that Ethiopian soils harbour diverse populations of rhizobia with distinct genomic composition which also differ in symbiotic effectiveness (Beyene et al., 2004; Degefu et al., 2013; Tena et al., 2017a, b; Wolde-Meskel et al., 2005). Hence, it could be logical to hypothesize that there is a large, undiscovered genetic diversity of rhizobia nodulating pigeon pea growing in Ethiopia, an acknowledged geographic centre of many leguminous plants (Lie et al., 1987). Furthermore, to improve productivity of the target crop (that is, pigeon pea), it is relevant to characterize the indigenous population of rhizobia compatible with cultivated crops and develop inoculants for use in legume production in various locations in the country and beyond. This fact necessitates investigation of rhizobia nodulating pigeon pea growing in various locations in the country.

As a first step to such approach, (either from field standing nodule or by trapping techniques) rhizobia nodulating pigeon pea growing on soils of six locations in Ethiopia was systematically recovered. Accordingly, the

investigations were primarily based on considering an array of morphophysiological features including cultural characterization, intrinsic antibiotic and heavy metal resistance, salt, pH and incubation temperature tolerance, carbon and nitrogen sources utilization ability of rhizobial test isolates recovered from surface-sterilized root nodules of pigeon pea grown at diverse locations in central and southern Ethiopia. Cluster analysis of the test isolates were carried out using numerical taxonomic approach where 91 features were examined. Furthermore, preliminary symbiotic potential of the test isolates were studied using nodule and leaf color.

MATERIALS AND METHODS

Rhizobial source and isolation procedure

The rhizobial strains were isolated from desiccated and fresh nodules obtained from pigeon pea grown at the different locations in central and southern Ethiopia (Table 1) following procedures detailed elsewhere (Somasegaran and Hoben, 1994). All isolates were stored in 20% glycerol stock at -21°C (Coutinho et al., 1999). Presumptive tests were also carried out based on the methods described earlier (Somasegaran and Hoben, 1994). Plates were incubated at 28°C, observed daily for colony appearance with their characteristics color (colorless on congo red, yellow for fast growers and blue for slow growers) (Jordan, 1982; Vincent, 1970).

Authentication of the test isolates

The ability of isolates to form nodules was determined by re-inoculating them into homologous host (pigeon pea). Authentication was carried out in modified Leonard jars constructed from plastic cups, which was sterilized after filling it with washed river sand. Surface-sterilized pre-germinated seedlings were aseptically transplanted into each Leonard jar. Pure single colony of each isolates was inoculated on Yeast Extract Manitol Broth (YMB) and from the logarithmic growth phase the seedlings were inoculated with 1 ml of each isolate (Somasegaran and Hoben, 1994). Positive control (none inoculated and supplied with N-solution where 0.1% KNO₃ was added to N-free solution) and negative control (none inoculated and supplied with N-free nutrient solution) were included during authentication. The plants were grown in triplicate under

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Table 2. Geographical origins, strain designations (alphabets along with numbers) and number of isolates obtained from each sampling sites.

Location	Isolate designation	Total No. of isolates
Ziway	PZ11, PZ81, PZ82	3
Badawacho	PBa32, PBa33, PBa92, PBa93	4
Bodity	PB86, PB87, PB88, PB89, PB90, PB91, PB94, PB96, PB97, PB98, PB99	11
Hawassa	PH104, PH105, PH106, PH107, PH127, PH128, PH147, PH148, PH149, PH150, PH151, PH152, PH153	13
Gofa	PG13, PG17, PG27, PG28, PG29, PG31, PG35, PG59, PG61, PG63, PG64, PG67, PG68, PG69, PG70, PG71, PG72, PG 73, PG74, PG75, PG76a, PG76b, PG79, PG80, PG84	25
Humbo	PHu34, PHu36, PHu49, PHu50, PHu100, PHu101, PHu108, PHu109, PHu110, PHu111, PHu112, PHu113, PHu114, PHu116, PHu117, PHu119, PHu120, PHu121, PHu122, PHu123, PHu124, PHu125, PHu126, PHu130, PHu131, PHu132, PHu133, PHu134, PHu135, PHu136, PHu137, PHu138, PHu139, PHu140, PHu141, PHu143, PHu144, PHu145, PHu146, PHu154, PHu156, PHu157, PHu159, PHu161, PHu162, PHu163, PHu164, PHu165, PHu166, PHu169, PHu170, PHu172, PHu173, PHu174, PHu175, PHu176, PHu177, PHu178, PHu179, PHu181	60

