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ADDIS ABABA UNIVERSITY

Addis Ababa Institute of Technology

School of Chemical and Bio-Engineering

Determine the potency of caffeine degradation microbes and process optimization.

A thesis submitted to the Research and Graduate School of Addis Ababa Institute of Technology, School of Chemical and Bio engineering in partial fulfillment of the requirements for the attainment of the Degree of Master of Science in Chemical Engineering under Biochemical Engineering Stream

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Addis Ababa University

Addis Ababa Institutes of Technology

School of Chemical and Bio Engineering

This is to certify that the thesis prepared by ZEYNU SHAMIL AWOL entitled: determine the potency of caffeine degradation microbes and process optimization. Submitted in partial fulfillment of the requirements for the Degree of Master of Science in biochemical Engineering, complies with the regulations of the University and meets the accepted standards with respect to the originality and quality.

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Abstract

Bioremediation being a problem solver for many different environmental and other major hazards, they play an important and cost effective role in the decaffeination process. Form collected environmental sample coffee husk. From CH caffeine can be eliminated. With this experimental work take two spp of microorganism that were bacillus subtilis and rhizobium from Ethiopia bio diversity institute, that can be capability of improving soil fertility. Could grow on the medium supplemented with 2.5g/L caffeine and could effectively degrade 2.5 g/L of caffeine in the liquid media as a sole source of carbon and nitrogen. Morphological and biochemical characteristics were maintained for re-affirmation the organism as bacillus subtilis and rhizobium. The degradation of caffeine capability was authenticated by growth curve. The next task were that those authenticated caffeine degrading bacteria applicable for conserving soil fertility by means of N-fix and p- solubilized potential then propagation and growth of those selective caffeine degrading bacteria was maintained by selective medium with and without supplement of 2.5g/l caffeine then optimized the factors that affecting cell growth with prepared broth with and without supplement of caffeine and determined interaction of each factor from experiment design software 6.0.8) the result would be temp; 35^{o}c, pH 7.07 incubation time 36hr from those optimal condition obtained maximum cell growth in number was 3.41995E+008 and 5.0707E+008 with and without caffeine respectively for bacillus subtilis and temp 34^{o}c, pH 6.6 and incubation time 28hrs optimum condition maximum cell growth 3.51146E+008 and 4.09131E+008 was obtained for rhizobium sp. of organism. That viable cell grown microorganism ready for applicable of as liquid bio fertilizer those were easiest way of production, handle, cost effective and keep soil health care method of production.

Keywords coffee husk caffeine rhizobium bacillus subtilis tannins YEM medium and PIKOVSKAYA medium

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DECLARATION

I declare that this thesis was my original work for fulfillment Degree of M.Sc from Addis Ababa University, I affirmed that have no previously been submitted for the degree at this or any other university, and that all resources of materials used in this thesis have been justly acknowledged.

Name: zeynu shamil awol date…… Signature ……

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ACRONYMS

ANOVA	analysis of variance
BF	bio fertilizer
CF	chemical fertilizer
Do	dissolve oxygen
OD	optical density
ETB	Ethiopian Birr
Ha	humic acid
K mobilizer	potassium mobilizer
LBF	liquid bio fertilizer
MPN	Most Probable Number
N fixer	nitrogen fixer
PGPR	plant growth promoting rhizobacteria
P solublizer	phosphors solubilize
SPP	species
T201	non-nodulating isolines
T202	nodulating isolines
NR	nitrate reduction
GP	gas production

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Chapter one

1.1 Background

1.1.1 Introduction

Every day, large quantity of waste is generated in all the developing and developed countries (Buerge IJ, Poiger T, Muller MD, Buser HR.2003). The highly toxic organic compounds in the form of solid, liquid and gas have been synthesized and released into the environment directly or indirectly over a long period of time by industrial and agricultural activities. The production and improper disposal of agro wastes has become a major pollution issue round the world.

Biological decomposition of organic waste such as fertilizers, pesticides and agro wastes are the most important and effective way to remove these compounds from the environment. Bacteria, actionmycetes, fungi, algae and protozoa are the major microorganisms found in soil which decompose soil organic materials, of which bacteria are most prominent and most abundant. Microbes use the waste for their own metabolism and finally produce some simple and useful compounds which are important for soil health, plant growth and overall eco-balance. Microorganisms have the ability to interact, both chemically and physically with substances, leading to the structural changes or complete degradation of the target molecules. Therefore, the present study was aimed to focus at the importance of characterization and identification of caffeine degrading bacteria from coffee husk. Bacteria constitute a large domain of prokaryotic microorganisms and Bacteria also live in symbiotic and parasitic relationships with plants and animals were among the first life forms to appear on Earth, and are present in most of its habitats typically a few micrometers in length, bacteria have a number of shapes, ranging from spheres to rods and spirals. However most bacteria have not been characterized, and only about half of the bacterial phyla have species that can be grown in the laboratory from environmental sample. (https://en.wikipedia.org/wiki/Bacteria)

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6 dione) was one of environmental sample a purine alkaloid, which acts as a stimulant of central nervous system and also has negative withdrawal effects and is present in different varieties of plants such as coffee plant, tea leaves, colanut, cocoa beans and other plant. It is also present in soft drinks and is being used extensively in human consumption and has in addition some therapeutic uses but in minimal

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amount. Evidence has proved the harmful effects of caffeine thus opening a path in the field of caffeine biodegradation. Biodegradation by bacteria is considered to be the most efficient technique in degrading caffeine within the environment. Even though there are available methods for the removal of caffeine using conventional methods such as water, supercritical and solvent decaffeination but they are lack of accuracy/specificity for the removal of caffeine and in addition to the existing caffeine which sometime remains (Blecher R, Lingens F1997). Caffeine content in regular and instant coffee ranges from 0.43 to 0.85 and from 0.61 to 0.82 mg/ml, respectively (Camargo MCR, Toledo MCF 1998). It stimulates the central nervous system, shows toxicity when fed in excess and is even mutagenic in vitro (Europaisches A. 1978- Pons FW, Muller P1990).Excessive consumption of caffeine through beverages is associated with a number of health problems like adrenal stimulation, irregular muscular activity (Essig D, Costill DL, van Handel PJ1980- Spriet LL, MacLean DA, Dyck DJ, Hultman E, Cederblad G, Graham TE.1992), cardiac arrhythmias (Kalmar JM, Cafarelli E1999) and increased heart output. Excess caffeine is reported to cause mutation (Essig D, Costill DL, van Handel PJ1980), inhibition of DNA repairs and inhibition of adenosine mono phosphor diesterase (Blecher R, Lingens F.1997)and during pregnancy causes malformation of fetus and may reduce fertility rates (Srisuphan W, Bracken MB 1986). Studies on caffeine degradation by microorganisms were not reported till 1970 probably because caffeine was regarded as toxic to bacteria (Sundarraj CV, Dhala S). Caffeine concentration greater than 2.5 mg/ml in the growth medium has been found to inhibit the growth of many bacterial species. Moreover, caffeine is also one of the major agro industrial wastes generated from the coffee and tea processing plants and these wastes are often released into the water bodies (Buerge IJ, Poiger T, Muller MD, Buser HR.2003).Therefore, decaffeination of waste is very necessary from the point of view of environmental conservation (Roussos S, Hannibal L, Aquiahuatla MA, Hernandez TMR, Marakis S.1994). At higher concentration, caffeine is toxic to saprophytic microorganisms that are concerned in the important biotransformation within environment, which causes disorderliness in environmental stability. Coffee waste disposal signifies vast pollution problem in the producer countries. The attempt to make use of the coffee pulp as an animal feed source has been made from the economic to the environmental view point. For this reason, the removal of anti-nutritional parts such as caffeine becomes essential (Dash SS, Gummadi SN.2010) As microorganisms have gained the capability to adapt itself to grow in harsh environments through evolutionary processes, identification of organisms from such environmental condition can yield

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interesting organisms with special characteristics. Generally caffeine hinders the growth of many organisms so, in the present work the source of bacterial identification was selected based on were the organism would have best adapted to uptake caffeine as a sole source of carbon and nitrogen, after processing coffee by dry method the coffee husk was or burnt to ash. (Kurtzman Jr RH, Schwimmer S.1971) Hence from that husk the organism capable of degrading caffeine can be identified efficiently. The present work deals with the identify of caffeine degrading organisms from coffee husk and investigating its ability to degrade caffeine by using different concentrations of caffeine in the medium provided for the growth of the organism and preparing broth for growth of cell as liquid bio fertilizer. The influence of the liquid bio-fertilizer inoculation viz. Rhizobium and phosphate solubilizing bacteria alone and in combination form. That can be maintaining the soil fertility effectively. The use of N fertilizer not only spoils the ground water of soil but also have deleterious effects by the emission of harmful gases. The chemical fertilizers should be replaced with the natural and organic fertilizers which can play a key role of the conservation of the environment. All the ingredients which were used in the present investigation for making liquid bio-fertilizer are easily available. In the present study of broth was tested for the identification of organism which was used as a liquid bio fertilizer. All the identify colonies were then inoculated on yema broth and pikovskaya ‘s broth with and without supplement of caffeine.

The Caffeine degradation by bacteria’s with their efficiency use of caffeine (g /100 ml) were Pseudomonas putida (0.03418), Alcaligenes xylosoxydans (0.6418), rhizobium sp(3.2) Pandoraea sp.(0.6216), Bacillus sp(2.75), Enterobacter aerogenes (10.1) and E.coli (6.12) the efficiency of caffeine degrade from bacteria were low because poison high concentration of caffeine. (Wang, D., Sakoda, A., & Suzuki, M. (2001). Environmental impact due to caffeine pollution was large concentrated form of caffeine can be poison the free living microbe from the soil. Those poison microbes have the potent to improve soil fertility by means of N fixing and P solubilizing for plant nutrient. Due to this negative impact plant were disappeared then change of climate and animal cultivation condition So need of use caffeine in adequate amount for use of microbes as substrate (Asano et al. 1993).

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1.2 Statement of problem

Currently biotechnological cultivation of microorganism has gained a prime position in the industries as it is cost effective and eco-friendly. However, the main problem of conventional biotechnological microorganism cultivation, one of the most serious obstacles for the future propagation of microorganism to compete with synthesis nutrient agar as substrate were its higher propagation/cultivation cost due to their grow of high cost medium and its potential impact on the human food chain due to their processing of food crops for preparations of nutrient agar. Therefore, for microorganism cultivation, low cost medium for it must be developing as soon as possible. In this case, caffeine removed from coffee husk represents a potentially inexpensive for the large-scale cultivation of MOs as substrate due to their huge abundance coffee suppliers' by-product dispose as waste because of Coffee was nowadays produced in a large number in countries approximately 37 million tone(according to Ethiopian commodity exchange Authority data sources in the year 2008 E.C) from this data 1.2 million tone was coffee husk as by product, it was expected that coffee husk which can be available in large amount in the coffee planted area(one third from total production of coffee) as waste dispose through environment which is the main cause of environmental pollution and eco-unfriendly.

From this assessments of cheap substrate are essential for the feasibility of the biotechnological cultivation of microorganism. Pharmaceuticals, waste water treatment from any factory, bio fertilizer producer and other industrial users usually require large quantities of microorganism at a relatively low cost supplied substrate. The use of low-cost, non-food materials for microorganism propagation substrate appears to be more attractive because they do not have any impact on the human food chain and environmental pollution. In addition to that biotechnological propagation of microorganism can keep its prime position over use of synthesis nutrient agar of microorganism propagation which has economical flair of industry. Because, the cultivation cost of microorganism cultivation can be significantly reduced if low cost cultivation substrate such as caffeine removed from coffee husk. Caffeine containing media could be used for the cultivation of microorganism especially rhizobium and bacillus sp of bacteria.

The demand for substrate of microorganism was depend on the development of the manufacturing sector, particularly the pharmaceuticals, food, soft drinks brewery and tannery sub sectors. Ethiopia was costing huge amount of money to import nutrient agar for substrate of

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microorganism. The country's requirement of substrate for microbe's media was entirely met through import.

1.3 Research hypothesis

Caffeine eliminated from coffee husk can be used for carbon and nitrogen source for rhizobium and bacillus spp of microbial growth with acceptable yield that bacterium has the potent to nitrify and phosphor solubilize for crops growth respectively.

1.3 General objective

The aim of this study was to evaluate the rate of caffeine degrading potency for rhizobium and bacillus bacterial strain.

1.4 Specific objective

- characterize physiochemical composition of coffee husk
- determine the removal/reduction rate of caffeine from coffee husk
- Characterization of rhizobium and bacillus sp of bacteria.
- Determine the effect and optimum of temperature, pH and incubation time for the maximum cell growth within and without caffeine contain media.

1.5 Significance of the study

Bio fertilizer plays a significant role in every cultivated crop that has the opportunity to be food for animals and human being. Today, Ethiopian use chemical fertilizer this chemical fertilizer has gradual impact on soil fertilities. In which it is known that, chemical fertilizer cover large surface area in farm land. And becoming gradual impact on fertility of soil the impact was that loss of naturally occurring bacteria those bacteria improve soil fertility without poison of the soil. Then manipulation of chemical fertilizer or chemical pesticide not recommended Because of toxicity and side reaction of chemical fertilizer with environment. If once poison naturally occurred nitrify microbes of farm land there was difficult to tolerate any growing crops. Until isolation and inoculation of microbes on the farm land so need of laboratory activity on those is nitrifying and phosphor solubilizing bacteria. So the main aim of this thesis was isolation of microbes and manipulation to the soil. Because of shelf life is more than one year easy to transport easy to handle and effective cost. Additionally it conserves soil fertility or keeping natural health care of the soil. By supplied caffeine as substrate to minimize the cost of culture.

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Chapter two

2 Literature review

2.1 Sources of caffeine:

Caffeine is found in about a hundred species of plants, but the most highly cultivated sources are the coffee beans, (Coffea Arabica or Coffea canephora, variety robusta), the leaves & leaf-buds of tea (The a sinensisor Camellia sinensis), cola nuts(Cola acuminate) and cacao beans (Theobroma cacao). Coffee and tea plants are the maor sources of natural caffeine and related compounds such as theophylline and the bromine are produced by a large number of plant species belonging to numerous genera, families, and orders it is believed that methyl xanthine-producing plants accumulate these substances as part of a chemical defense system against pests and herbivores. Interestingly, a very large proportion of the non-alcoholic beverages used in social settings contain caffeine. The most important beverages and foods containing caffeine are coffee, tea, guarana, maté, cola nuts, cola drinks, cocoa, chocolate, yaupon and yoco. The amount of caffeine found in these products varies, but is generally high. Based on dry weight, the highest amounts are found in guarana (4-7%). (ISSN:2455-944XInt .JCurrRes Biol.Med(2016).1(9):8-4411) Tea leaves contain approximately 3-5% caffeine, coffee beans 1.1-2.2 %(Saldana et.al 2000), cola nuts1.5%, and cocoabeans 0.03%(Bogo and Mantle, 2000; Kretschmar and Baumann, 1999). Cocao beans in addition contain about 1.8-2.5%\\theobromine. Caffeine also occurs in certain soft drinks, energy drinks, and so called “smart” drinks, as well as in medicinal drugs. In these cases, however, purified or synthesized caffeine has often been added to the products.

2.2 History of coffee plantation

Coffee began to be savored in Europe in 1615, brought by travelers. Germans, Frenchmen, and Italians were looking for a way of developing the plantation of coffee in their colonies. But it was the Dutchmen who got the first seedlings and who cultivated them in the stoves of the botanical garden of Amsterdam, a fact that made the drink one of the most consumed in the old continent and becoming a definitive part of the habits of the Europeans. Next, the Frenchmen were given a plant of coffee by the major of Amsterdam, and they began to cultivate in the islands of Sandwich and Bourbon (Neves 1974). With the Dutch and French experiences, the coffee cultivation was taken to other

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European colonies. The European market growth favored the expansion of the plantation of coffee in African countries and was also through the European colonists that coffee reached Puerto Rico, Cuba, Suriname, São Domingos, and Guianas. Through the Guianas, coffee arrived to the north of Brazil. Then, the secret of the Arabs was spread by the entire world (Taunay 1939). The coffee tree or shrub belongs to the family Rubiaceae. Coffee beans are produced from the plant Coffea L., of which there are more than 70 species. However, only two of these species are commercially explored worldwide: Coffea arabica (Arabica), considered as the noblest of all coffee plants and providing 75% of world's production; and Coffea canephora (Robusta), considered to be more acid but more resistant to plagues, and provides 25% of world's production (Belitz et al. 2009; Etienne 2005). C. arabica is a bush originally from Ethiopia and develops well in high altitudes (600–2,000 m), while C. canephora plantations adapt well in altitudes below 600 m (Comité Français du Café 1997).

2.3 Coffee World Production

World coffee production has grown more than 100% from 1950 to 1960, and there was a prediction to grow more 0.5–1.9% by 2010 (Fujioka and Shibamoto 2008). Coffee is nowadays produced in a large number of countries worldwide. Nevertheless, the ten largest coffee producing countries are responsible for approximately 80% of the world production. Of this percentage, South America participates with around 43%, Asia with 24%, Central America 18%, and Africa with 16%. Brazil, Vietnam, Colombia, and Indonesia are respectively the first, second, and third largest world producers, responsible for more than half of the world supply of coffee (Table 2). According to the International Coffee Organization (ICO 2010), in 2009 Brazil produced approximately 40 million bags of coffee (Table 2). The world consumption of coffee in 2007, estimated by the International Coffee Organization, has been around 124,636 million bags of 60 kg, representing an increase of 2.88% regarding the 121,150 million sacks consumed in 2006 (ICO 2010*).* Despite the financial crisis, the world consumption of coffee in 2008 was

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Table 1 Annual worldwide coffee production (million bags of 60 kg)(From ICO(2010))

Countries	Production					
	2004	2005	2006	2007	2008	2009
Brazil	39.272	32.944	42.512	36.070	45.992	39.470
Vietnam	14.370	13.842	19.340	16.467	18.500	18.000
Colombia	11.573	12.564	12.541	12.504	8.664	9.500
Indonesia	7.536	9.159	7.483	7.777	9.350	9.500
Ethiopia	4.568	4.003	4.636	4.906	4.350	4.850
India	4.592	4.396	5.159	4.460	4.372	4.827
Mexico	3.867	4.225	4.200	4.150	4.651	4.500
Guatemala	3.703	3.676	3.950	4.100	3.785	4.100
Peru	3.425	2.489	4.319	3.063	3.872	4.000
Honduras	2.575	3.204	3.461	3.842	3.450	3.750
Côte d'Ivoire	2.301	1.962	2.847	2.598	2.353	1.850
Nicaragua	1.130	1.718	1.300	1.700	1.615	1.700
El Salvador	1.437	1.502	1.371	1.621	1.547	1.500
Other countries	15.713	15.779	16.019	16.138	15.680	15.455
Total	116.062	111.463	129.138	119.396	128.181	123.002

Around 128 million bags. According to ICO, the consumption of coffee was not affected by the crisis. The consumers will not stop drinking coffee, but instead of drinking high quality coffee, people will start to take coffee of middle quality.

2.4 Anatomy of the coffee fruit

Inside the skin, the epicarp, is a sweet-tasting mesocarp called pulp. Within the mesocarp is a thin layer of endocarp called parchment. The endosperm, the coffee bean, is also covered with a spermaderm called silver skin. The bean consists of two hemispheres with flattening adjacent sides. Each bean has an inner layer of silver skin while the parchment both covers the spheres and separated them from each other (Figure 1). There are two species which provides almost 100% of the production in the world: Coffea arabica and Coffee canephora, which are commonly referred to as Arabica and Robusta (Berlitz et al., 2009; Mussatto et al., 2011b). The chemical composition differ between the green beans from the two species table 2

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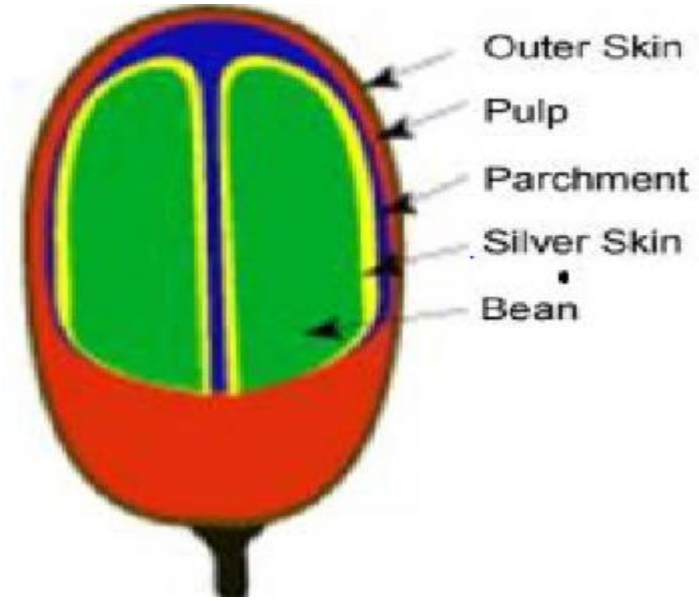

Fig 1 the anatomy of the coffee fruit (Murthy and Naidu, 2012a)

Table 2 Chemical composition of green beans from Arabica and Robusta bertilz et al 2009) in % of solid

	Arabica		*Robusta*
Soluble carbohy-drates	9.0- 12.5		6.0- 11.5
Monosaccharides		0.2-0.5	
Oligosaccharides	6.0- 9.0		3.0- 7.0
Polysaccharides		3-4	
Insoluble carbohy-drates	46- 53		34- 44
Hemicellulosa	5.0- 10		3.0- 4.0
Cellulosa	41- 43		32-40
Chlorogenic acid	6.7- 9.2		7.1- 12.1
Lignin		1- 3	
Lipids	15- 18		8.0- 12
Protein		8.5- 12	
Caffeine	0.8- 1.4		1.7- 4.0
Minerals		3.0- 5.4	

However, coffee bean is constituted by several other components, including cellulose, minerals, sugars, lipids, tannin, and polyphenols. Minerals include potassium, magnesium, calcium, sodium, iron, manganese, rubidium, zinc, copper, strontium, chromium, vanadium, barium, nickel, cobalt, lead, molybdenum, titanium, and cadmium. Among the sugars, sucrose, glucose, fructose, arabinose, galactose, and mannose are present. Several amino acids such as alanine, arginine, asparagine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine can also be found in these beans (Belitz et al. 2009; Grembecka et al. 2007; Santos and Oliveira 2001). Additionally, coffee

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beans contain vitamin of complex B, the niacin (vitamin B3 and PP), and chlorogenic acid in proportions that may vary from 7% to 12%, three to five times more than the caffeine (Belitz et al. 2009; Lima 2003; Trugo 2003; Trugo and Macrae 1984). Table 1 shows the chemical composition of coffee beans from Arabica and Robusta varieties. Among the substances present in the chemical composition of coffee, only caffeine is thermostable, i.e., it is not destroyed by excessive roasting. Other substances such asproteins, sugars, chlorogenic acid, trigonelline, and fat may be preserved or even destroyed and transformed into reactive products during the coffee roasting process (Ginz et al. 2000; Lima2003; Rawel and Kulling 2007; Trugo 2003; Trugo and Macrae 1984).

2.5The by-products and their applications

During the different processes to obtain the beans, large amounts of by-products are generated (Mussatto et al., 2011b; Nabais et al., 2008) as approx. 50% of the coffee fruit is not used for the production of green coffee beans (Berlitz et al., 2009; Esquivel and Jimenez, 2012). The by-products are mainly used as fertilizers (Berlitz et al., 2009), but the usage for that is limited due to the high amount of organic material. The high concentrations of caffeine, tannins and polyphenols (Mussatto et al., 2011b; Pandey et al., 2000) make the by-products highly pollutant (Fan et al., 2006; Mussatto et al., 2011b) and unsuitable as animal feed in larger quantities (Delfiol et al., 2012; Orozco et al., 2008). The main by-product from the dry method is the coffee husk which is composed of the dried skin, pulp and parchment (Esquivel and Jimenez, 2012). Of each ton harvest coffee fruit, 0.18 ton of coffee husk are produced. From the wet method, the by-products are the coffee pulp and the coffee silver skin. Another by-product which is obtained after brewing is spent coffee grounds (Murthy and Naidu, 2012a), also called coffee extract residue (Tehrani et al., 2015)

2.5.1 Residues Generated in the Coffee Industry

The generation of residues and by-products is inherent in any productive sector. The agro-industrial and the food sectors produce large quantities of waste, both liquid and solid. Coffee is the second largest traded commodity in the world, after petroleum, and therefore, the coffee industry is responsible for the generation of large amount of residues (Nabais et al. 2008). In the last decade, the use of such wastes has been subject of several studies, but this concern did not exist in past decades (1930 to 1943) when 77 million bags of green coffee were simply burned and released to the sea and in landfills (Cunha 1992). However, this is an important topic explored nowadays. Coffee silverskin (CS) and spent coffee grounds (SCG) (Fig. 2) are the main coffee

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industry residues. CS is a tegument of coffee beans obtained as a by-product of the roasting process. It is a residue with high concentration of soluble dietary fiber (86% of total dietary fiber) and high antioxidant capacity, probably due to the concentration of phenolic compounds in coffee beans, as well as to the presence of other compounds formed by the Maillard reaction during the roasting process, such as melanoidins (Borrelli et al. 2004). Microscopic examination (Fig. 2b) shows the presence of fibrous tissues from the surface layers of the CS. The main components of these fibrous tissues are cellulose and hemicellulose. Glucose, xylose, galactose, mannose, and arabinose are the mono saccharides present in CS; glucose being found in major amounts. Proteins and extractives are also fractions present in significant amounts in this coffee waste. SCG is a residue with fine particle size (see Fig. 2d, which is 50-fold, magnified), high humidity (in the range of 80% to 85%), organic load, and acidity, obtained during the treatment of raw coffee powder with hot water or steam for the instant coffee preparation. Almost 50% of the worldwide coffee production is processed for soluble coffee preparation (Ramalakshmi et al. 2009). Therefore, SCG is generated in large amounts, with a worldwide annual generation of 6 million tons (Tokimoto et al. 2005). Numerically, 1 ton of green coffee generates about 650 kg of SCG, and about 2 kg of wet SCG are obtained to each 1 kg of soluble coffee produced (Pfluger 1975). Chemical composition of this residue is shown in Table 4. It can be noted that SCG are richer in sugars than CS, among of which mannose and galactose are the most abundant. Proteins constitute also a significant fraction in SCG (Mussatto et al. 2011). CS neend of treat is was residue is high concentration of soluble dietary fiber.

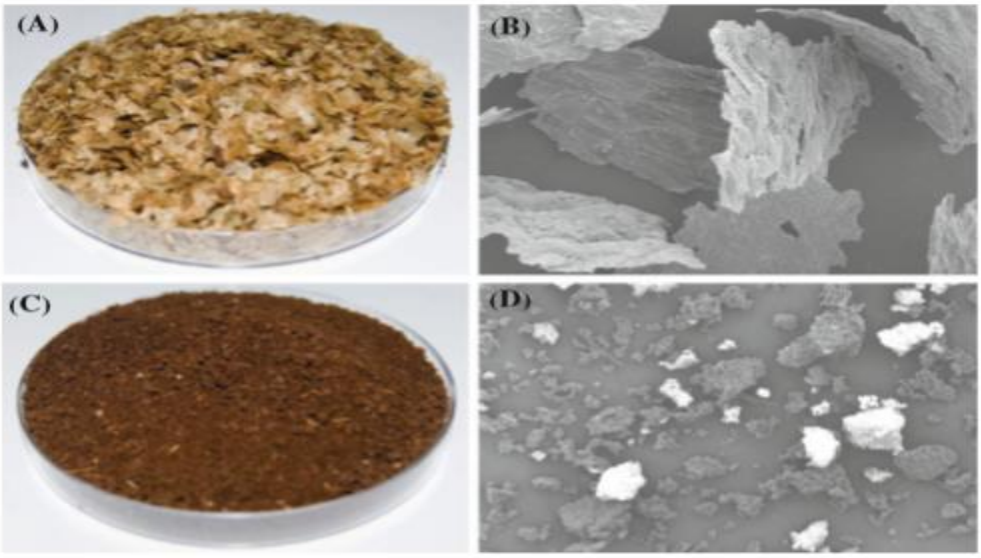

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Fig 2 Appearance of coffee silverskin (a, b) and spent coffee grounds (c, d). Scanning electron microscopy (b, d) of particles at 50-fold magnification

2.5.2 Coffee Industry Residues Applications

Nowadays, there is great political and social pressure to reduce the pollution arising from industrial activities. Almost all developed and underdeveloped countries are trying to adapt to this reality by modifying their processes so that their residues can be recycled. Consequently, most large companies no longer consider residues as waste, but as a raw material for other processes (Mussatto et al. 2006). Due to the presence of organic material, CS and SCG are highly pollutant residues, and demand great quantities of oxygen to degrade (Silva et al. 1998).

2.5.3 Proposed Utilization of Coffee Industrial Residues

Chemical composition of CS and SCG, based on cellulose, hemicellulose, and protein, opens up possibilities for application of these residues in the production of different value-added compounds. Cellulose, for example, is a linear homo polymer of repeated glucose units extensively used for the pulp and paper production. Besides this potential application, cellulose can be converted to sugars such as polysaccharides, oligosaccharides, and mono saccharides by different treatment processes using acids or enzymes as catalysts (Mussatto et al. 2008a; Rinaldi and Schüth 2009). The conversion of cellulose to glucose is the first step in the large-scale chemical utilization of cellulose since this sugar may be subsequently converted to several products of interest such as ethanol (Mesa et al. 2010; Shen and Agblevor 2010), butanol (Qureshi and Ezeji 2008), hydrogen (Pan et al. 2010; Ren et al. 2009), organic acids (Mussatto et al. 2008b; Zhuang et al.2001), glycerol (Taherzadeh et al. 2002), and hydroxyl methyl furfural (Huang et al. 2010), among others. Hemicelluloses are heteropolymers constituted by 5-carbon sugars such as xylose and arabinose, and 6-carbon sugars including mannose, galactose, and others. As well as glucose can be released from cellulose, these pentose and hexose sugars may also be released from the hemicellulose structure by means of some chemical or enzymatic pretreatment (Mussatto and Roberto 2004). Furfural once served as the raw material for nylon until displaced by butadiene, a chemical currently derived from petroleum (Wang and Huffman 1981). Xylose can also be used for the production of ethanol (Silva et al. 2010) as well as mannose and other hexose sugars (Jorgensen et al. 2010; Machado 2009). Low molecular weight aliphatic compounds (ethylene and propylene) could be derived from ethanol produced by fermentation of these

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sugars (Mussatto et al. 2006). Mannose may be used also for the production of polyols like mannitol, which has great application in the food industry (Ghoreishi and Shahrestani 2009). Figure 4 summarizes other potential applications for cellulose and hemicellulose fractions present in CS and SCG. In addition to cellulose and hemicellulose, the protein content is also significant in CS and SCG. Therefore, and also due to the large continuous supply and relative low cost, both coffee industry residues could be considered as adjunct for human food. Other protein-rich residues like brewer's spent grain have been successfully evaluated for nutritional enrichment of food products (Miranda et al. 1994). Incorporation of CS and SCG for the manufacture of flakes, breads, biscuits, and aperitif snacks would be an interesting alternative for application of these materials and should be also evaluated. Besides the aforementioned applications, it is worth mentioning that the simultaneous presence of polysaccharides, proteins, and minerals makes CS and SCG substrates of high biotechnological value, which might be used, for example, as substrates or solid supports in fermentative processes, for microorganisms' cultivation, and extraction/ production of compounds with applications in the food and pharmaceutical industries. Selection of the most potential microorganisms' strains and the establishment of the best process conditions are the main challenges to efficiently convert those components into value-added products. In fact, there are a number of feasible uses for CS and SCG, but up till now, these residues remain few explored as raw material for the recovery and/or production of value added compounds, as well as nutritional source for food products (Murthy and Naidu 2010b). This is a research area with great potential to expand in the future for economic and environmental viewpoints. Attention should also be paid to finding economical methods for drying SCG since this material in the wet form (approximately 80% moisture content) requires high cost of transportation.

2.6 Coffee husks

Coffee husks are the major solid residues from the handling and processing of coffee, since for every kg of coffee beans produced, approximately 1 kg of husks are generated. Proposed alternative uses for coffee husks include employing this solid residue as a supplement for animal feed, direct use as fuel, fermentation for the production of a diversity of products (enzymes, citric acid and flavoring substances), use as a substrate for growth of mushrooms/bacteria and use as adsorbents (Franca and Oliveira 2009). However, considering the high amounts generated, there is still a need to find other alternative uses for this solid residue.

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Coffee husk has properties that could be utilized in a food perspective. Its high concentration of caffeine and tannins, which are negative in environmental perspective, could be extracted for use in “energy drinks” or “energy bars A further application for coffee husk is as brewed for tea, called coffee cherry tea (Pabari, 2014), the name depending in which country the coffee husk are brewed. The coffee husk is brewed alone or with spices, e.g. cinnamon. The taste is described as fruity, with notes from watermelon and blackcurrant (Pabari, 2014) to strawberries and raisins (Wiser, 2011). Recurring are notes of citrus and cherry. Pabari (2014) suggests that the tea contains caffeine, based on the perceived energized experience after consumption. However, no scientific studies have been performed on the subject yet.

2.7 The chemical composition of coffee husk

The proximate composition of the coffee husks employed in the present study was determined as 15.0% moisture, 5.4% ash, 7.0% protein, 0.3% lipids and 72.3% carbohydrates. The high contents of carbohydrates are expected, given the origin of such solid residue, i.e., fruit pulp and outer skin. Cellulose, hemi-cellulose and lignin contents were 16, 11 and 9% dry basis, respectively. Such levels are low or at most similar to other agricultural residues considered as alternatives for ethanol production, including sugarcane bagasse, barley and wheat straws, and rice husks, among others. This is an indication that this solid residue seems quite promising for ethanol production, given the high percentage of sugars that are readily available for fermentation. It is noteworthy to mention that the coffee husks employed in the present study are denominated sticky coffee husks. Some attributes that differentiate this specific type of coffee husks from the regular dry processed ones include its higher density, protein contents and lower fiber contents. A comparison with chemical composition data obtained from the literature is presented in Table 3 However, the major difference lies in its higher cellulose contents (see Table 3), which were approximately twice the amount

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Table 3 Chemical composition of coffee husks (% dry basis)

	Coffee husks	Sticky coffee husks
Protein	8–11	9–10
Lipids	0.5–3	0.7–1.2
Minerals	3–7	5–6
Total carbohydrates	58–85	83–85
Cellulose	43	16–25
Hemicellulose	7	9–11
Lignin	9	6–10
Caffeine	~1	0.6
Tannins	~5	0.8–1.2

4.8 Caffeine

The physiological role of this well-known alkaloid of the methylated purine type is well documented, and no attempt will be made to review it here. However, three factors appear to be important in relation to coffee pulp and the effects observed in various animals: the relatively, high concentration of nitrogen in caffeine;, Its known effect of stimulating increased activity; and its well-known diuretic effect. Caffeine contains 26.38% N and is found in an average concentration of 1.0% in dehydrated coffee pulp. This means that caffeine nitrogen is present in amounts of about 0.26%, which is equivalent to a crude protein value of 1.6%. On the other

4.9 Tannins

The phenolic compounds discussed in the previous section are monomeric compounds, but in plant tissue, there are also two groups of polymeric substances of a phenolic nature. Lignin, derived from polymerization of phenyl propanoid units, and cellulose constitute the structural material of all higher plants. The second group of polymeric phenolic substances is known under the poorly defined name of tannins. Chemically, tannins may be grouped into two types: the hydrolysable tannins, which yield gallic acid and sugars on hydrolysis; and the condensed tannins, which are derivatives of flavonoid monomers. The possible role of hydrolysable tannins as related to the adverse effects of coffee pulp on animal performance has already been discussed. The most important characteristic of tannins is probably their high capacity to bind proteins, making them unavailable to the organism, but they also act as enzyme inhibitors.

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Dietary proteins can, by complexing with tannins, be protected from hydrolysis by proteolytic enzymes in the rumen (Leroy et al. 967). These polymeric compounds can, therefore, interfere with animal performance by lowering the availability of the protein that is consumed, by inactivating enzymes, or by acting as sources of free phenolic compounds. Evidence of their particular role is not available; however, dehydrated coffee pulp contains about 50% of its protein in a lignified form.

2.10 Decaffeination

Decaffeination is defined as the act of removing caffeine from coffee beans and tea leaves (http://en.wikipedia.org/wiki/Decaffeination). Most decaffeination processes are performed on unroasted (green) coffee beans, but the methods vary somewhat. It generally starts with the steaming of the beans. They are then dipped into solvent for several hours. The process is repeated for 8 to 12 times until it meets either the international standard of having removed 97 % of the caffeine in the beans or the EU standard of having the beans 99.9 % caffeine free by mass.

The first commercially successful decaffeination process was invented by Ludwig Roselius and Karl Wimmer in 1903. It involved steaming coffee beans with a brine (salt water) solution and then using benzene as a solvent to remove the caffeine. Coffee decaffeinated this way was sold as Cafe sanka in France and later as Sanka brand coffee in the US Due to health concerns regarding benzene, this process is no longer used commercially and Sanka is produced using a different process. Three different methods of decaffeination, widely used, are; 'Water decaffeination', 'Solvent decaffeination' and 'Carbon dioxide decaffeination'. Although caffeine is water soluble above 175^{o} F, water alone is generally not used to decaffeinate coffee because it strips away too many of the essential flavor and aroma elements.

Decaffeination by solvents can be through two methods: direct and indirect contact. In the first the beans come directly in contact with the decaffeinating agents, after being softened by steam. In the latter method, a water/coffee solution is normally used to draw off the caffeine; after being separated from the beans, the solution containing the caffeine is then treated with a decaffeinating agent. In both methods, the agent is removed from the final product.