*PZ: Pigeon pea Ziway, PBa: Pigeon pea Badawacho, PB: Pigeon pea Bodity, PH: Pigeon pea Hawassa, PG: Pigeon pea Gofa, PHu: Pigeon pea Humbo

greenhouse condition. The modified Leonard jars were arranged in complete randomized design (CRD) and fertilized with quarter strength Jensen's N-free medium. Seedlings were checked for nodules after 45 days and roots from the plants were gently washed and nodules were carefully removed. Nodule number per plant and internal color (pink, white and green) were scored.

Characterization of the isolates

Morphological characteristics

The colony morphology was observed on Yeast extract mannitol agar (YMA) after incubation for 2 to 13 days at 28°C. Colony appearance was characterized based on their color, shape, size, texture and ability to produce extracellular polysaccharide (Somasegaran and Hoben, 1994).

Salinity tolerance

Isolates were tested for salt tolerance in YMA supplemented with NaCl (Bouhmouch et al., 2001), temperature (Chen et al., 2002) and pH tolerance (Kishinevsky et al., 2003). The results were recorded as (+) for growth and (-) for no growth.

Carbon sources utilization

Carbon source (Somasegaran and Hoben, 1994) and amino acid utilization (Amarger et al., 1997), intrinsic heavy metal resistance (IHR) (Zhang et al., 1991), intrinsic antibiotic resistance (IAR) (Lindström and Lehtomäki, 1988), phosphate solubilizing ability (PSA) (Alikhani et al., 2006) for the entire test isolates were carried out following the methods described in their respective references.

Numerical analysis

Characters were coded 1 for positive and 0 for negative. Cluster analysis for the final matrix containing 123 strains and 91 features was carried out using similarity coefficient and a phenotypic dendrogram was constructed by the unweighted pair group method

with arithmetic means average (UPGMA) clustering method using Numerical Taxonomic Analysis System NTSYSpc version 2.

RESULTS AND DISCUSSION

In a systematic collection of rhizobia nodulating pigeon pea growing in central and southern Ethiopia, a total of 116 rhizobial isolates were recovered from root nodules of *C. cajan* grown in Ziway (3 strains), Badawacho (4 strains), Bodity (11 strains), Hawassa (13 strains), Gofa (25 strains) and Humbo (60 strains) (Table 2). In order to investigate their diversity and relationships with the reference strains, they were characterized based on various morphophysiological features. From the total isolates, 20% were found to be fast grower, as the first colony was found to appear between 3 and 5 days, while 80% of the isolates were slow growers (colony appeared between 5 and 10 days). This indicated that both fast and slow growing rhizobia are the natural endophytes nodulating pigeon pea in the study sites. The results from this study was in agreement with results generated elsewhere, which reported that fast-growing root nodule bacteria form visible colonies on YMA within 3 to 5 days, and slow growers needs 5 to 7 days when incubated under 28°C (Jordan, 1984). In another study of similar nature, it was clearly shown that pigeon pea was found to be nodulated by both fast and slow growers (Anand and Dogra, 1991), indicating that pigeon pea is non-selective to make symbiotic association with rhizobia for biological nitrogen fixation.

With the respect to presumptive test, it has been well documented that rhizobia showed little or no congo red (CR) absorption when incubated in the dark, and they showed no growth or poor growth on PGA-BCP (Jordan, 1984; Somasegaran and Hoben, 1994). In agreement

Table 3. Summary table on the overall symbiotic effectiveness of the isolates.

No. of Isolates	Leaf color	Nodule color	Remark
51	Deep green	R/P	Effective
52	Green	P	Effective
13	Pale green	P/W	Ineffective

*R: Red, P: pink, W: white.

Table 4. Colony characteristics, acid/base reaction, of the test isolates.

property		Ziway n=3	Badawacho n=4	Bodity n=11	Hawassa n=13	Gofa n=25	Humbo n=60	Total No. of isolate	% of isolates
Colony size mm	<1.0	3	3	7	6	20	37	76	66
	1-1.5	-	-	4	7	5	22	38	32
	>1.5	-	1	-	-	-	1	2	2
Colony color	WT	-	3	5	12	16	55	91	78
	M	3	-	6	1	9	3	22	19
	WO	-	1	-	-	-	-	1	1
	Y	-	-	-	-	-	2	2	1
Colony shape	C	3	3	11	13	25	59	114	98
	F	-	1	-	-	-	1	2	2
Colony texture	B	3	3	11	13	24	59	144	98
	E	-	1	-	-	1	1	2	2
EPS		-	1	-	-	1	1	3	3
Acid/Base reaction	Al	2	3	10	12	59	59	107	92
	A	1	1	1	1	1	1	9	8
Date first colony appeared	3-5 days	-	2	11	-	6	6	23	20
	5-10 days	3	2	-	13	54	54	93	80

*n: Number of isolates, M: milky, WT: watery translucent, WO: white opaque, Y: yellow, B: buttery, E: elastic, C: convex, F: flat, Al: alkaline, A: acid, EPS: exopolysaccharide production.

with this, the present study clearly showed that, all isolates did not absorb CR, and they did not grow on PGA-BCP, confirming that the test isolates were all rhizobia. Furthermore, Gram reaction of the isolates was detected, and all were found to be Gram negative.

Based on the authentication test, 116 isolates were found to form nodule with the host plant. The preliminary symbiotic effectiveness test (based on nodule and leaf color) indicated that more than 44% of the test isolates were found to produce leaf (with green or deep green color) and red/pink nodule, when inoculated to pigeon pea, whereas the rest produced leaf (with pale green color) (Table 3). It can be concluded that, the isolates with deep green and green leaf color as well as with red/pink nodule color were effective strains, whereas, the isolates with pale-green leaf color can be categorized as being ineffective.

Colony morphology

As shown in Table 4, the isolates were found to vary in morphology; 66% formed small colonies (<1.0 mm), whereas 32% had diameter ranging between 1 and 1.5 mm, and only strains designated as PHu110 (from Humbo) and PBa93 (from Badawacho) had diameters of 4.9 and 5.5 mm, respectively. PHu110 and PBa93 had flat shape and produced copious EPS, but the rest of the isolates had convex or domed shape and did not produce EPS. Previous report indicated that fast growing rhizobia produce excessive EPS within 3 to 5 days, while slow growing isolates produce less or no mucus and do not grow more than 1 mm size within ten days of incubation (Jordan, 1984). The majority of the isolates (78%) have expressed watery translucent, while 19% have shown milky appearance on YMA. In agreement with the

Table 5. Salt tolerance of the isolates NaCl% (W/V).

Isolate origin	NaCl%										
	0.5	1.0	1.5	2	2.5	3.0	3.5	4.0	4.5	5.0	5.5
Ziway (n=3)	+	+	+	+	1	1	-	-	-	-	-
Badawacho (n=4)	+	+	+	3	2	2	-	-	-	-	-
Bodity (n=11)	+	+	+	+	7	7	1	1	-	-	-
Hawassa (n=13)	+	+	+	+	2	1	-	-	-	-	-
Gofa (n=25)	+	23	23	18	11	6	3	3	2	2	2
Humbo (n=60)	+	52	51	44	17	3	1	1	1	1	1

*n: Number of isolates, '+'=growth, '-'=no growth. Figures in the table indicate the number of test isolates, which were able to grow on the indicated salt concentration.

findings, all isolates recovered from Kenyan soils fell into watery, milky translucent and curdle milk color type (Odee et al., 1997). PBa93 has produced white opaque colonies while, PHu110 and PHu114 (isolated from Humbo) were found to appear as yellow colonies. Taken together, the color of the colonies investigated in the study is a characteristic feature of rhizobia (Somasegaran and Hoben, 1994).