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2.10.1 Solvent decaffeination:

A solvent is used for decaffeination in this technique. There are criteria in choosing the right solvent for this process. According to Katz (1987), some of the criteria include: Safety, cost, caffeine solubility, ease of solvent removal and recovery, toxicity and chemical reactivity, and environmental effects. The common solvents used are methylene chloride and ethyl acetate. However, methylene chloride is mostly used in the industry. This chemical is more selective to remove caffeine without removing the taste and aroma of coffee. According to the United States Food and Drug Administration (FDA), most decaffeinated coffee has less than 0.1 parts per million residual methylene chlorides. The process of solvent decaffeination involves steaming, pre-wetting, caffeine extraction, steam stripping, and drying. Green coffee beans are transferred into an extractor, steamed to make the surface more permeable so that the caffeine can be easily extracted when the solvent comes in contact with the caffeine. After steaming the beans are steeped in water to increase their moisture content to 40 % by weight Pre wetting water and solvent (methylene chloride or ethyl acetate) are added together in this step. The ratio of solvent to beans is 4:1 (Pintauro, 1975). Caffeine in the beans is extracted by heating the solvent, at a temperature of 150°F. The caffeine extraction step takes about 10 hours to be completed. About 97 % of the caffeine in the green coffee beans is extracted in this step. Solvent stripping or steam stripping is then done on the green coffee beans. The main purpose of this step is to get rid of any residual methylene chloride or solvent. The coffee beans are then dried and stored. However, due to health concerns the use of solvent decaffeination has greatly decreased in recent years.

2.10.2 Water decaffeination:

Water decaffeination, uses water to extract caffeine from the green coffee beans the water decaffeination is probably the most widely accepted method used to decaffeinate coffee. This method is based on the natural ability of water to make caffeine soluble. However, in this process the water acts non-selectively on the raw coffee, extracting all of the soluble components, like the aromas and the flavor. In order to prevent the extraction of all water-soluble components of coffee beans, the extraction water contains essentially equilibrium quantities of the non-caffeine soluble solids (Katz, 1987). The coffee beans are kept in the extractor for about 8 hours to remove about 98 % of the original caffeine. The extract water with caffeine, coffee solids, coffee aroma and flavor is subjected to caffeine extraction by solvents. The decaffeinated coffee beans are then washed, dried and stored. Another form of water decaffeination is the Swiss Water

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Decaf method .This method is also based on the theory of the caffeine being soluble in water; however it is not necessary to return the other soluble components to the bean. Swiss water decaffeination is a relatively simple process. The coffee beans are extracted in hot water, removing the caffeine and the flavor components of the bean into the water. After the water has been saturated, the caffeine is removed by passing the water through carbon filters. Caffeine is adsorbed on to the carbon filters and the caffeine free extract is reabsorbed by the beans, which are dried and roasted.

2.10.3 Supercritical carbon dioxide decaffeination:

The supercritical carbon dioxide decaffeination is considered to be a safer process than the solvent decaffeination. By using only carbon dioxide and water this method has gained acceptance as being a natural method of decaffeination. The supercritical carbon dioxide decaffeination uses carbon dioxide gas that has been compressed and subjected to high temperature. The combination of high temperature and pressure enables carbon dioxide to become a solvent. The decaffeination process begins with pre wetting the beans with steam, loading of the prewetted coffee beans into an extractor and at the same time solid absorbent (activated carbon adsorber) is loaded into a vessel moist carbon dioxide is also loaded into the vessel that contains coffee beans and the solid absorbent. The supercritical carbon dioxide is then circulated between the extractor and solid adsorber vessel. As the carbon dioxide passes through the extractor, caffeine is extracted and the caffeine rich carbon dioxide flows to the adsorber where the caffeine is adsorbed. The caffeine-free carbon dioxide then goes through the cycle again. This process is continued till the desired level of decaffeination is achieved. The beans are then dried and stored.

The advantage of this process is that no flavor elements are lost from the coffee and 98% of caffeine can be removed from the coffee beans. However, there are a couple of disadvantages. The first disadvantage is that due to the high pressure used, the equipment is costly and only batch processing can be done (Katz, 1987). The second disadvantage is that the average concentration of caffeine in the carbon dioxide is low; therefore a large quantity of carbon dioxide is needed. This might also be costly. The disadvantages might cause the price of the decaffeinated coffee beans to be higher than solvent decaffeinated and water decaffeinated coffee beans. Moreover, the use of membranes or carbon filters in caffeine removal processes will be

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very expensive and the commercialization of the process becomes less viable.In lieu of the disadvantages of the existing processes of decaffeination research there has been an increasing emphasis of developing greener and economic methods of decaffeination.

Biotechnological decaffeination methods are the only alternatives, which offer safe, economical and greener routes of decaffeination of beverages. Moreover biological means of decaffeination have a wider reach of application even to pollution abatement due to coffee and tea processing wastes.

2.10.4 Bio decaffeination: A natural route of decaffeination:

Bio decaffeination can be defined as the removal of caffeine from coffee, tea and other caffeine containing materials by the action of externally added microbial cells or enzymes. The concept of bio decaffeination is a relatively new area of decaffeination and there is a growing interest in this area of biotechnology due the advantages it offers like being environmentally safe, economical and in preserving the quality of the beverages. Development of biological or enzymatic methods of decaffeination demands a deep understanding of the caffeine metabolism in microbial, plant and animal systems. A thorough knowledge of the caffeine metabolism, the enzymes involved and various factors involved in the caffeine degradation in different living systems will give deep insights into the development of efficient bio decaffeination processes. Detailed information on different enzymes involved in the degradation of caffeine in different organisms could help in developing an enzymatic process for caffeine removal.

Bacteria can be used in reducing the caffeine content in caffeine bearing plants. It has been found that leaf surface play a vital role in Agrobacterium infection in tea plants (Kumar et. al., 2004). A method has been proposed for producing tea leaves with less caffeine content by growing caffeine degrading bacteria on the surface of the leaf. Ramarethinam and Rajalakshmi (2004) found in situ lowering of caffeine in tea leaves without affecting the quality of the other tea components when tea plants were sprayed with a suspension of Bacillus licheniformis. Anaerobic fermentation of coffee pulp resulted in about 13–63% reduction of caffeine in 100 days (Porres et. al., 1993). In contrast, aerobic fermentation resulted in 100% degradation of caffeine in 14 days (Rojas, et. al., 2003).

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Several studies were carried out to investigate the use of purines, including caffeine, as a source of energy for microorganism growth (Schwimmer and Kurtzman., 1971; Woolfolk, 1975; Woolfolk and Downard, 1977; Middelhoven and Bakker, 1982; Mazzafera et. al.,1994a). A comprehensive review on purine utilization by microorganisms was published by Vogels and Drift (1976). Although fungi growing on caffeine have been isolated, most of the studies were done with bacteria isolated from soil, mainly those belonging to the Pseudomonads group, with particular attention to Pseudomonas putida (Burr and Caesar, 1985). Madyastha et. al., (1998, 1999) have reported the degradation of caffeine by a consortium of bacteria belonging to Klebsiella and Acinetobacter species.

2.11 Immobilization

Bio decaffeination of tea, coffee and other caffeine containing materials is gaining importance due to the increasing demand for the decaffeinated products and the consumer preference towards naturally bio decaffeinated products. Development of bio decaffeination processes for caffeine containing materials requires the employment of microorganisms or enzymes capable of degrading caffeine. Immobilized cells have advantages over free cells due to the retention of the cells in the matrix enabling reuse of the immobilized cells and multi enzymes involved in sequential degradation of caffeine to NH3 and CO2. The conversion of caffeine to its metabolites is primarily brought about by N-demethylases (such as caffeine 1Ndemethylase and 3N-demethylase), xanthine oxidase, uricase, urease etc., that are produced by several caffeine-degrading bacterial species such as Pseudomonas putida, Serratia, Alcaligenes, Rhodococcus, Klebsiella, etc. Development of bio decaffeination techniques using whole cells offers an attractive alternative to the present existing chemical and physical methods removal of caffeine, which are costly, toxic and nonspecific to caffeine.

The technique used for the physical or chemical fixation of cells, organelles, enzymes, or other proteins (e.g. antibodies), Nucleic acids (DNA, RNA) onto a solid support, into a solid matrix or retained by a membrane, in order to increase their stability and make possible their repeated or continued use making the process economical. The industrial biotechnology processes using microorganisms generally involve the cells suspended in the fermentation medium. The classical fermentations suffer from various constrains such as low cell density, nutritional limitations, and batch-mode operations requiring high power input. It has been well recognized that the microbial

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cell density is of prime importance to attain higher volumetric productivities. The continuous fermentations with free-cells and cell recycle options aim to enhance the cell population inside the fermentor During the last 20–25 years, the cell immobilization technology, with its origins in enzyme immobilization, eliminates most of the constrains faced with free-cell systems and has attracted the attention of several research groups. The remarkable advantage of immobilized cell based system is the freedom it has to determine the cell density prior to fermentation. It also facilitates operation of microbial fermentation on continuous mode without cell washout. Since the early 70s, when Chibata's group (Chibata et.al., 1974 a&b) announced successful operation of continuous fermentation of l-aspartic acid, numerous research groups have attempted various microbial fermentations with immobilized cells. Several process based on immobilized microbial cells have been developed.

2.11.1 Immobilization methods:

Many methods namely adsorption, covalent bonding, cross-linking, entrapment, and encapsulation are widely used for immobilization (Groboillot, et.al.1994). Every method has its own advantages and disadvantages and the immobilization method varies from process to process.

2.11.1.1 Adsorption:

Adsorption of cells to surfaces is a mild process, and suitable for obtaining viable cells and the adsorption is based on non-covalent forces such as ionic interactions. Ion-exchange materials such as Dowex-1 and DEAE-cellulose have proved useful (West and Strohfus, 1996). Cell immobilization by this method depends on a number of factors and one of the most important is the charge on the support material.

The disadvantage of adsorption is cell leaching, and this would cause serious problems if the cell continues growing downstream of the reaction or provides a source for bacterial growth or releases contaminating proteins and biochemical when the cell is disrupted (Yaskovich, 1998). Micro carriers (MC) are known to be the best supports for adsorption of cells.

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2.11.1.2 Covalent bonding:

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (crosslinking) agent. For covalent linking, chemical modification of the surface is necessary. Cells of S. cerevisiae were immobilized by coupling silanized silica beads (Novarro and Durand, 1977). The reaction requires introduction of reactive organic group on inorganic silica surface for the reaction between the activated support material and yeast cells. a -amino propyl triethoxy silane is generally used as the coupling agent (Marek et. al., 1986). This inorganic functional group condenses with hydroxyl group on silica surface. As a result, the organic group is available for covalent bond formation on the surface of silica. Covalent bonding can also be achieved by treating the silica surface with glutaraldehyde and isocyanate (Kennedy and Cabral, 1985). A system of more general interest has been developed by Kennedy and Cabral (1985), using inorganic carrier system. The addition of Ti4+ or Zr4+ chloride salts to water results in pH-dependent formation of gelatinous polymeric metal hydroxide precipitates wherein the metals are bridged by hydroxyl or oxide groups. By conducting such a precipitation in a suspension of microbial cells, the cells have been entrapped in the gel-like precipitate formed. In continuous operation, titanium hydroxide immobilized cells of Acetobacter were employed to convert alcohol to acetic acid.

2.11.1.3 Entrapment:

The most extensively studied method in cell immobilization is the entrapment of microbial cells in polymer matrices. The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. Among the above matrices, polyacrylamide has been widely used by several workers (Martinsen, et.al., 1989). The entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium while still allowing penetration of substrate. The other procedures for network formation for cell entrapment are precipitation, ion exchange gelation, and polymerization. The precipitation techniques are exemplified by collagen (Kurosawa, et al., 1989),cellulose and carrageenan (Axelsson, et.al., 1994). Entrapment of cells in alginate gel is popular because of the requirement for mild conditions and the simplicity of the used procedure. Several reports on alginate gel are available (Jamuna, et.al., 1992).

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κ -carrageenan is one of the earliest gel materials used for cell immobilization for continuous production of lactic acid by Escherichia coli (Ogbonna et.al., 1989).The immobilization procedure is similar to alginate. Using k -carrageenan, Takata et al. (1978) reported that the immobilized Brevibacterium flavum attained high stability against several denaturing chemicals. The rate of cell leakage could be lowered by hardening the gel with potassium cations. Similarly several other natural polymers such as agar, agarose, pectin and gelatin were also employed for cell immobilization. The reversible network formed is affected by certain calciumchelating agents like phosphates, Mg2+, K+ and EDTA and the gel integrity was poor.

2.11.2 Application of immobilized cells for bio decaffeination:

Caffeine is an alkaloid naturally occurring in coffee, cocoa beans, cola nuts and tea leaves, and is a central nervous system stimulant. It is known to show toxicity when fed in excess and is even mutagenic in-vitro (Friedman and Waller, 1983a and b). Excessive consumption of caffeine through beverages is associated with a number of health problems (Friedman and Waller, 1983a and b, Srisuphan and Bracken, 1986, Dlugosz et.al., 1996). Increasing knowledge of the effects of caffeine on human health led to the development of processes for decaffeination using solvents which are considered unsafe for humans. Biotechnological means of decaffeination have been considered as safe alternatives for the conventional decaffeination processes. Since 1970's several studies have been conducted by several groups in the world on the identification of caffeine degrading organisms for possible use in the development of bio decaffeination technologies.

Caffeine degrading bacteria and fungi have immense potential in the decaffeination processes for utilization of coffee, tea and other caffeine containing wastes which are otherwise unusable and pose sever health and environmental problems (Roussos, et.al., 1995). Although several reports on the use of free cells of bacteria and fungi for the degradation of caffeine are available, they are limited to solid state fermentation of caffeine containing agro wastes (Jarquín, 1987). Caffeine is now being determined as a marker for contamination of water and processes involving decontamination of caffeine laden waste waters have high environmental significance. Literature in this area is scanty. Middelhoven and Beckker- (1982) report the immobilization of a caffeine-resistant strain of Pseudomonas putida isolated from soil in agar gel particles which were continuously supplied with a caffeine solution in a homogeneously mixed aerated reaction

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vessel. The caffeine degradation was monitored in this reactor system. No other reports are available on the immobilization of microbial cells for the degradation of caffeine. Caffeine degrading microorganisms utilizing caffeine as the sole source of carbon and nitrogen have been isolated and characterized which have enzymes that bring about the actual degradation of the substrate. In this thesis, we report the determination of an effective caffeine-degrading microbe bacillus sp. and rhizobium from soil, its growth and decaffeination studies followed by process optimization.

2.12 Caffeine degradation by bacteria:

However, some microorganisms have the ability to grow in the presence of caffeine and survival would be related to their capacity to degrade the alkaloid (Sundarraj and Dhala, 1965). Actually, it is not rare to find bacterial strains resistant to caffeine (Woolfolk, 1975). Some microorganisms, e.g., Klebsiella pneumoniae, bacillus sp and rhizobium sp can utilize purines as carbon or nitrogen sources (Vogels and Drift, 1976.)

First report on caffeine degradation by microorganisms was in the early 1970s (Kurtzman and Schwimmer, 1971). Since then progress has been achieved on using caffeine as source for microbial growth (Schwimmer and Khurtzman, 1971; Vogels and Drift, 1976; Roussos et. al., 1995). A few reports in the literature have already described the isolation of bacteria strains from soil with the ability to degrade caffeine (Wool folk, 1975; Blecher and Lingens, 1977; Gluck and Lingens, 1987; Mazzafera et.al, 1994a). Bacterial strains capable of degrading caffeine belonged to Pseudomonas and Serratia genus. Caffeine concentration greater than 2.5 mg/ml in the growth medium has been found to inhibit the growth of many bacterial species. Synergistic effect has been observed when caffeine is added to antimicrobial agents like chloramphenicol (Sundarraj and Dhala, 1965). Attempts were made for biological production of caffeine catabolic intermediates with the help of inhibitors. Asano et. al., (1993) reported the production of theobromine using Pseudomonas strain for the first time.

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Chapter three

3. 1Experiment Location, reagent, Material and method

3.1.1 Experiment Location

This thesis determination of caffeine degrading microbes and process optimizations experiment were done from the institute ESA (Ethiopia standard agency) the experiments were absorbance of caffeine at 600nm for both sp. of bacteria with spectroscope and physiochemical property of coffee husk was analyzed , Ethiopia biodiversity institute and from Addis Ababa institute of technology done most of the activity were shaking incubator of fermentation serial dilution count of viable cell(spread plate techniques) with school of chemical and bio engineering within bio laboratory were carried out.

3.1.2 Materials (equipment) are used in this thesis work

Apparatus used for this research were include compound microscopy, electronic beam balance, filter paper, fennels, beakers, pipettes (volumetric), volumetric flasks(100ml) , pH meter. petri dish bean, Vacuum apparatus separator funnels, beaker, heater, and 250 mL round-bottom flask bakers, cylinder shaker and Laminar flow or biological safety cabinet for the experiment run on Burner: Conical flask: Test tube, Glass rod: the revolving equipment that used to make the mixture homogenous during dilution Glass bottle to cultivate(grow) the microorganism Autoclave: to sterilize the petri dish, conical flask etc. Incubator: used for microorganism growth under controlled conditions. Aluminum foil and cotton: to cover the conical flask during sterilization of the prepared distilled, agar and broth nutrient.

3.1.3 Reagent (chemical) use in this thesis work

The reagents used in this research are Coffee husk used as raw material, water, distilled water ,hydrogen peroxide, H_2O_2, dichloromethane (CH2Cl2), Calcium carbonate (CaCO3) , anhydrous magnesium sulfate (MgSO4), Phenolphthalein indicator, caffeine , buffer solution, nutrient agar and broth. The phosphoric acid methyl red

3.2 Method

3.2.1Coffee husk sampling method

Coffee husk samples were collected to analysis of the selected physic-chemical parameters after one week of harvesting coffee bean clutch dried coffee husk. The coffee beans collected from

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SNNP region in sidama zone bansa kebele coffee plantation site of farmer dried coffee husk after one week of harvest of coffee used. This sample collected in the material of chemically sterilized plastic pot. All coffee husk samples can be collected on the same time at the day of morning and immediately taken to the Laboratory for physical-chemical analysis.

3.2.2 Sample Preparation and analysis

5kg of single dried coffee husk sample were collected from SNNP in sideman zone bansa town coffee plantation of farmer site the collected coffee husk samples were kept in an incubator before being prepared for analysis because protecting from other unwanted microbes side effect(conversion) the moisture contents were analyzed and also physical observation of coffee on plantation site is must be maintained. Washed by distil waters for dry experiment followed by immediate drying after there were leads to size reduction by disk mill with diameter of particle size <<<0.2mm.

3.2.43 physicochemical value of coffee husk

Physicochemical analysis of coffee husk tests measure the microbial composition and activity of different CH(coffee husk) horizons. The physicochemical characteristics of CH influence the rate of biomass production and the activity and composition of microorganisms. Seasonal changes in CH moisture, CH temperature, pH organic carbon, nitrogen, lignin, starch, cellulose and hemicelluloses can have a large effect on CH microorganisms, which, in turn, affect the ability of the CH to supply nutrients to plants through the turnover of CH organic matter. Therefore,the physiochemical analiysis of the collected CH samples were extremely important and should always be performed. pH was measured using pH meter, where as the CH temperature was measured using thermometer. The moisture content was analyzed by weighing the CH sample before and after oven drying. Spectrophotometric analysis as done in order to find nitrogen, cellulose and starch content. The standard titration method as well as laboratory analytical method was followed in order find the organic content, lignin and hemicelluloses content.

Moisture content

To determine the moisture content of coffee husk, AOAC, (1995) method 925.09B were used. Well mixed 2g sample coffee husk was dried at 130oC ±3oC for one hour in an air draft drought oven and the mass loss on drying was determined as % moisture as follows:

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Where:- M initial =m2-m1; M dried= m3-m1, m2 is mass of sample and its crucible before drying, m1 is mass of crucible and m3 is mass of sample and crucible after drying.

$$Moisture\ (\%) = \frac{M_{initial} - M_{dried}}{M_{initial}}$$

Ash content

The ash content was determined by the gravimetric method as described in the AACC (2000) method No. 08-03. Three gram of coffee husk sample was placed in to pre-weighed crucible (mL). The sample was dried at 120 ^{o}C for 1 hour in drying oven. Sample dish was removed from the oven and carbonized by the blue Bunsen burner by placing the sample dish on wire guaze or triangle. After heating gently the dish with its content was transferred in to a muffle furnace and ignite at about 520-530oC until free from carbon and the residue appears grayish-white (about 8 hrs). Then the dish was removed from the furnace and placed in a desiccator until temperature assumes ambient and weighed. The ash (%) was determined as follows:

$$Ash(\%) = \frac{M_3 - M_1}{M_2 - M_1} x100$$

Where: - M3 = mass of crucible and sample after ashing

M2 = mass of crucible and sample before ashing

M1 = mass of crucible

M3 - M1 = mass of sample after ashing

M2 - M1 = mass of sample before ashing

Crude protein content (N x 6.25)

Protein content was determined using the Kjeldahl method AACC (1995) Method No. 46-11, of nitrogen analysis. About 0.3 g of ground (1 mm), dried coffee husk samples was measured in to digestion flask containing catalyst, (1 g of mixture Na2SO4 mixed with anhydrous CuSO4 in 10:1) and 5 mL of concentrated H2SO4. Digestion was carried out using digestion apparatus. The sample was digested to temperature of 350oC until digestion was complete and the digest

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were clear. The acid digest was allowed to cool to room temperature. Distillation was performed by adding 30 mL of distilled water, 25 mL (40%NaOH) to Kjeldahl flask and connecting it to distillation apparatus whose out let tube is immersed in 25 mL of 4% boric acid solution. About 150 mL distillate was collected and titrated by standard acid (0.1N HCl). The volume of HCl consumed was taken from the burette reading, and then the %N was calculated by the equation described below. Urea was used as control in the analysis.

$$N(\%) = (\frac{V_{HCl}\ in\ L x N_{HCl}(ca\ 0.1) x 14}{Sample\ weight\ in\ g\ \ dry\ matter\ \ basis} x100$$

Where: VHCl= Volume of HCl in liter-

NHCl =Normality of HCl

F=Conversion factor using for coffee husk 6.25.

14= Molecular Weight of Nitrogen

Crude fat content

Crude fat was determined by Soxhlet method of fat extraction according to AACC (2000) method No. 32-10. About 5 g of flour was extracted with about 150 mL petroleum ether for a minimum period of 8 hrs in the Soxhlet extractor. The solvent is then evaporated by heating on a steam bath. The flask containing the extracted fat was dried for about one hour in a drying oven (103 ºC).

$$Crude\ \ fat\ (db)\ percent\ by\ weight = \frac{w_2 - w_1}{w} x100$$

Where: - W1 = weight of the extraction flask (g)

W2 = weight of the extraction flask plus the dried crude fat (g)

W = weight of sample coffee husk (g)

Total utilizable carbohydrate

This was determined by subtracting the sum of other constituents from 100.

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% Total utilizable carbohydrate = 100 – (% moisture content + % crude protein + % crude fiber + % crude fat + % ash).

3.2.5 Extraction of caffeine (decaffeination)

We used 250 mL of distil water and 20 g of pre-ground coffee husk in the espresso maker to make the coffee. After we collected the coffee into a beaker that had a 6.0 g of CaCO3 in it, we heated the mixture to about 70 °C for about 10 minutes on a hot plate to make digestion and cold down up to room temperature. Then we used the funnel, for filter aid inside the filter to filtration. Foaming is occurred; vacuum removed right away until foaming disappeared.

Afterwards, the filtrate was transferred evenly into 2 separator funnels, and extracted with 80mL of dichloromethane (CH2Cl2). The separator funnels were rolled carefully and not shaken. After the extractions, the organic (bottom) layer was collected. The combined organic layer then was dried with anhydrous magnesium sulfate (MgSO4). The insoluble solids were filtered and removed.

The filtrate then was transferred into a 250 mL round-bottom flask. Then a rotary evaporator was used to evaporate the solution. This then formed the crude caffeine. The purpose is to isolate caffeine from coffee husk by using the organic solvent dichloromethane (CH2Cl2) that is also referred to as direct contact. Calcium carbonate (CaCO3) is used to remove the tannins from the coffee husk. Afterwards an extraction is used to separate the organic layer (bottom) from the aqueous layer (upper). By using an evaporator, it evaporates the organic solvent and forms the crude caffeine. Afterwards we used filtration for recrystallization and to obtain pure caffeine.

3.2.6 Screening of caffeine degrading bacteria

Using coffee husk sample from Ethiopia biodiversity institute initially before serial dilution 1g of coffee husk sample was inoculated into enrichment media (g/l) containing Disodium hydrogen phosphate (0.12), Potassium dihydrogen phosphate (1.3), Calcium chloride (0.3), Magnesium sulphate (0.3), and ferrous sulphate (0.02). From that medium 1ml was taken and transferred to serial dilution tubes and organisms were isolated by spread plate technique up to 8 strain of bacteria isolated from coffee husk. (From interview of Ethiopia biodiversity institute microbial staff) from those isolated strain of bacteria from Ethiopia biodiversity institute we take rhizobium and bacillus strains of bacteria for this experiment

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Organisms selected from the primary media were then inoculated into secondary screening media with different concentrations of caffeine was used. The strain capable of growing 2.5g/100ml concentration of caffeine was selected and transferred to the enrichment broth with supplement of caffeine (2.5g/L) and incubated at 37˚C in shaking incubator 120 rpm, for 48 hrs.

3.2.6.1 Analytical methods

Analytical method was methods used for this experiment for determination of potent or potential of microbes (rhizobium and bacillus spp) to degrade caffeine analyzed by growth curve experiment.

3.2.6.2 Growth curve experiment

The growth curve experiment was carried out to understand the effect of caffeine on the growth kinetics of the organism. In the control flask the organism was inoculated into enrichment media without caffeine so that the normal growth of the organism can be analyzed and the test flask media was supplemented with Caffeine (2.5g/L) deflection of growth curve were occurred to determine caffeine degrading capacity (see appendence C) the same result observed with sneha nayak 2010.

3.2.7 Viable cell counting

3.2.7.1 Serial dilution

54ml (i.e.9ml for each test tube) of distilled water was added into 6 test tubes to dilute the sample. The prepared distilled water were foiled with aluminum and then sterilized at 121^{o}C for 15 psi and for 1hrs the sterilized distilled water was then cooled. Then serial dilution was done. Then 54ml of distilled water were shared for 6 test tube up to 9ml of distilled water then1ml of samples filtrate of caffeine were taken and added into first distilled water test tube label 10^{-1} using pipette to dilute the sample. Then 1ml droplet of solution was taken from each of the1st test tube, and added to each 2nd test tube label 10^{-2}. This procedure was continued up to the 6th test tube of sterilized distilled water for each sample (i.e., you make 10^{-1}-10^{-6} dilution for each sample) one after the other. 0.1ml of diluted sample was taken from each of 12 test tubes and inoculated into labeled agar plate (inoculation of the sample occur after agar solution were solidified on plate. This solidification processes may be take up to 20min) then by using sterilized spreader the sample was distributed. Finally viable cell counted

The bacteria can be counted by calculating C.F.U. i.e. Colony Forming Unit.

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C.F.U. = no, of colonies/inoculums size (ml) X dilution factor C.F.U/ml

3.2.7 Characterization of screening caffeine degrading bacteria.

The caffeine samples serial dilutions were done. The organism was identified by the analysis of the characteristics according to the Morphological and Biochemical characteristics. The various biochemical tests conducted were citrate utilization, catalase, urease, indole, methyl red, nitrate reduction test and carbohydrate fermentation such as lactose maltose sucrose were performed and confirmed. Then using the specific medium agar(YEMA and pikovaskiya media supplement with/without caffeine) for rhizobium and bacillus subtilis were used to grow the organism for the mass production.

3.2.7.1 Gram staining

Gram stain The Gram stain is almost always the first step in the preliminary identification of a bacterial organism. A smear was prepared by placing a drop of water on the slide and then transferring microorganism to the drop of water with a sterile cooled loop. It was mixed and spread by means a circular motion of the inoculating loop. Smear was air dried Crystal violet is the primary stain used first and stains all cells purple. Its function is to impart its color to all cells in order to establish a color contrast. Grams iodine, used as mordent in which this reagent is not only a killing agent, but also serves as a mordant a substance that increases the cells affinity for a stain. It does this by binding to the primary stain, thus forming an insoluble complex. The resultant crystal violet iodine complex serves to intensify the color of the stain. At this point, all cells will appear purple black. Ethyl alcohol, 95% is used as decolorizing agent in which this reagent serves as a dual function as a protein dehydrating agent and as a lipid solvent. By using this Gram negative bacteria become colorless. Safranin is used to stain red those cells that have been previously decolorized. Thus safranin is a counter stain since only gram negative cells undergo decolourization, they may now absorb the counterstain. Gram positive cells retain the purple color of the primary stain and gram negative bacteria appear as pink color using compound microscope 40* lenses.

3.2.8 Caffeine degradation by bacteria.

Some microorganisms have the ability to grow in the presence of caffeine and survival would be related to their capacity to degrade the alkaloid (Sundarraj and Dhala, 1965). Actually, it is not rare to find bacterial strains resistant to caffeine (Woolfolk, 1975). Some microorganisms, e.g.,

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Klebsiella pneumoniae, bacillus and rhizobium can utilize purines/caffeine as carbon or nitrogen sources (Vogels and Drift, 1976.). So the experiment looks likes follows to determine bacteria use caffeine as substrate.

Prepare the 50ml broth of microorganism (rhizobium, and, bacillus) using YEMA (look table 5) and PIKOVASKIYA (look table 6) medium for rhizobium and bacillus sp. of bacteria respectively. Similar procedure is followed for preparing 50ml of broth which supplemented with 2.5g/l of caffeine. These both of the mixture were ready to use as liquid bio fertilizer. Each individual microorganism inoculated the respective microorganism and shakes vigorously now this broth was ready to use for liquid bio-fertilizer then investigated the effect and the optimum of those selected factor PH, temperature and incubation time for response of maximum cell growth with and without caffeine containing broth by means of counted bacterial cell.

3.2.9 Ingredients For preparation of broth

Table 5 Ingredients for Rhizobium Broth (YEM medium)

Sr no,	Ingredients	GM/mL
1	K_2HPO_4	0.5
2	$MgSO_4$	0.1
3	Nacl	0.2
4	Yeast extract	10
5	Manitol	10

Table 6 Ingredients for bacillus broth (PIKOVSKAYA medium)

Sr no,	Ingredients	GM/L
1	Dextrose	10
2	Calcium phosphate	5
3	Ammonium sulphate	0.5
4	Potassium chloride	0.2

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5	**$Mgso_4$**	**0.1**
6	**Manganese sulphate**	**0.001**
7	**Ferrous sulphate**	**0.01**

3.2.10 Experimental design analysis

Experimental data analysis was analyzed by the software Design-Expert 6.0-8.0. The experimental design selected for this study type was response surface method, and design model was quadratic model, Box-Behnken Design and the response variable was count number of bacteria cell growth. for each of with and without caffeine supplemented media broth (with caffeine use 2.5g/l) for both of bacteria spp(bacillus and rhizobium) The three independent variables studied to determine the response factor cell growth(number of bacteria) was temperature, incubation time and PH (acid base status media) and those independent factor was the condition that can affect the growth of bacteria. There was level for each of condition (temp, pH and incubation time) shown as the following table

Table 7 experimental factor selected levels

FACTORS		**LEVEL**	
temperature(ºC)	27	37	47
pH	6	7	8
Incubation time(hrs)	10	25	40

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Chapter four

4.1 Results and discussion

Physicochemical analysis of coffee husk based on the physicochemical analysis of dry coffee husk samples the result was tabulated in the table 8. Moisture content, pH, Temperature, play a major role in the increase of the microbiological activity of the environmental sample. So before identifying or supplemented to the bacteria, the physical and chemical properties of the coffee husk sample must be analyzed properly. So as shown table 3 the caffeine content of dry coffee husk was 0.9% whereas the current individual study finding shown on the table 8 was 0.6% this shows that the study had a little bit of difference due to the increment of total carbohydrate and protein content of dry coffee husk.

Sr no,	Physicochemical properties	Dry coffee husk
1	PH	6.9
3	total Carbon	49
4	Nitrogen	7
5	Moisture	28
6	Starch	11
7	Protein	9
8	Lipids	1.5
9	Minerals	4.3
10	Total carbohydrate	69
11	Cellulose	42
14	hemicellulose	6.5
15	Lignin	18
17	ash content	5
18	caffeine	0.6

Extracted Caffeine from this experiment were 0.9% per gram of sample were crude caffeine was obtained from rotary evaporator extracted cured caffeine was again subjected to recrystallization(drying) there was 0.6% of pure caffeine were obtained. Those extracted percent of caffeine was more related with table 3 then we used those extracted caffeine for bacterial substrate as carbon/ nitrogen source.

The collected strain of bacteria from Ethiopia biodiversity institute that were isolated from coffee husk were revealed or authenticated by means of morphological and biochemical test see from table 9

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Table 9 Morphological and Biochemical test

Test	rhizobium sp.	bacillus sp.
shape	strait rode	rode
Gram staing	-ve	+ve
Indole	-ve	-ve
Methyl red	+ve	-ve
Citrate	-ve	+ve
Gas production	-ve	-ve
Catalase	+ve	+ve
Urease production	+ve	-ve
NR	+ve	+ve
	Carbohydrate fermentation	
Lactose	-ve	-ve
Maltose	+ve	+ve
Sucrose	+ve	+ve

From the two identity microbes as caffeine degrading bacteria was taken for secondary screening both rhizobium and bacillus was able to tolerate to 2.5 g/L of caffeine supplemented with other media components. The two identify bacteria were inoculated into enrichment broth with different concentration of caffeine to analyze how much amount of caffeine was degraded by the organism within 24hrs of cell grow with growth curve analysis see above table 9. Identified or authenticated (revealed) as bacillus spp and rhizobium spp.by means of morphological and biochemical test. Organism growth curve to study the effect of caffeine (caffeine degrading capacity) were done on the growth pattern of the organism. The organism was inoculated into medium containing caffeine and a control medium without caffeine being inoculated with the same organism; the results were shown in figure appendix C growth of the organism was monitored by growth curve experiment. The control flask showed a normal growth of the bacterium and there were changes in the growth pattern of organism on the test flask. This was due to the fact that caffeine contributed the cellular metabolism of the bacteria. So that the log phase was promoted lag phase along with high cell density was observed.

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4.1.3 Statistical Analysis of the Experimental Results for 50mL of broth for rhizobium bacteria (without supplement of caffeine)

Experimental results of three factors with three levels, one replicate and the following tables show statistical analysis of the experimental results by using Design-Expert Soft-ware (version 6.0.8). Summary of the model details is that study type is response surface method design Box-Behnken center point is zero design model is quadratic number of experiment is 17.

Table10 response of cell growth for rhizobium

std	Run	Block	Factore1 temp(^{o} c)	Factore 2 pH	Factore 3 incubation time	Cell growth
12	1	Block 1	37.00	8.00	40.00	3.2E+008
1	2	Block 1	27.00	6.00	25.00	3.994E+007
3	3	Block 1	27.00	8.00	25.00	7.99E+007
7	4	Block 1	27.00	7.00	40.00	1.45E+008
4	5	Block 1	47.00	8.00	25.00	8.9E+008
11	6	Block 1	37.00	6.00	40.00	2.6E+008
15	7	Block 1	37.00	7.00	25.00	4.23E+008
14	8	Block 1	37.00	7.00	25.00	4E+008
6	9	Block 1	47.00	7.00	10.00	1.42E+008
9	10	Block 1	37.00	6.00	10.00	2E+008
17	11	Block 1	37.00	7.00	25.00	4.99E+008
5	12	Block 1	27.00	7.00	10.00	1E+008
13	13	Block 1	37.00	7.00	25.00	3.46E+008
2	14	Block 1	47.00	6.00	25.00	4E+007
16	15	Block 1	37.00	7.00	25.00	4.62E+008
10	16	Block 1	37.00	8.00	10.00	2.22E+008
8	17	Block 1	47.00	7.00	40.00	1.99E+008

Analysis of variance (ANOVA) In ANOVA analysis the model was significant when fisher's F test with a very low probability value. From table 11 observed that the Model F-value of 3.6 implies the model is significant. There is only a 4.42% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A,B AB, are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The coefficient was linear effect of temperature and pH and incubation time was least significant

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Table11 ANOVA of rhizobium without supplement of caffeine

Source Model	**Sum of Square**	**DF**	**Mean Squares**	**F Value**	**Prob > F**	
	5.758E+017	8	7.198E+016	3.60	0.0442	significant
A	1.026E+017	1	1.026E+017	5.14	0.0332	
B	1.181E+017	1	1.181E+017	5.91	0.0411	
C	8.450E+015	1	8.450E+015	0.42	0.5337	
A^2	7.534E+016	1	7.534E+016	3.77	0.0881	
B^2	3.732E+015	1	3.732E+015	0.19	0.6770	
C^2	8.942E+016	1	8.942E+016	4.48	0.0673	
AB	1.640E+017	1	1.640E+017	8.21	0.0210	
AC	3.600E+013	1	3.600E+013	1.802E-003	0.9672	
Residual	1.598E+017	8	1.998E+016			
Lack of Fit	1.461E+017	4	3.653E+016	10.66	0.0208	significant
Pure Error	1.371E+016	4	3.427E+015			
Cor Total	7.356E+017	16				

The “Lack of Fit F-value” 10.66 implies the Lack of Fit significant relative to the pure error. There is a 2.08% chance that a “Lack of Fit F-value” this large could due to noise

Std. Dev.	1.413E+008	R-Squared	0.7828
Mean	2.805E+008	Adj R-Squared	0.5655
C.V.	50.40	Pred R-Squared	-1.5322
PRESS	1.863E+018	Adeq Precision	6.768

The "Pred R-Squared" of -1.5322 is not as close to the "Adj R-Squared" of 0.5655 as one might normally expect. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 6.768 indicates an adequate signal. This model can be used to navigate the design space.