Acid/Base reaction of the isolates on YMA-BTB

Acid and alkaline production has been used as a tool to elucidate the general characteristics of rhizobia. Generally, slow-growing rhizobia produce alkaline, while fast-growing rhizobia produce acid (Jordan, 1984). However, in another study of similar nature, it was reported that slow growth and alkali production were not mutually inclusive characteristics (Kennedy and Greenwood, 1982). In the present study, only 8% of the isolates were acid producing strains when inoculated and incubated on YMA containing BTB, while the others were alkali-producing strains (Table 4), which is in agreement with other studies conducted elsewhere (Hernandez and Focht, 1984; Moreira et al., 1993; Wolde-meskel et al., 2004), and that our test isolates represent both fast and slow growing groups.

Salinity tolerance

The characteristic salt tolerance profile of the test isolates is presented in Table 5. Accordingly, 80% of the test isolates were able to grow in YMA with 2% NaCl, while 41% at 2.5% NaCl and only 17% of the test isolates were able to grow at salt concentration of 3%. Isolates PG73 (from Gofa) and PB87 (from Bodity) continued to grow at 4% NaCl, while PG68 and PG80 (from Gofa), and PHu162 (from Humbo) tolerated salt concentration of 5.5%. In previous undertakings, rhizobia were reported to grow at salt concentration of 2% (Jordan, 1984), 3% (Odee et al., 1997), 4% (Swelim et al., 1997) and 5% (Surange et al., 1997). Successful rhizobium-legume

symbiosis under salt stress require isolates that could resist higher NaCl concentration, thus the presence of high salt concentration tolerant isolates in the collection could potentially offer a possibility for developing inoculant for legume production in sites where salinity is a problem.

pH and temperature tolerance of the isolates

The pH and temperature tolerance of the test isolates is presented in Table 6. The isolates were shown to have wide diversity with respect to their pH and temperature requirements. With the exception of the following isolates including PG27, PG29 and PG74 (which failed to grow at acidic and/or alkaline pH), most of the tested isolates were shown to have wider range of pH profile for their growth. This was to some extent in agreement with other study (Kalita and Matek, 2004). Soil acidity is a significant problem for agricultural production in many areas of the world and limits legume productivity (Bordeleau and Prévost, 1994). Isolates with high adaptability to acidic and alkaline pH can be used as inoculant for legume production where acidity and alkalinity is a problem.

With respect to the temperature tolerance of the test isolates, the optimum growth temperature was found to range between 20 and 30°C, which is in line with results reported elsewhere (Jordan, 1984). The exceptions to this finding was that, the following test isolates including PG59, PG 68, PB87, and PBa93 were able to grow at extreme temperatures (at 5, 40, and 45°C). In previous studies, rhizobia were reported to grow at temperature values of 44°C (Zhang et al., 1991) and 50°C (Surange et al., 1997). It has been reported that high temperatures decrease rhizobial survival and establishment in tropical soils (Hungria and Vargas, 2000). Nevertheless, the high temperature tolerant rhizobial isolates investigated in this study might be regarded as good opportunity to develop efficient inoculum for legumes production tropical soils, where high temperature is a common phenomenon. Furthermore, isolates that tolerated a wide range of temperature can be used for production of effective inoculum for legumes grown in different eco-climatic

Table 6. pH and temperature tolerance of the isolates.