Final Equation in Terms of Actual Factors:

cell growth= +6.70931E+008 -3.19402E+007 temp -2.11012E+008 pH +3.38111E+007 incubation time -1.33770E+006 $temp^2$ -2.97700E+007 pH^2-6.47689E+005 incubation $time^2$ +2.02510E+007 temp * pH +20000.00000 temp * incubation time

Diagnostic test for the responses

All diagnostic plots are also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs factor, box cox plot, studentized residuals, leverage, etc.). From the plot as shown below, the normal probability plot indicates the residuals

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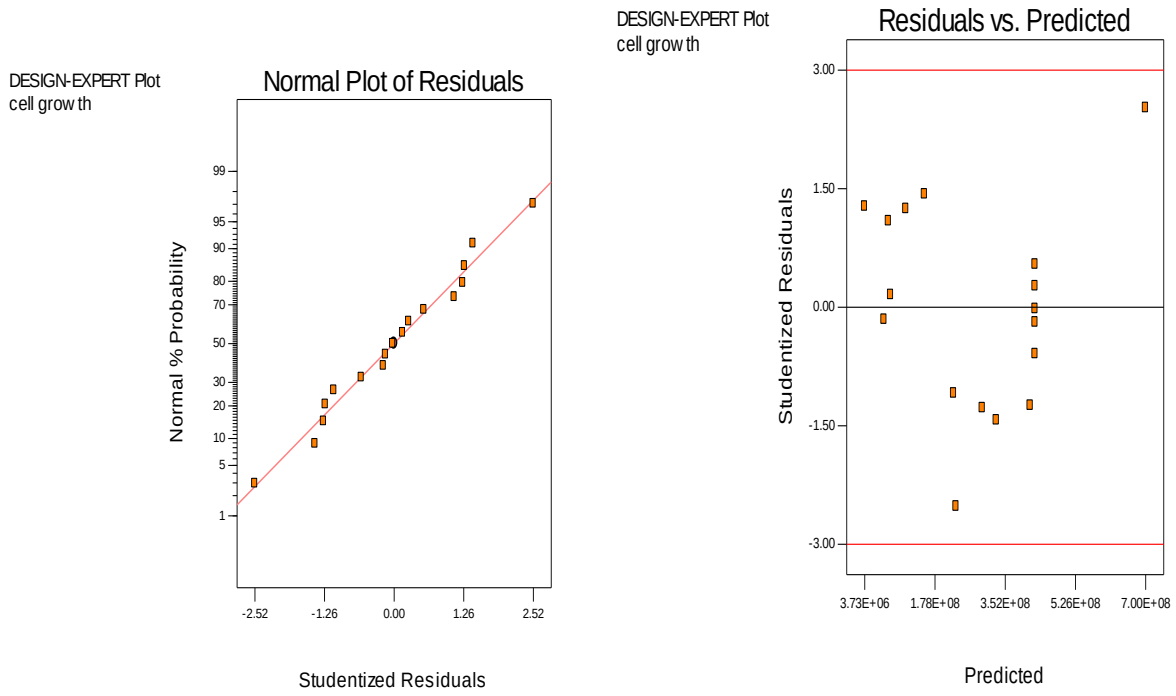
following by the normal % probability distribution, in the case of this experimental data the points in the plots shows fitted to the straight line in the figure 3, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal.

Figure 3 Diagnostic tests for the responses for 50mL of broth for rhizobium bacteria (without caffeine)



4.1.3.1 Interaction Effect of Experimental Variables on Cell Growth (50ml broth) without caffeine supplement rhizobium bacteria

The following Figure (4 and 5) show that an interaction effect of temperature & pH In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum factors. When temperature increase cell growth also increase at PH of 7.

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Figure4 shows Contour plots of the effect of temperature and pH on the cell growth at constant incubation time for rhizobium bacteria and 50ml of broth

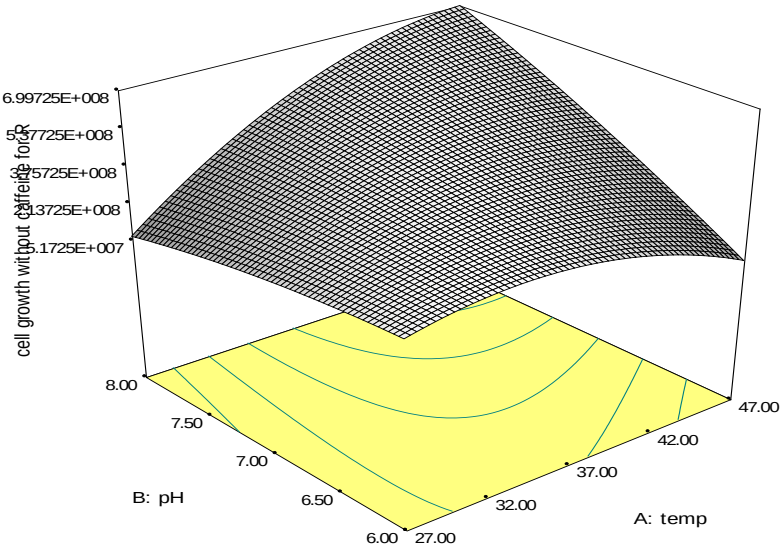
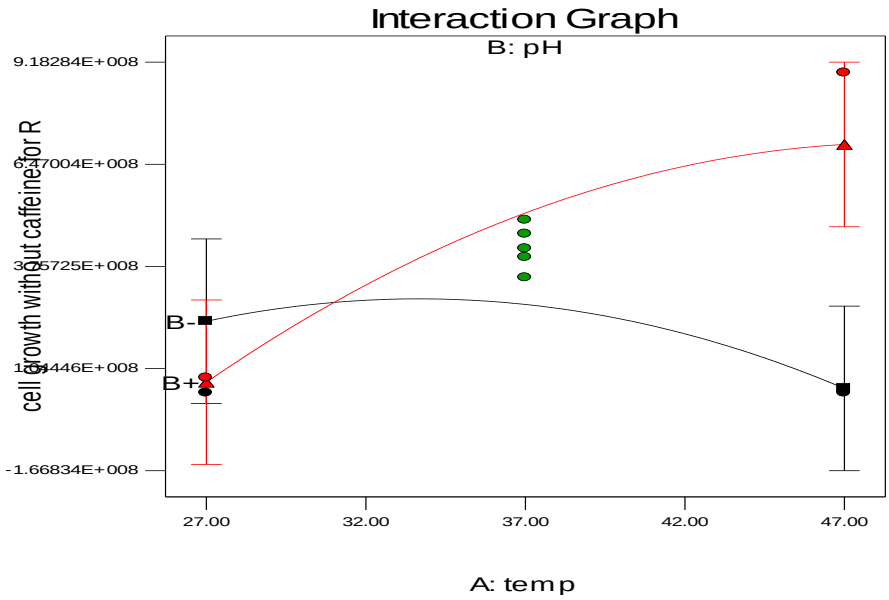


Figure 5 Effect and interaction of temperature and PH (fixed) on the incubation time for rhizobium bacteria growth.



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The following Figure (6 and 7) show that an interaction effect of temperature & incubation time have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum in both factors. The effect of temprature and incubation Time on the cell growth it show 37 ^{o}C of temperature of media with microbes inter in to shaking incubator for 21 hrs. give high amount of cell growth see figure 6.

Figure 6 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH rhizobium bacteria (50ml of rhizobium

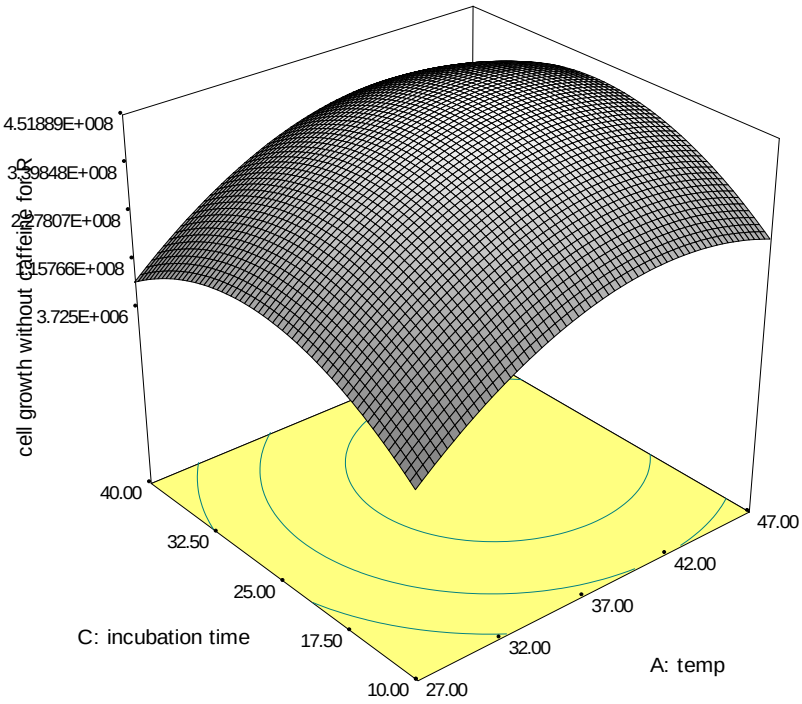

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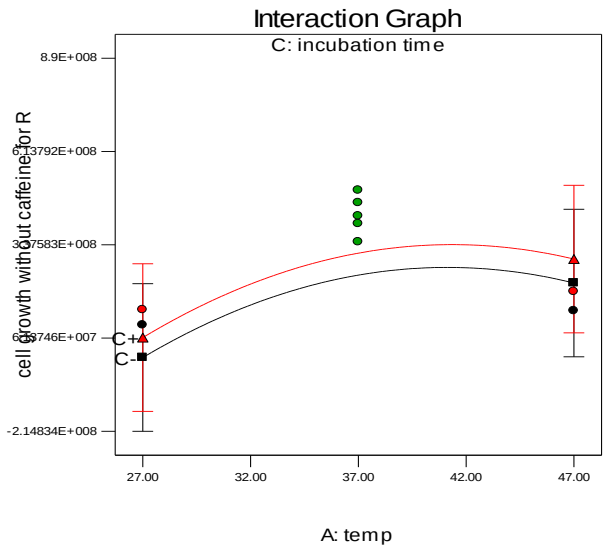
Figure 7 Effect and interaction of temperature and incubation time (fixed) on the pH For rhizobium bacteria growth.



The following Figure (8 and 9) show that an interaction effect of pH & incubation time have no significant quadratic effects ($P > 0{\cdot}05$), indicating no presence of an optimum for both factors. The effect of PH and incubation Time on the cell growth it show 7 PH of media with microbes inter in to shaking incubator for 20hr give high cell growth(center).

Figure 8 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH rhizobium bacteria (50ml of rhizobium)

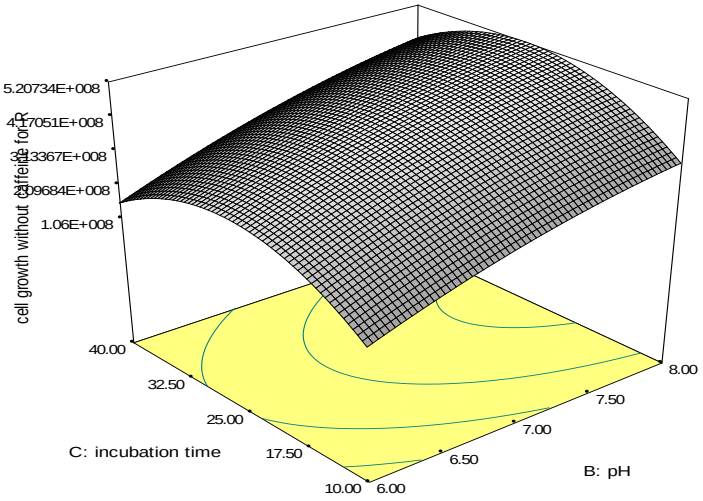


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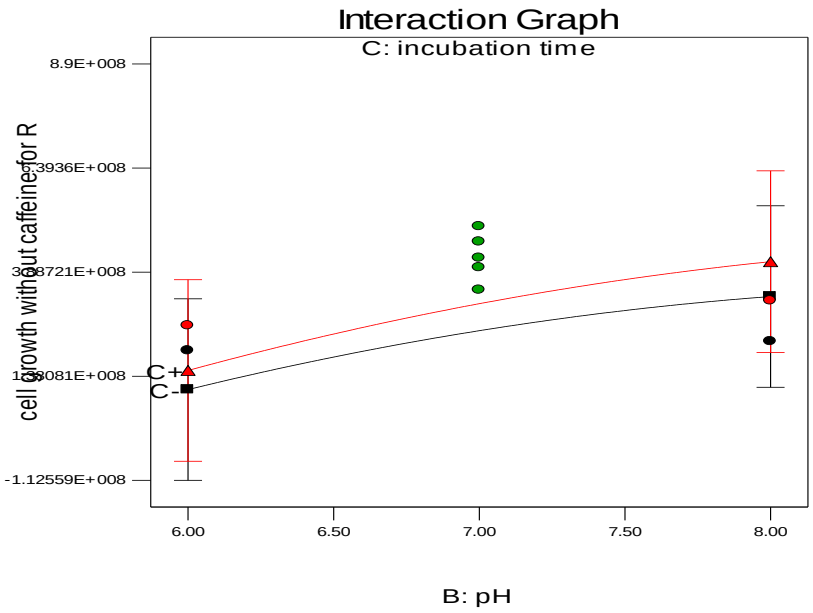
Figure 9 Effect and interaction of temperature and incubation time (fixed) on the pH For rhizobium bacteria growth.



4.1.4 Statistical Analysis of the Experimental Results of media supplemented with 2.5g/l of caffeine for rhizobium bacteria

Experimental results of three factors with three levels and one republican and the following tables show statistical analysis of the experimental results by using Design-Expert Soft-ware (version 6.0.8). Summary of the model details is that study type is response surface method box-Behnken design model is quadratic number of experiment is 17

Table 12 response of cell growth for rhizobium media supplemented with caffeine

Std	Run	Block	Factore 1 temp(^{o}c)	Factore 2 pH	Factore3 incubation time	Cell growth
4	1	Block 1	47.00	8.00	25.00	9.4E+007
15	2	Block 1	37.00	7.00	25.00	4.63E+008
10	3	Block 1	37.00	8.00	10.00	2.62E+008
11	4	Block 1	37.00	6.00	40.00	3E+008
1	5	Block 1	27.00	6.00	25.00	4.394E+007
6	6	Block 1	47.00	7.00	10.00	1.82E+008
16	7	Block 1	37.00	7.00	25.00	5.02E+008
3	8	Block 1	27.00	8.00	25.00	8.39E+007
9	9	Block 1	37.00	6.00	10.00	1.89E+008
14	10	Block 1	37.00	7.00	25.00	4.4E+008

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13	11	Block 1	37.00	7.00	25.00	3.86E+008
8	12	Block 1	47.00	7.00	40.00	2.39E+008
7	13	Block 1	27.00	7.00	40.00	1.85E+008
17	14	Block 1	37.00	7.00	25.00	5.39E+008
2	15	Block 1	47.00	6.00	25.00	4.4E+007
12	16	Block 1	37.00	8.00	40.00	3.6E+008
5	17	Block 1	27.00	7.00	10.00	1.4E+008

Analysis of variance (ANOVA) In ANOVA analysis the model was significant when fisher's F test with a very low probability value. From table 13 observed that the Model F-value of 19.32 implies the model is significant. There is only a 0.04% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A^2, B^2, are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The coefficient was linear effect of temperature and pH and incubation time was not significant.

Table 13 Analysis of variance (ANOVA caffeine supplemented rhizobium

Source Model	**Sum of Square**	**DF**	**Mean Squares**	**F Value**	**Prob > F**	
	4.046E+017	9	4.496E+016	19.32	0.0004	significant
A	*1.409E+015*	*1*	*1.409E+015*	*0.61*	*0.4621*	
B	*6.214E+015*	*1*	*6.214E+015*	*2.67*	*0.1463*	
C	1.209E+016	1	1.209E+016	5.19	0.0567	
A^2	2.536E+017	1	2.536E+017	108.93	< 0.0001	
B^2	1.000E+017	1	1.000E+017	42.98	0.0003	
C^2	4.897E+015	1	4.897E+015	2.10	0.1902	
AB	2.520E+013	1	2.520E+013	0.011	0.9200	
AC	3.600E+013	1	3.600E+013	0.015	0.9045	
BC	4.225E+013	1	4.225E+013	0.018	0.8966	
Residual	1.629E+016	7	2.328E+015			
Lack of Fit	2.584E+015	3	8.612E+014	0.25	0.8572	not significant
Pure Error	1.371E+016	4	3.427E+015			
Cor Total	4.209E+017	16				

The "Lack of Fit F-value" 0.25 implies the Lack of Fit significant relative to the pure error. There is a 85.72% chance that a "Lack of Fit F-value" this large could due to

Std. Dev.	4.825E+007	R-Squared	0.9613
Mean	2.619E+008	Adj R-Squared	0.9115
C.V.	18.42	Pred R-Squared	0.8509

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PRESS	6.276E+016	Adeq Precision	11.841

The "Pred R-Squared" of 0.8509 is not as close to the "Adj R-Squared" of 0.9115 as one might normally expect. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 11.841 indicates an adequate signal. This model can be used to navigate the design space.

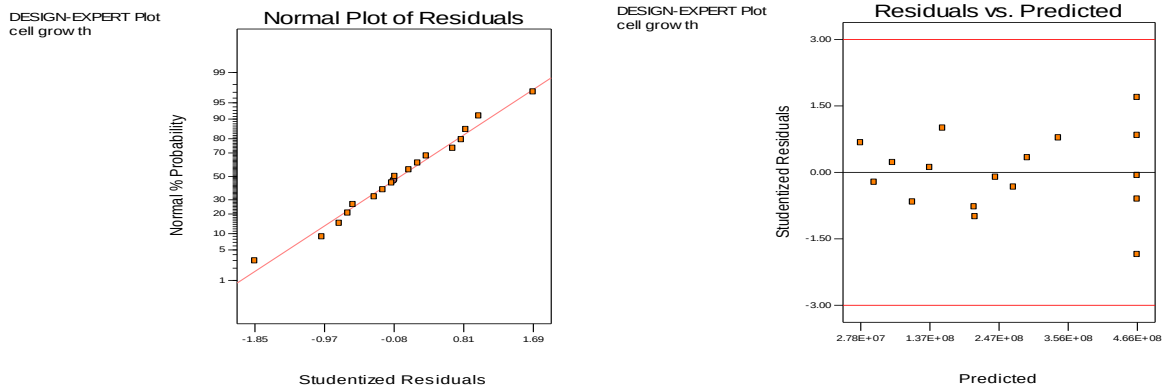
Final Equation in Terms of Actual Factors:

cell growth = -1.08047E+010+1.80662E+008 temp+2.18203E+009 pH+1.09472E+007 incubation time -2.45395E+006 temp^2-1.54145E+008 pH^2-1.51578E+005 incubation time^2 +2.51000E+005 temp * pH+20000.00000 temp * incubation time-2.16667E+005pH * incubation time

Diagnostic test for the responses

All diagnostic plots are also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs factor, box cox plot, studentized residuals, leverage, etc.). From the plot as shown below, the normal probability plot indicates the residuals following by the normal % probability distribution, in the case of this experimental data the points in the plots shows fitted to the straight line in the figure 10, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal.

Figure 10 Diagnostic tests for the responses of caffeine supplemented media for rhizobium



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4.1.3.3 Interaction Effect of Experimental Variables on Cell Growth (media supplemented with2.5g/l of caffeine) rhizobium bacteria

The following Figure (11 and 12) show that an interaction effect of temperature & pH on cell growth. In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum factors. The effect of temperature and PH on the cell growth it show 37 ^{o}C of temperature of media with microbes inter in to shaking incubator by PH of 6.8 give high cell growth.

Figure 11 Contour plots of the effect of temperature and pH on the cell growth at fixed incubation time rhizobium bacteria (media supplemented by caffeine)

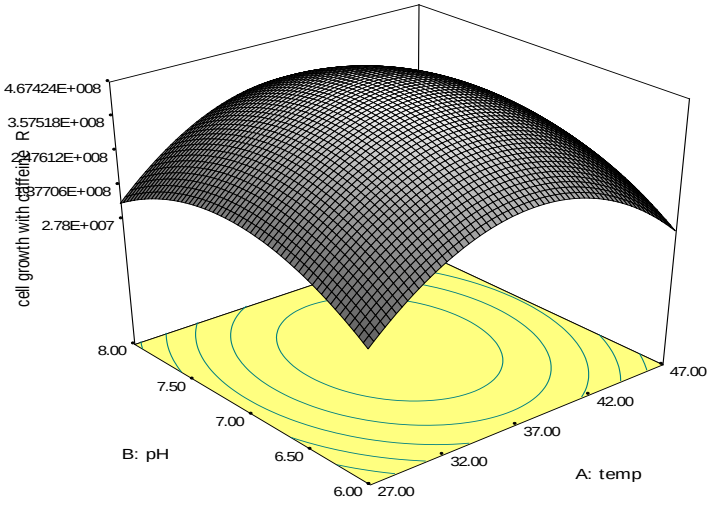
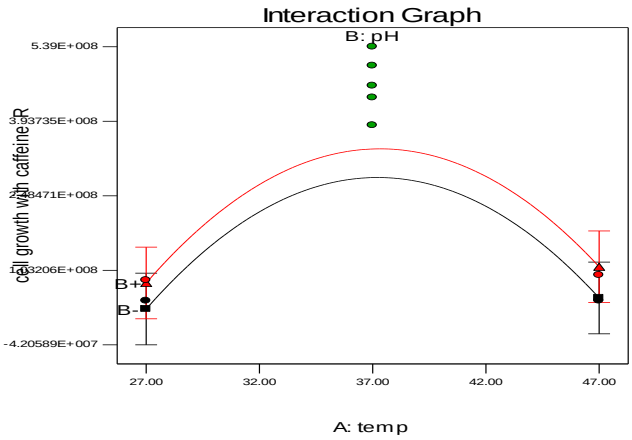


Figure 12 Effect and interaction of temperature and PH (fixed) on the incubation time for rhizobium bacteria growth.



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The following Figure (13 and 14) show that an interaction effect of temperature & incubation time on cell growth. In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum factors. The effect of temprature and incubation Time on the cell growth it show 37 ^{o}C of temperature of media with microbes inter in to shaking incubator for 18 hrs give high cell growth.

Figure 13 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH rhizobium bacteria (media supplemented with caffeine)

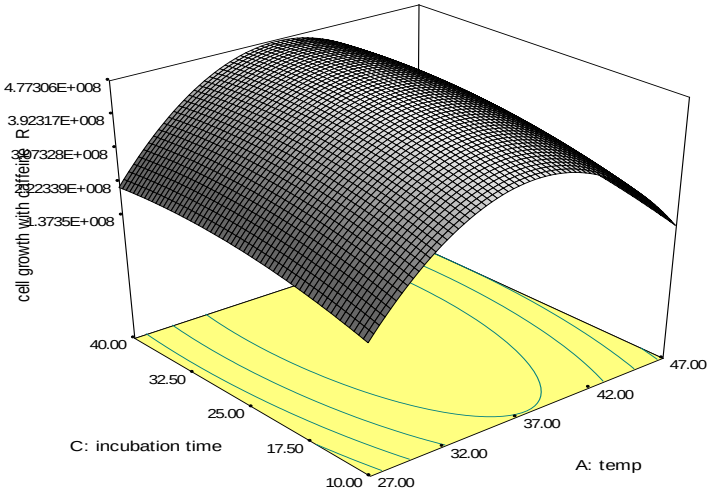
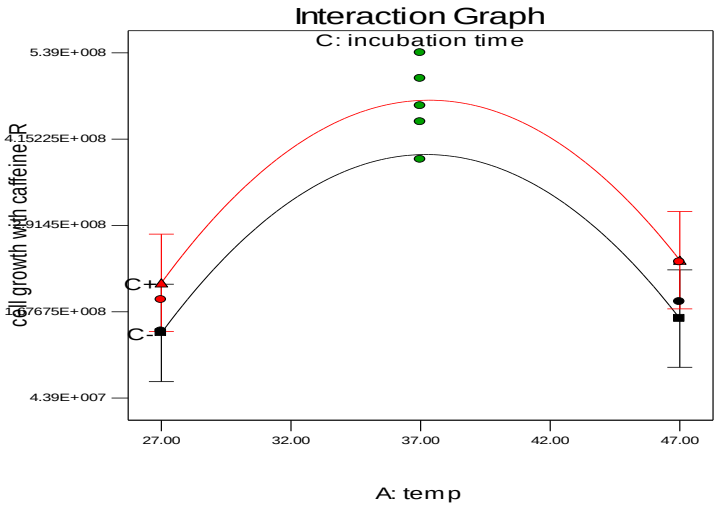


Figure 14 Effect and interaction of temperature and incubation time (fixed) on the pH For rhizobium bacteria growth.



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The following Figure (15 and 16) show that an interaction effect of pH & incubation time on cell growth. In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum for both factors.

Figure 15 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH rhizobium bacteria (media supplemented by caffeine)

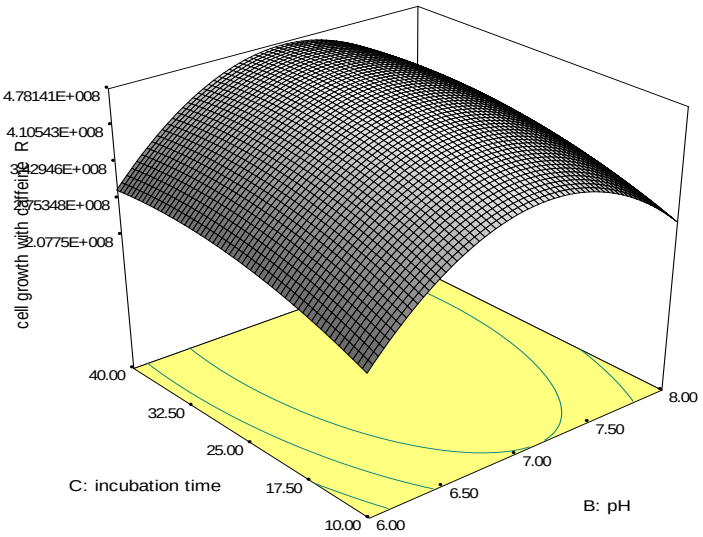
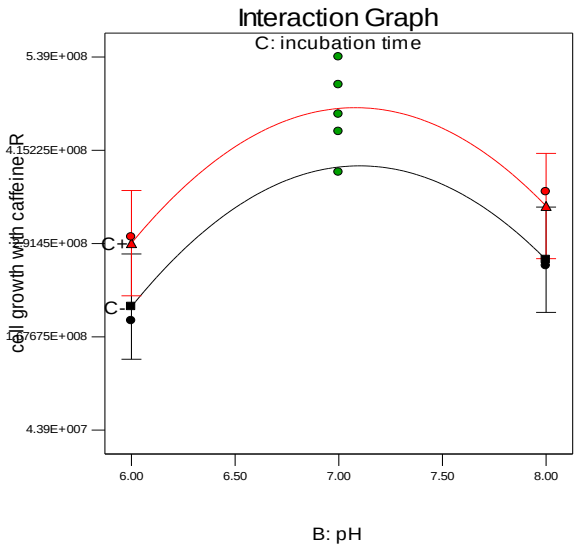


Figure 16 Effect and interaction of temperature and incubation time (fixed) on the pH For rhizobium bacteria growth.



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4.1.5 Statistical Analysis of the Experimental Results media without supplement of caffeine for bacillus subtilis

Experimental results of three factors with three levels and one republican and the following tables show statistical analysis of the experimental results by using Design-Expert Soft-ware (version 6.0.8). Summary of the model details is that study type is response surface methodology Box-Behnken design model is quadratic number of experiment is 17

Table 14 number of cell without media supplemented use caffeine for bacillus

Std	Run	Block	Factore1 temp(^{o}c)	Factore 2 pH	Factore 3 incubation time (hr)	Cell growth
10	1	Block 1	37.00	8.00	10.00	2.22E+008
16	2	Block 1	37.00	8.00	25.00	4.62E+008
11	3	Block 1	37.00	6.00	40.00	2.6E+008
3	4	Block 1	27.00	8.00	25.00	7.99E+007
9	5	Block 1	37.00	6.00	10.00	2E+008
4	6	Block 1	47.00	8.00	25.00	9E+007
12	7	Block 1	37.00	8.00	40.00	3.2E+008
6	8	Block 1	47.00	7.00	10.00	1.42E+008
2	9	Block 1	47.00	6.00	25.00	4E+007
13	10	Block 1	37.00	7.00	25.00	3.46E+008
17	11	Block 1	37.00	7.00	25.00	4.99E+008
1	12	Block 1	27.00	6.00	25.00	3.994E+007
8	13	Block 1	47.00	7.00	40.00	1.99E+008
5	14	Block 1	27.00	7.00	10.00	1E+008
15	15	Block 1	37.00	7.00	25.00	4.23E+008
7	16	Block 1	27.00	7.00	40.00	1.45E+008
14	17	Block 1	37.00	7.00	25.00	4E+008

Analysis of variance (ANOVA) In ANOVA analysis the model was significant when fisher's F test with a very low probability value. From table 15 observed that the Model F-value of 17.99 implies the model is significant. There is only a 0.05% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A^2, B^2, are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The coefficient was linear effect of temperature and pH and incubation time was least significant.

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Table15 ANOVA of bacillus media without supplement of caffeine

Source Model	**Sum of Square**	**DF**	**Mean Squares**	**F Value**	**Prob > F**	
	3.477E+017	9	3.863E+016	17.99	0.0005	Significant
A	1.409E+015	1	1.409E+015	0.66	0.4446	
B	3.696E+015	1	3.696E+015	1.72	0.2309	
C	8.450E+015	1	8.450E+015	3.94	0.0877	
A^2	2.301E+017	1	2.301E+017	107.16	< 0.0001	
B^2	7.091E+016	1	7.091E+016	33.02	0.0007	
C^2	8.805E+015	1	8.805E+015	4.10	0.0825	
AB	2.520E+013	1	2.520E+013	0.012	0.9168	
AC	3.600E+013	1	3.600E+013	0.017	0.9006	
BC	3.610E+014	*1*	3.610E+014	0.17	0.6940	
Residual	1.503E+016	*7*	2.147E+015			
Lack of Fit	1.321E+015	*3*	4.403E+014	0.13	0.9383	not significant
Pure Error	1.371E+016	*4*	3.427E+015			
Cor Total	*3.627E+017*	*16*				

The “Lack of Fit F-value” of 0.13 implies the Lack of Fit not significant relative to the pure error. There is a 93.83% chance that a “Lack of Fit F-value” this large could due to noise. Non-significant lack of fit is good – we want the model to fit.

Std. Dev	4.634E+007	R-Squared	0.9586
Mean	2.334E+008	Adj R-Squared	0.9053
C.V.	19.85	Pred R-Squared	0.8827
PRESS	4.256E+016	Adeq Precision	11.137

The "Pred R-Squared" of 0.8827 is not as close to the "Adj R-Squared" of 0.9053 as one might normally expect. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 11.137 indicates an adequate signal. This model can be used to navigate the design space.

Final Equation in Terms of Actual Factors:

cell growth = -9.94779E+009 + 1.79386E+008 temp +1.95175E+009 pH + 9.35556E+006 incubation time - 2.43670E+006 $temp^2$ - 1.39670E+008 pH^2 - 2.47244E+005 $incubation\ time^2$ +2.51000E+005 temp * pH +20000.00000 temp * incubation time + 6.33333E+005 pH * incubation time

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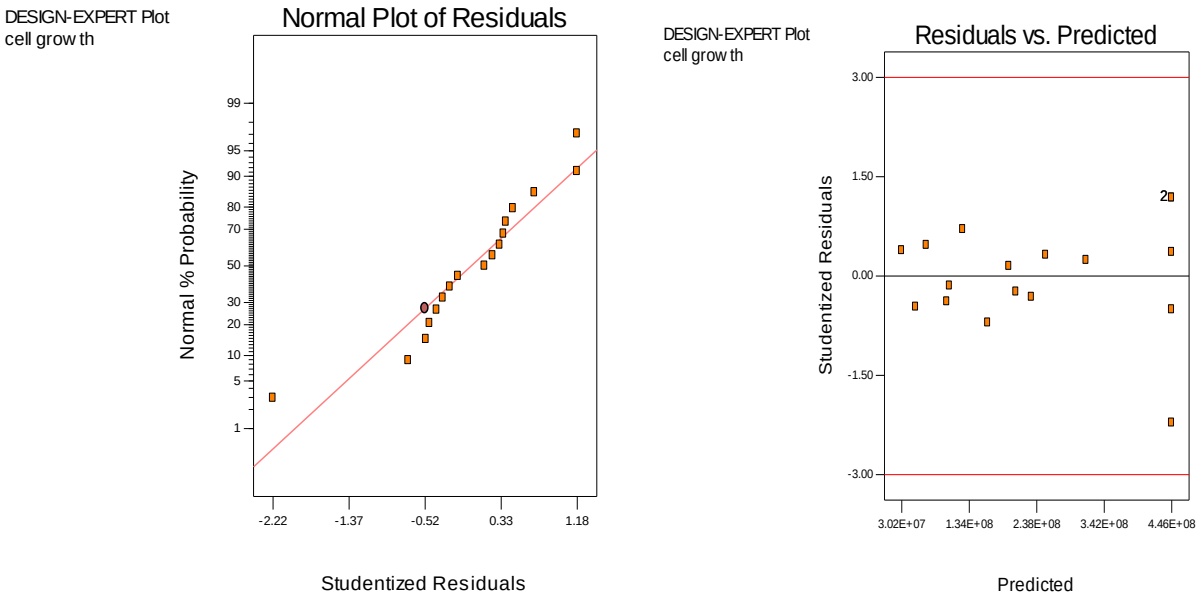
Diagnostic test for the responses

All diagnostic plots are also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs factor, box cox plot, studentized residuals, leverage, etc.). From the plot as shown below, the normal probability plot indicates the residuals following by the normal % probability distribution, in the case of this experimental data the points in the plots shows fitted to the straight line in the figure 17, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal. The following Prediction graph shows that constant range of residuals across the graph which is justifiable no need for a transformation to minimize personal error.

Figure 17 Diagnostic tests for the responses for medium without of supplement of caffeine for bacillus bacteria



4.1.5.3Interaction Effect of Experimental Variables on Cell Growth

The following Figure(18,19,20,21,22 and 23) show that an interaction effect of temperature & pH on cell growth. In this case, both temperature and pH have significant quadratic effects ($P <$ 0·05), indicating the presence of an optimum both factors for without supplement of caffeine for bacillus sp.

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Figure 18 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH For *Bacillus subtilis.(for 50ml broth bacillus*

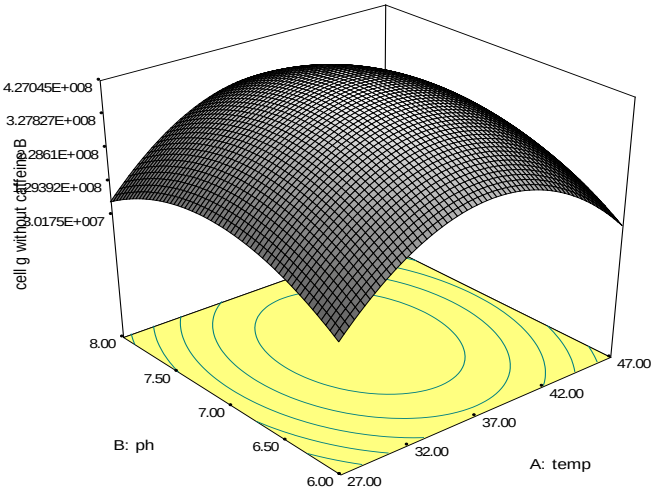
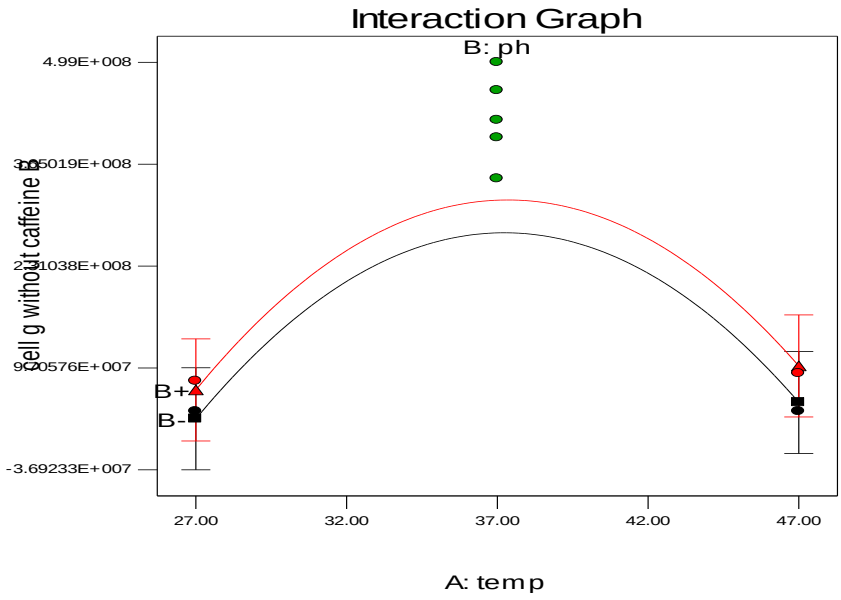


Figure 19 Effect and interaction of temperature and pH (fixed) on the incubation time For *Bacillus sp.* bacteria growth.



The following figure (20 and 21) show that an interaction effect of temperature & incubation time on cell growth. In this case, both temperature and pH have significant quadratic effects (*P*

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< 0·05), indicating the presence of an optimum for both of the factors for without supplement of caffeine for bacillus sp.

Figure 20 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH For *Bacillus subtilis*

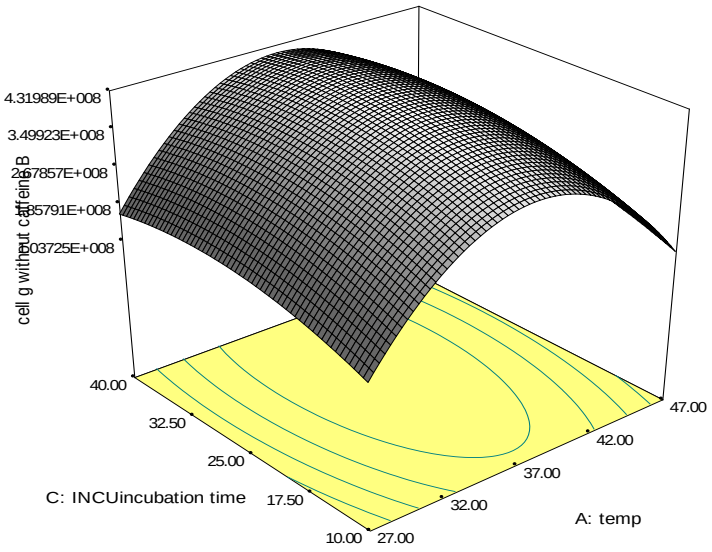
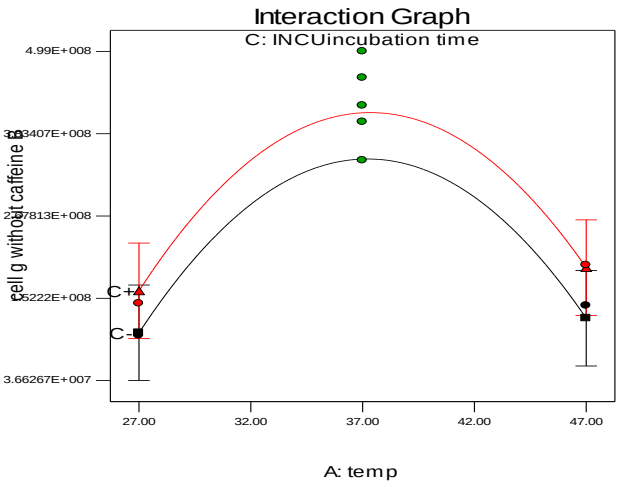


Figure 21 Effect and interaction of temperature and pH (fixed) on the incubation time For Bacillus subtilis bacteria growth



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The following figure (22 and 23) show that an interaction effect of pH & incubation time on cell growth. In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum for both factors for without supplement of caffeine for bacillus sp.