Isolate	pH					Temperature (°C)			
	4.0	4.5	5.0-9.5	10.0	10.5	5	20-30	40	45
PZ (n=3)	-	1	+	+	+	-	+	-	-
PBa (n=4)	2	2	+	+	+	1	+	1	1
PB (n=11)	8	9	+	+	+	1	+	1	1
PH (n=13)	8	11	+	+	+	-	+	-	-
PG (n=25)	9	14	+	23	22	2	+	2	2
PHu (n=60)	30	45	+	+	+	7	+	-	-

*n: Number of isolates, '+'=growth, '-'=no growth. The figures in the table show the number of test isolates which tolerated the tested parameter.

regions.

Carbon and amino acids sources utilization pattern of the isolates

With respect to carbon source utilization (Table S1), it was observed that there is only a slight difference among the isolates. All isolates were able to grow on the 17 carbon sources provided, while 88% of the isolates utilized arabinose. Several studies reported that most carbon sources were utilized by rhizobia (Amarger et al., 1997; Workalemahu and Assefa, 2007; Zhang et al., 1991). Sixty four percent of the isolates utilized dulcitol and only 15% were able to metabolize fructose. Strain selection to produce inoculum requires effective isolates with high resistance to various environmental constraints and competence for resources. Since the isolates in this study have utilized a wide range of carbon sources, they will have a selective advantage over those with limited carbon source utilization ability, thus paramount importance for production of inoculum that can be useful in soil with different carbon sources.

Regarding the amino acids source utilization (Table S2), most of the amino acids were utilized by about 95% of the isolates. However, the most selective amino acids were Glycine and D-serine. Of the total 116 isolates tested, only 13 strains, namely, PG27, PHu50, PG59, PG68, PG72, PG76b, PG79, PG80, PG84, PB87, PBa93, PHu114, and PHu117 were able to utilize Glycine. On the other hand, isolates designated as PG 27, PG68, PG72, PG79, PG80, PG84, PB87, PHu114, PHu179, and PHu181 have utilized D-serine as N sources. The overall results presented in S2 have shown that the isolates of *C. cajan* in this study had an ability to utilize a wider range of nitrogen sources. It can thus be concluded that the test isolates in this study were not fastidious in their amino acids requirement; rather they were able to grow on wide range of amino acids. The ability of the test isolates to utilize a wide range of amino acid sources would be selected for a system thus can survive and establish themselves in an environment where nitrogen sources are a limiting factor for growth.

IAR, IHMR, and PSA of the test isolates

The intrinsic antibiotic resistance (IAR; Table S3), intrinsic heavy metal resistance (IHMR; Table S4) and phosphate solubilizing ability (PSA) clearly indicated the existence of variations among the test isolates. Accordingly, isolates of *C. cajan* showed high degree of resistance to the tested concentration of antibiotics, and this is in agreement with results of other several investigations (Eaglesham, 1987; Elkan, 1992; Jordan, 1984; Zhang et al., 1991). Similarly, the tested isolates resisted different concentration of heavy metals including Pb, Zn, Cu, and Al (Table S4), which is in agreement with other study of similar nature (Zhang et al., 1991). In the present study, cobalt seems to be selective in that only 43% of the isolates have resisted $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 100 $\mu\text{g/ml}$. It is known that heavy metals are regarded as persistent in soils. Therefore, isolates with high intrinsic heavy metal resistance, is very useful for production of inoculum, which can be applicable in polluted soils with heavy metals released from industries.

Phosphorus is one of the limiting factors to crop production in many tropical soils (Singleton et al., 1985). In the present study, 68% of the isolates had solubilized phosphate in medium containing tricalcium phosphate (TCP). Therefore, it can be concluded that, in addition to their beneficial nitrogen fixing activity with legumes, the tested rhizobial strain can potentially improve plant P nutrition by mobilizing inorganic and organic P. Phosphate solubilizing ability is a good attributes for the strain to be used in soil with limited phosphorus source.