Figure 22 Contour plots of the effect of PH and incubation time on the cell growth at fixed temperature For *Bacillus sp.*

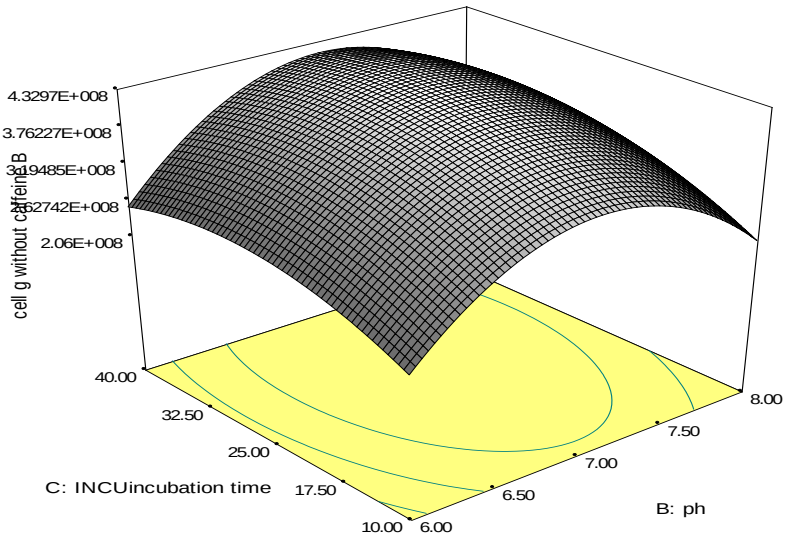
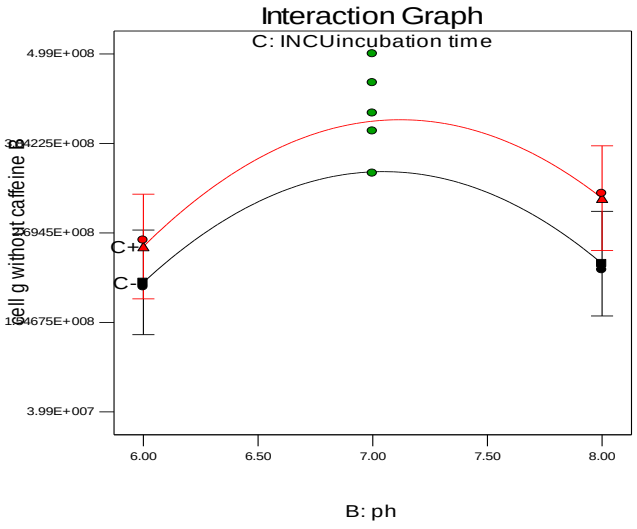


Figure 23 Effect and interaction of PH and incubation time (fixed) on the temperature For *Bacillus* subtilis bacteria growth.



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4.1.6 Statistical Analysis of the Experimental Result of media supplemented with 2.5g/l of caffeine for bacillus bacteria

Experimental results of three factors with three levels and one republican and the following tables show statistical analysis of the experimental results by using Design-Expert Soft-ware (version 6.0.8). Summary of the model details is that study type response surface box-Behnken design model is quadratic number of experiment is 17

Table 16 number of cell for media supplemented with caffeine for bacillus bacteria

std	Run	Block	Factore 1 temp(^{0}c)	Factore 2 pH	Factore 3 incubation time	Cell growth
2	1	Block 1	47.00	6.00	25.00	5.5E+007
8	2	Block 1	47.00	7.00	40.00	3.49E+008
9	3	Block 1	37.00	6.00	10.00	3.5E+008
12	4	Block 1	37.00	8.00	40.00	4.7E+008
4	5	Block 1	47.00	8.00	25.00	4.1E+008
10	6	Block 1	37.00	8.00	10.00	3.72E+008
14	7	Block 1	37.00	7.00	25.00	5.5E+008
13	8	Block 1	37.00	7.00	25.00	4.96E+008
6	9	Block 1	47.00	7.00	10.00	2.92E+008
1	10	Block 1	27.00	6.00	25.00	5.49E+007
11	11	Block 1	37.00	6.00	40.00	4.1E+008
5	12	Block 1	27.00	7.00	10.00	2.5E+008
15	13	Block 1	37.00	7.00	25.00	5.73E+008
7	14	Block 1	27.00	7.00	40.00	2.95E+008
17	15	Block 1	37.00	7.00	25.00	6.49E+008
3	16	Block 1	27.00	8.00	25.00	9.49E+007
16	17	Block 1	37.00	7.00	25.00	6.12E+008

Analysis of variance (ANOVA) In ANOVA analysis the model was significant when fisher's F test with a very low probability value. From table 17 observed that the Model F-value of 12.21 implies the model is significant. There is only a 0.17% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, A^2, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The coefficient was linear effect of temperature and pH and incubation time was least significant

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Table 17 Analysis of variance (ANOVA for media supplemented with caffeine for bacillus

Source Model	**Sum of Square**	**DF**	**Mean Squares**	**F Value**	**Prob > F**	
	5.079E+017	9	5.643E+016	12.21	0.0017	significant
A	2.114E+016	*1*	2.114E+016	*4.57*	*0.0698*	
B	2.844E+016	*1*	2.844E+016	*6.*15	*0.0422*	
C	8.450E+015	*1*	8.450E+015	*1.83*	*0.2184*	
A^2	2.916E+017	*1*	2.916E+017	63*.08*	*< 0.0001*	
B^2	1.066E+017	*1*	1.066E+017	*23.07*	*0.0020*	
C^2	1.126E+015	*1*	1.126E+015	0.24	0.6368	
AB	2.481E+016	*1*	2.481E+016	5.37	0.0537	
AC	3.600E+013	*1*	3.600E+013	7.789E-003	0.9321	
BC	3.610E+014	*1*	3.610E+014	0.078	0.7880	
Residual	3.235E+016	7	4.622E+015			
Lack of Fit	1.864E+016	*3*	6.215E+015	1.81	0.2845	not significant
Pure Error	1.371E+016	*4*	3.428E+015			
Cor Total	5.402E+017	16				

The “Lack of Fit F-value” of 0.078 implies the Lack of Fit not significant relative to the pure error. There is a 28.45% chance that a “Lack of Fit F-value” this large could due to noise. Non-significant lack of fit is good – we want the model to fit.

Std. Dev	6.799E+007	R-Squared	0.9401
Mean	3.696E+008	Adj R-Squared	0.8631
C.V.	18.40	Pred R-Squared	0.4082
PRESS	3.197E+017	Adeq Precision	9.767

The "Pred R-Squared" of 0.4082 is not as close to the "Adj R-Squared" of 0.8631as one might normally expect. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 9.767 indicates an adequate signal. This model can be used to navigate the design space.

Final Equation in Terms of Actual Factors:

cell growth = -9.36305E+009 +1.44246E+008 temp +1.98052E+009 pH +6.26667E+005 incubation time -2.63150E+006 $temp^2$-1.59150E+008 pH^2-72666.66667 $incubation\ time^2$ +7.87500E+006temp * pH +20000.00000 temp * incubation time +6.33333E+005 ph * incubation time

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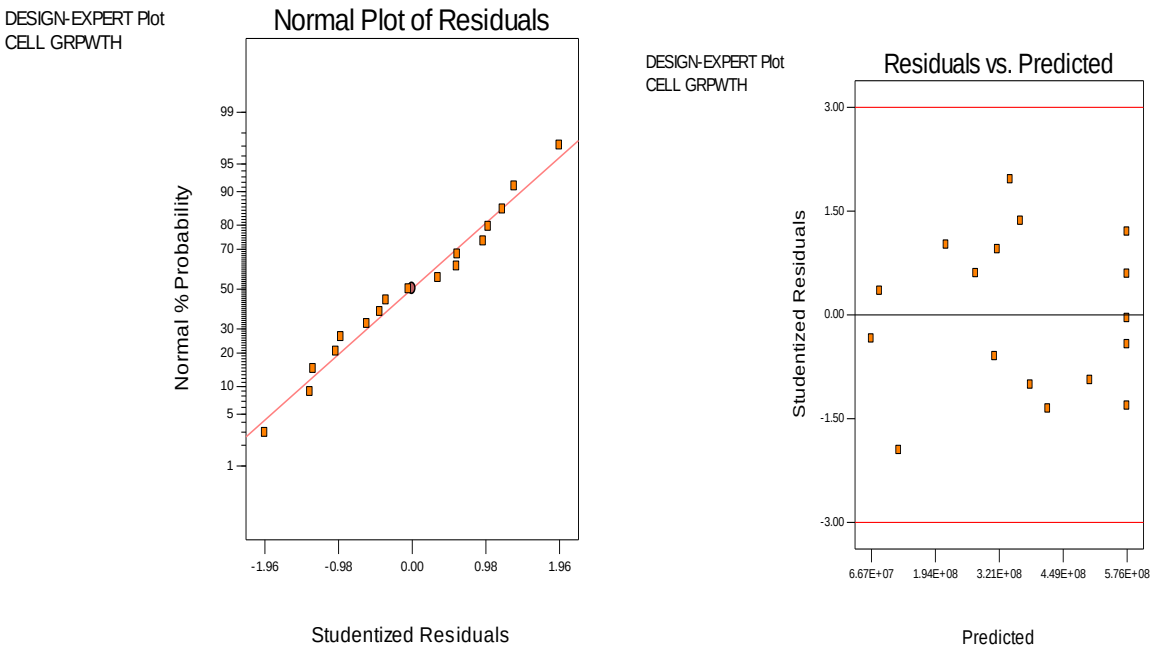
Diagnostic test for the responses

All diagnostic plots are also tested for all responses for adequacy of the models (normal plot of residuals, residuals vs predicted value, residuals vs. factor, box cox plot, studentized residuals, leverage, etc.). From the plot as shown below, the normal probability plot indicates the residuals following by the normal % probability distribution, in the case of this experimental data the points in the plots shows fitted to the straight line in the figure 24, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal.

Figure 24 Diagnostic tests for the responses of caffeine supplemented media for bacillus bacteria



4.1.6.3 Interaction Effect of Experimental Variables on Cell Growth (media supplemented with 2.5l/g of caffeine) bacillus bacteria

The following Figure (25 and 26) show that an interaction effect of temperature & pH on cell growth. In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum factors.

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Figure 25 Contour plots of the effect of temperature and pH on the cell growth at fixed incubation time rhizobium bacteria (media with caffeine for bacillus bacteria)

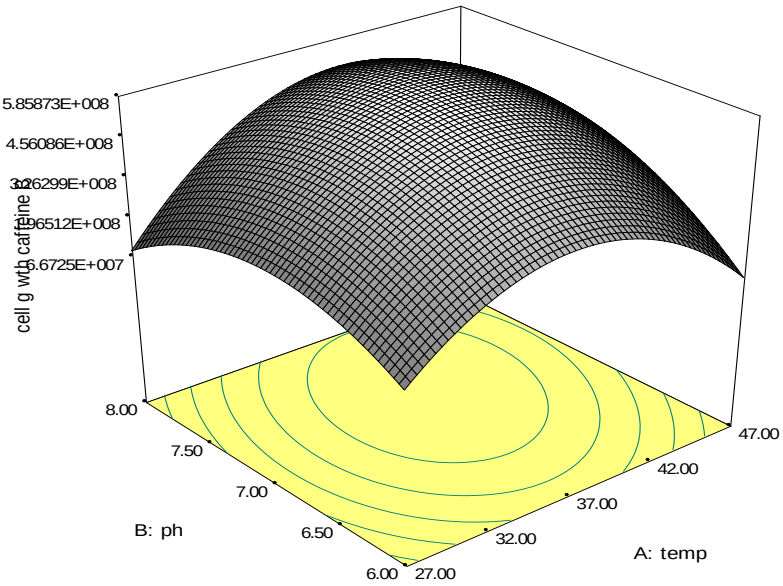
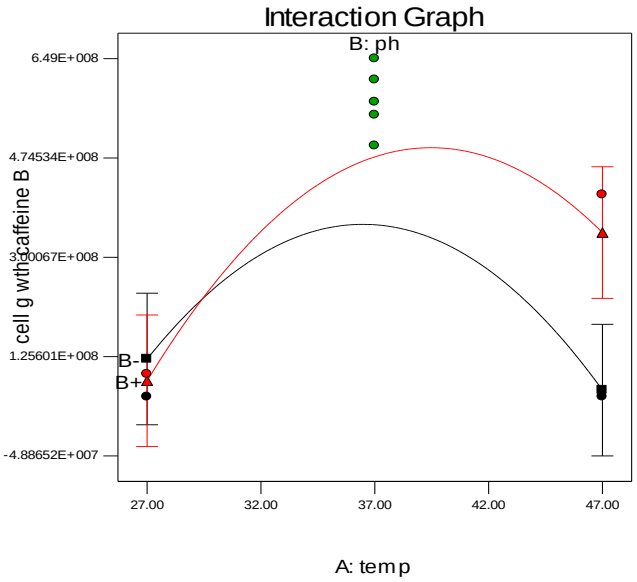


Figure 26 Effect and interaction of temperature and PH (fixed) on the incubation time for rhizobium bacteria growth.



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The following Figure (27 and 28) show that an interaction effect of temperature & incubation time on cell growth. In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum factors.

Figure 27 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH rhizobium bacteria (with caffeine for bacillus bacteria)

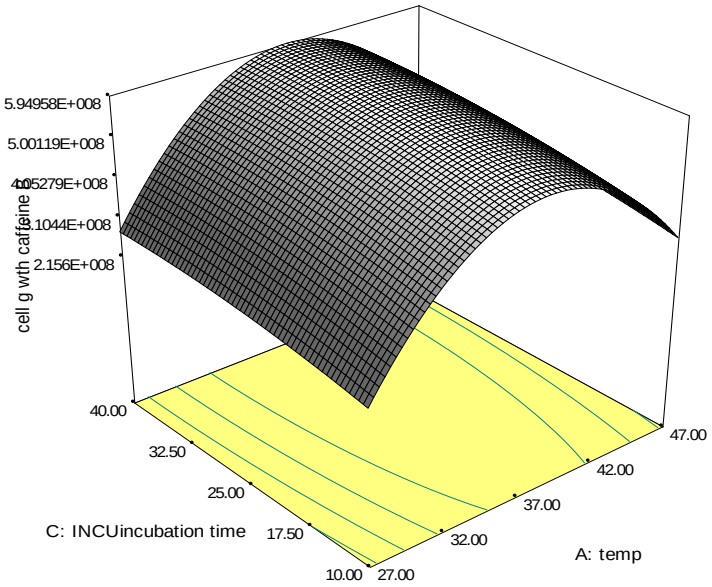
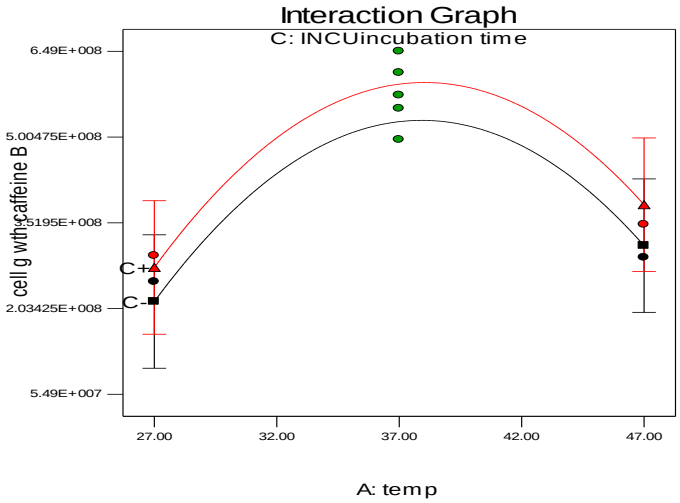


Figure 28 Effect and interaction of temperature and incubation time (fixed) on the pH for rhizobium bacteria growth.



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The following Figure (29 and 30) show that an interaction effect of incubation time & pH on cell growth. In this case, both temperature and pH have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum factors.

Figure 29 Contour plots of the effect of temperature and incubation time on the cell growth at fixed pH rhizobium bacteria (media with caffeine for bacillus bacteria)

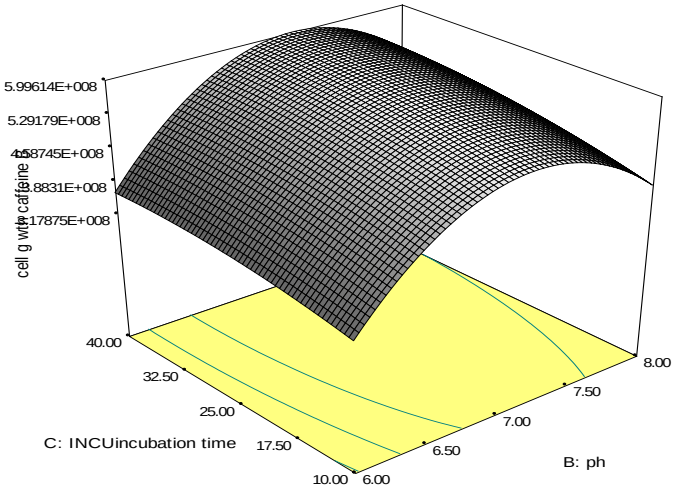
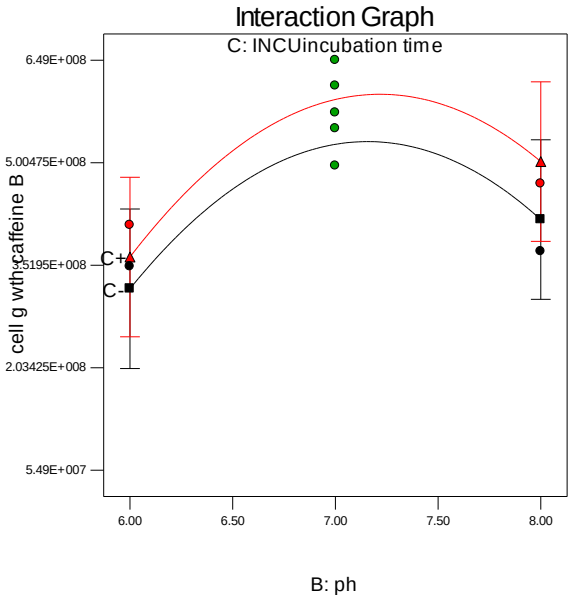


Figure 30 Effect and interaction of temperature and incubation time (fixed) on the pH For rhizobium bacteria growth.