Numerical analysis

Cluster analysis of the 116 isolates and 7 reference strains was performed based on 91 phenotypic characteristics. The phenogram shows six phenon and eight unclustered positions that were separated at a similarity cut point of 80% (Figure 1). Phenon I contained majority of the isolates (83%) representing fast and slow growing rhizobia, and grouped with *Bradyrhizobium japonicum* (HAMBI 2314¹) and *Bradyrhizobium elkanii*

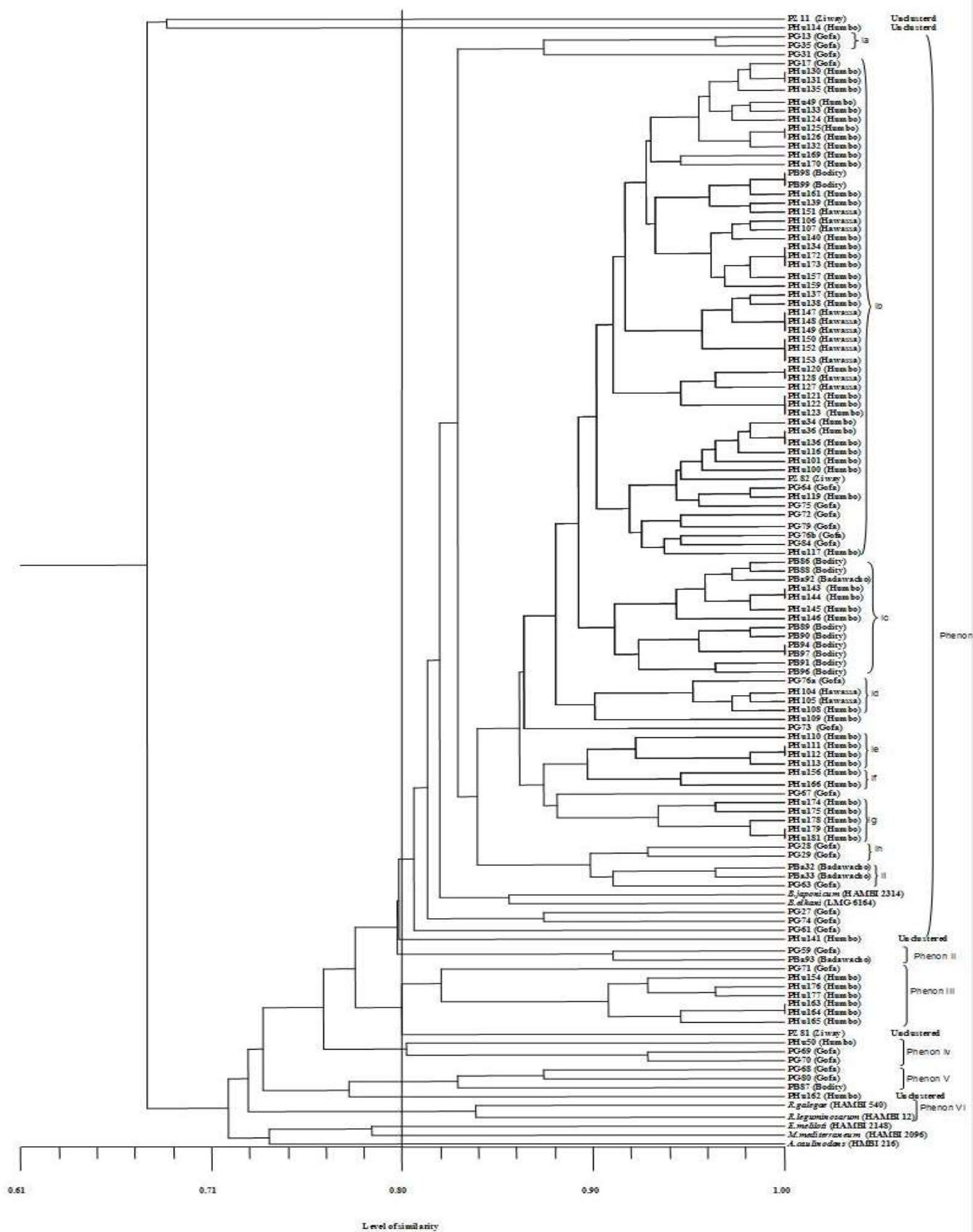


Figure 1. Phenogram showing similarity between the tested isolates and the reference species.

(LMG 6164). This phenon was very heterogeneous with 9 subphenons at a similarity of 90%. It was reported that a significant number of slow-growing isolates of *C. cajan* are phylogenetically related to *B. elkanii* and *B. japonicum* strains (Ramsubhag et al., 2002). In addition, the existence of fast as well as slow-growing strains of rhizobia infecting *Cajanus* species were reported (Anand and Dogra, 1991). From this study, it was observed that pigeon pea strains isolated from the study area were diverse, and thus still there are untapped rhizobial resources in Ethiopian soils.

In conclusion, this preliminary study based on morphophysiological features has provided well characterized and preserved collection of rhizobial strains from nodules pigeon pea, and has explored the presence of indigenous rhizobia nodulating pigeon pea in the study areas. This study recommends to characterize the test isolates further using modern molecular techniques in order to elucidate the proper identity of the strains. Moreover, symbiotic effectiveness under greenhouse and field conditions, as well as evaluation of the competitiveness against indigenous strains should be studied to exploit the benefits these test isolates could offer for pigeon pea production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table S1. Carbon sources utilization of pigeon pea isolates.

Carbon source	PZ (n=3)	PBa (n=4)	PB (n=11)	PH (n=13)	PG (n=25)	PHu (n=60)	% of utilizing isolates
Maltose, Sorbitol, Trehalose, Raffinose, Xylitol, Myo-inositol, Sucrose, Glucose, Mannitol, Starch, Galactose, Mannose, Ribose, Rhamnose, Dextrin, Inulin, Ribose, and Xylose	+	+	+	+	+	+	100
Arabinose	-	2	+	+	17	+	88
Dulcitol	+	-	3	2	23	56	64
Fructose	1	-	-	-	12	4	15
Citric and malonic acid	-	-	-	-	-	-	0

*n: Number of isolates, '+'=growth, '-'=no growth. Figures in the table show the number of the test isolates which were able to grow on the tested parameter.

Table S2. Amino acid utilization pattern of the isolates.

Characteristic	PZ (n=3)	PBa (n=4)	PB (n=11)	PH (n=13)	PG (n=25)	PHu (n=60)
L-Phenylalanine	+	+	+	+	+	+
L-Histidine	+	+	+	+	24	+
L-Pyroglutamic acid	2	+	+	+	+	59
L-Theronine	2	+	+	+	+	+
L-Alanine	+	+	+	+	+	+
L-Aspartic acid	2	+	+	+	24	59
L-Arginine	+	+	+	+	+	+
L-Proline	2	+	+	+	+	59
L-Asparagine	+	+	+	+	24	+
L-Gutamic acid	+	+	+	+	+	+
Inosine	+	+	+	+	+	+
Leucine	+	+	+	+	+	59
Thymidine	+	+	+	+	+	+
Uridine	+	+	+	+	+	+
Glycine	-	1	1	-	8	3
D-serine	-	-	1	-	6	3

*n: Number of isolates, '+'=growth, '-'=no growth. Figures in the table show the number of the test isolates which were able to grow on the tested parameter.

Table S3. Intrinsic antibiotic resistance of the isolates (IAR).

Antibiotic	Concentration ($\mu\text{g/ml}$)	Resistant isolates (%)
Lincomoycin	100	100
Trimtoprin	500	100
Streptomycin	10	83
Erythromycin	10	95
	20	95
Novobiocin	0.5	100
	1.5	100
Spectinomycin	2.5	94
	5	94
Kanamycin	5	91
	15	74
Neomycin	5	100
	20	98
Chloramphenicol	5	98
	15	98

Table S4. Intrinsic heavy metal resistance of the isolates.

Heavy metal	Concentration ($\mu\text{g/ml}$)	Resistant isolates (%)
$\text{Pb}(\text{CH}_3\text{COO})_2$	500	83
ZnCl_2	100	95
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	100	43
CuCl	100	96
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	500	99
$\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$	500	79



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