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Chapter five

5.1 Optimizations of factor parameter

Finally optimization is important in order to select the most effective use of a situation or resource. So, the following tables show the process of optimization with the help of Design-Expert Soft-ware (version 6.0.8) for optimization

Optimization for Maximum Cell Growth for Rhizobium bacteria (without caffeine supplement)

Table-16 presents optimization for maximum cell growth alone i.e. the maximum growth of bacteria that will be produced at optimum operating temperature, pH, and incubation time without considering the other response variables. The maximum number of bacteria that will be produced were 7.01886E+008 cells at 47.00 ^{o}C, 8.00 & 26.79-hr with 0.779 desirability; 7.01834E+008cells at 47.00 ^{o}C, 8.00 & 26.54-hr with 0.779 desirability; 7.01736E+008 cells at 47.00^{o} C, 8.00 & 27.31-hr with 0.779 desirability; and 7.01036E+008 cells at 46.84 ^{o}C, 8.00 & -27.00hr with 0.778 desirability; 7.01886E+008cells at 47.00 ^{o}C, 8.00 & 26.79-hr with 0.779 desirability were selected as optimum temperature, pH, and incubation time respectively due to its high desirability and number of cells

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight		Upper Weight	Importance
temp	is in range	27	47	1		1	
pH	is in range	6	8	1		1	
incubation time	is in range	10	40	1		1	
cell growth without caffeine for R	maximize	3.99E+007	8.9E+008	1		1	

Solutions

Number	temp	Ph	incubation time	cell growth without caffeine for R	Desirability	
1	**47.00**		**8.00**	**26.79**	**7.01886E+008**	**0.779**	**Selected**
2	47.00		8.00	26.54	7.01834E+008	0.779	
3	47.00		8.00	27.31	7.01736E+008	0.779	
4	46.84		8.00	27.00	7.01036E+008	0.778	

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5.1.1 Maximum Cell Growth for rhizobium bacteria media supplemented with caffeine

Table-16 presents optimization for maximum cell growth alone i.e the maximum growth of bacteria that will be produced at optimum operating temperature, pH, and incubation time without considering the other response variables. The maximum numbers of bacteria that will be produced were 4.78413E+008 cells at 37.31°C, 7.09, & 33.49-hr with 0.878 desirability; optimum temperature, pH, and incubation time respectively. And 4.78413E+008 37.31oC, 7.09, 33.49-hr with 0.878 desirability were selected as optimum temperature, pH, and incubation time respectively due to its high desirability and number of cells.

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
temp	is in range	27	47	1	1
pH	is in range	6	8	1	1
incubation time	is in range	10	40	1	1
cell growth with caffeine R	maximize	4.39E+007	5.39E+008	1	

Solutions

No,	temp	pH	incubation time	cell growth with caffeine R	Desirability	
1	37.31	7.09	33.49	4.78413E+008	0.878	Selected

5.1.2 Optimization for Maximum Cell Growth for bacillus bacteria for media without caffeine

Table-16 presents optimization for maximum cell growth alone i.e the maximum growth of bacteria that will be produced at optimum operating temperature, pH, and incubation time without considering the other response variables. The maximum number of bacteria that will be produced were 4.332E+008cells at 37.32oC, 7.10, & 30.52-hr with 0.857desirability and 4.332E+008 at 37.32°C, 7.10, 30.52-hr with 0.857 desirability were selected as optimum temperature, pH, and incubation time respectively due to its high desirability and number of cells

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
temp	is in range	27	47	1	1
ph	is in range	6	8	1	1
incubation time	is in range	10	40	1	1
cell g without caffeine	maximize	3.99E+007	4.99E+008	1	1

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Solutions						
Number	**temp**	**pH**	**incubation time**	**cell g without caffeine B**	**Desirability**	
1	**37.32**	**7.10**	**30.52**	**4.332E+008**	**0.857**	**Selected**

5.1.3 Optimization for Maximum Cell Growth for media supplemented with 2.5g/l caffeine for bacillus bacteria

Table-16 presents optimization for maximum cell growth alone i.e the maximum growth of bacteria that will be produced at optimum operating temperature, pH, and incubation time without considering the other response variables. The maximum numbers of bacteria that will be produced were 6.04699E+008_cells at 38.41 ^{o} C, 7.25, & 40.00-hr with 0.925 optimum temperature, pH, and incubation time respectively. Which was selected as optimum temperature pH, and incubation time due to its high desirability and number of cells

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
temp	**is in range**	**27**	**47**	**1**	**1**
ph	**is in range**	**6**	**8**	**1**	**1**
incubation time	**is in range**	**10**	**40**	**1**	**1**
cell g wth caffeine B		**maximize**	**5.49E+007**	**6.49E+008**	**1** **1**

Solutions

Number	temp	ph	incubation time	cell g wth caffeine B	Desirability	
1	**38.41**	**7.25**	**40.00**	**6.04699E+008**	**0.925**	**Selected**

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5.1.4 Optimization for Maximum Cell Growth (rhizobium) in the given. Ranges of Experimental Variables

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
temp	is in range	27	47	1	1
pH	is in range	6	8	1	1
incubation time	is in range	10	40	1	1
cell growth without caffeine for R	maximize	3.99E+007	8.9E+008	1	1
cell growth with caffeine R	maximize	4.39E+007	5.39E+008	1	1

Solutions

Number	temp	pH	incubation time	cell growth without caffeine for R	cell growth with caffeine R	Desirability	
1	39.63	7.48	28.21	4.37573E+008	5.23666E+008	0.673	Selected

Optimization for Maximum Cell Growth with the Objectives of Minimizing Temperature and PH (rhizobium bacteria)

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
temp	minimize	27	47	1	1
pH	minimize	6	8	1	1
incubation time	is in range	10	40	1	1
cell growth without caffeine for R	maximize	3.99E+007	8.9E+008	1	1
cell growth with caffeine R	maximize	4.39E+007	5.39E+008	1	1

Solutions

Number	temp	pH	incubation time	cell growth without caffeine for R	cell growth with caffeine R	Desirability	
1	34.08	6.58	27.78	3.51146E+008	4.09131E+008	0.593	Selected

The desirability function was the solution for multy-response optimization. Desirability is an objective function that ranges from zero outside of the limit to one at the goal. The numerical

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optimization finds a point that maximizes the desirability function. The desirability value of 0.631 corresponded to the cell numbers, and in the given range of parameters. The following histogram shows how each variable satisfied the criteria: values near one are good.

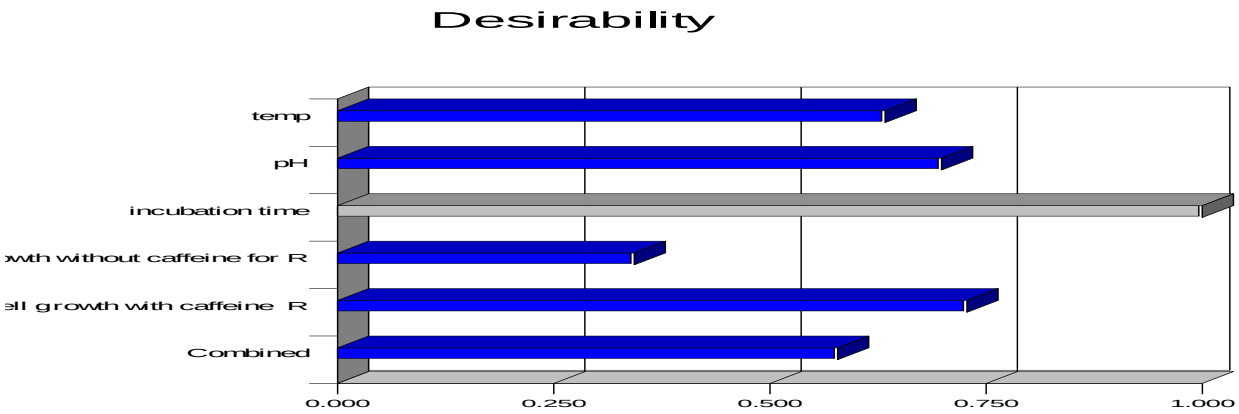

Figure 2 historical diagram of desirability

5.1.7 Validation of the analysis for rhizobium

Prepare 50ml of broth with and without supplemented of 2.5gr/l of caffeine for growth medium of microorganism's bacillus those bacteria serve as liquid bio-fertilizer by inoculated the identify colonies of organism in to the broth and incubated at 34.08 ˚C for 27.58 hrs. and buffering PH of 6.58 followed by shaking vigorously. We obtain maximum cell growth 4.09131E+008 with supplement of caffeine and without supplement of caffein maxiume cell growth was 3.51146E+008 those cell were use as liquid bio fertilazer so additional number of cell obtained that 5.7985E+007 this show that this microbes use caffeine as substrate.

5.2.1 .Optimization for Maximum Cell Growth (rhizobium) in the given. Ranges of Experimental Variables (bacillus spp)

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
temp	is in range	27	47	1	1
ph	is in range	6	8	1	1
incubation time	is in range	10	40	1	1
cell g without caffeine B	maximize	4.99E+007	5.99E+008	1	1
cell g wth caffeine B	maximize	5.49E+007	6.49E+008	1	1

.

Solutions

Number	temp	pH	incubation time	cell g without caffeine B	cell g wth caffeine B	Desirability	
1	**37.75**	**7.16**	**40.00**	**5.58009E+008**	**6.02614E+008**	**0.924**	**Selected**
2	37.84	7.14	40.00	5.58047E+008	6.02469E+008	0.924	
3	37.80	7.18	40.00	5.57391E+008	6.03146E+008	0.924	

5.2.2 Optimization for Maximum Cell Growth (bacillus) with the Objectives of Minimizing Temperature and incubation time

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	
temp	**minimize**	**27**	**47**	**1**		1
ph	**is in range**	**6**	**8**	**1**		1
incubation time	**minimize**	**10**	**40**	**1**		
cell g without caffeine B		**maximize**	**3.99E+007**	**4.99E+008**	**1**	1
cell g wth caffeine B		**maximize**	**5.49E+007**	**6.49E+008**	**1**	1

Solution

Number	temp	pH	incubation time	cell g without caffeine B	cell g wth caffeine B	Desirability	
1	34.60	7.07	11.25	3.41995E+008	5.0707E+008	0.739	Selected

The desirability function was the solution for multy-response optimization. Desirability is an objective function that ranges from zero outside of the limit to one at the goal. The numerical optimization finds a point that maximizes the desirability function. The desirability value of 0.729 corresponded to the cell numbers, and in the given range of parameters. The following histogram shows how each variable satisfied the criteria: values near one are good.

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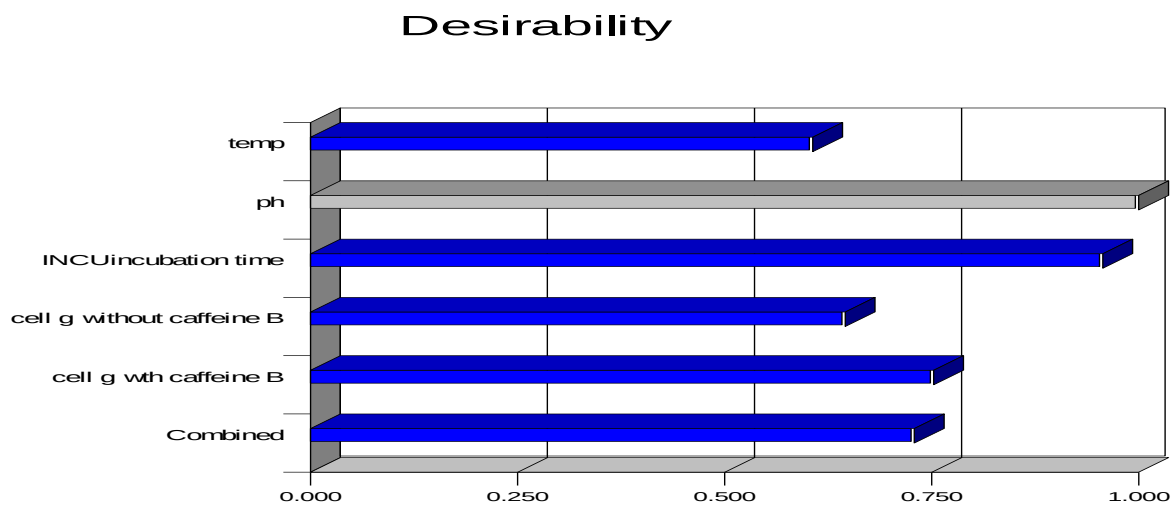

Figure 3 histogram diagram of desirability of bacillus SPP

5.2.4 Validation of analysis for bacillus

Prepare 50ml of broth with and without supplemented of 2.5gr/l of caffeine for growth medium of microorganism's bacillus those bacteria serve as liquid bio-fertilizer by inoculated the identify colonies of organism in to the broth and incubated at 34.6 ˚C for 11.25hrs and buffering PH of 7.07 followed by shaking vigorously. We obtain maximum cell growth 5.0707E+008 with supplement of caffeine and without supplement of caffein maxiume cell growth was 3.41995E+008 those cell were use as liquid bio fertilazer so additional number of cell obtained that 1.6508E+008 this show that this microbes use caffeine as substrate.

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Chapter six

6.1 CONCLUSIONS AND RECOMMENDATION

6.1.1 CONCLUSIONS

The environment where we live is the habitat for various microorganisms; mostly bacteria which are used for various industrial applications like enzymes production, fabric manufacturing, bioremediation, like bio fertilizers, pharmaceutical production, etc Microorganisms play an important role in composting of organic waste and can be an important contributor to optimal agricultural waste. This study revealed the identification of N2 fix (rhizobium) bacteria and phosphor solubilized bacteria like Bacillus species continue to be solubilizing phosphate for plant growth nutrient. Those bacteria are used for preparation of liquid bio fertilizer mostly there was diversity of microorganisms which are present in coffee husk as habitat. The present investigation focuses on the identification of Caffeine degrading bacteria from the environmental sample coffee husk where caffeine could be the sole source of carbon and nitrogen. Rhizobium and bacillus spp of bacteria identify from coffee husk can be caffeine capable of degrading caffeine up to 2.5 g/l efficiently with an incubation time of 24 hrs. The main study evidence that lower concentration was found to show promoting effect on growth and productivity of plants. The fertilizing efficiency broth is due to the presence of micro and macro nutrients, at preferential levels. It can be concluded that the broth prepared using two types of organism which were identify can be used as environment friendly liquid bio-fertilizers to replace hazardous chemical fertilizers. This will keep the fertile of soil conditions intact and maintain it for longer duration of time. Media supplemented with 2.5g/l of caffeine then optimized the factor like temperature pH and incubation time that affect the response of cell growth for both species of bacteria supplemented and without supplemented caffeine. The ability of the organism to degrade caffeine was determined by comparing the spectrum of caffeine with that of the sample. And also release best organism that degrade caffeine with this analytical method. 0.6% of caffeine eliminated from coffee husk from this research used species of coffee bean coffee husk then supplemented to microbes effectively degraded them as carbon nitrogen source. Because high content of carbon and nitrogen shown in physiochemical analysis. Then those microbes. Authenticated by different morphological and biochemical test with those test we observe that most of test were active. And also effect of temperature (mesospheric regiom) and PH (weak acidic region) and incubation time analysis.

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6.1.2 Recommendation

According to this investigation of research was recommended for further researcher should be study will need that the effectively of the influence of the liquid bio-fertilizer inoculation of those bacteria Rhizobium (nitrogen fix) and bacillus (phosphate solubilizing bacteria). Alone and in combination with recommended dose of liquid bio-fertilizer on coriander, peas and fenugreek crop will be need taste at specific interval of time. The result revealed significant improvement in growth of those important microbes special supplement of caffeine was obtained more microbes (N solubilized and p-solubilize).there will need five sets for investigation of effectively in which first pot cultivated coriander, in second pot cultivated peas, third pot cultivated fenugreed, in fourth fifth pots also cultivated fenugreed. In fourth pot chemical fertilizer is use and fifth one is control. Investigation will best growth is see in liquid bio fertilizer using microorganism. The second recommended matter that studying caffeine degrading performance of those bacteria with more advanced technology analytical method like UV spectrometry mean that samples from the enrichment broth were retrieved at different intervals time and checked for absorbance at 275nm in UV- Spectrometry, were caffeine was used as control.

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Appendix

Appendix A

Definition of terminology

Disinfection is a process in which vegetative, nonsporing microorganisms are destroyed. Agents that cause disinfection are called disinfectants or germicides. Such agents are used only on inanimate objects because they are toxic to human and animal tissues.

Tabletop Disinfection. The first chore of the day will be to sponge down your desktop with a disinfectant. This process removes any dust that may be present and minimizes the chances of bacterial contamination of cultures that you are about to handle.

Sterilization is a process in which all living microorganisms, including viruses, are destroyed. The organisms may be killed with steam, dry heat, or incineration. If we say an article is sterile, we understand that it is completely free of all living microorganisms. Generally speaking, when we refer to sterilization as it pertains here to laboratory safety, we think, primarily, in terms of steam sterilization with the autoclave. The ultimate method of sterilization is to burn up the infectious agents or incinerate them. All biological wastes must ultimately be incinerated for disposal.

Caffeine= stimulating component of coffee and cocoa beans

Colony forming unit The bacteria can thus be isolated and counted by calculating C.F.U. i.e., Colony Forming Unit.

C.F.U. = no, of colonies/inoculums size (ml) X dilution factor C.F.U/ml

Bio fertilizers from microorganisms can replace chemical fertilizers to increase crop production. In principle, bio fertilizers are less expensive and are more environmentally-friendly than chemical fertilizers.

Liquid bio fertilizer are are bio fertilizers of microorganism that can be grow in liquid medium

Methyl red is the reagent that can detect acid base states of medium

Kovacvs reagent is the reagent used for detect idole formation of the medium

.

Moisture content it indicates how much substance wter holding

Initial weighing of substance –weighing after drying the difference divided by 100 the result will be %moisture content

Colony formed by microorganism with selective medium in the form of drove/herd of bacterial cell

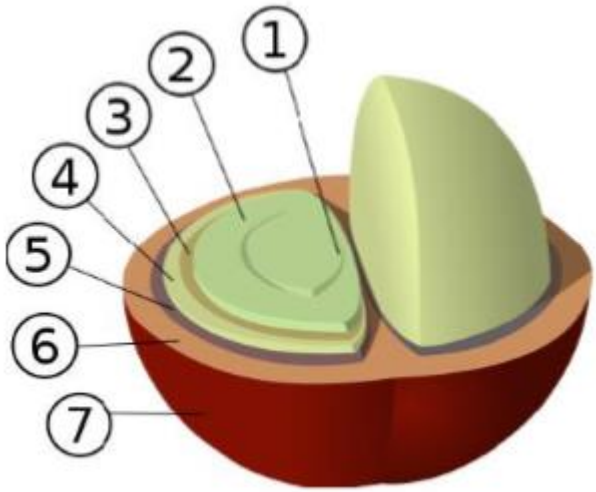
Aseptic techniques refer to those practices that are used by microbiologists to exclude all organisms from contaminating media or contacting living tissues.

Antiseptics are chemical agents (often dilute disinfectants) that can be safely applied externally to human tissues to destroy or inhibit vegetative bacteria.

Bunsen Burner Usage When using a Bunsen burner to flame loops, needles, and test tubes.

Rhizobium species of bacterial strain that can fix atmospheric nitrogen

Bacillus subtilis species of bacterial strain that solubilize un soluble form of phosphors



Structure of coffee cherr

Where;1=centre cut; 2= bean (endosperm) 3= **silver skin** (testa, epidermis) 4**=(parchment** (hull, endocarp) 5=pectin layer 6=pulp (mesocarp) 7= outer skin (pericarp, exocarp).

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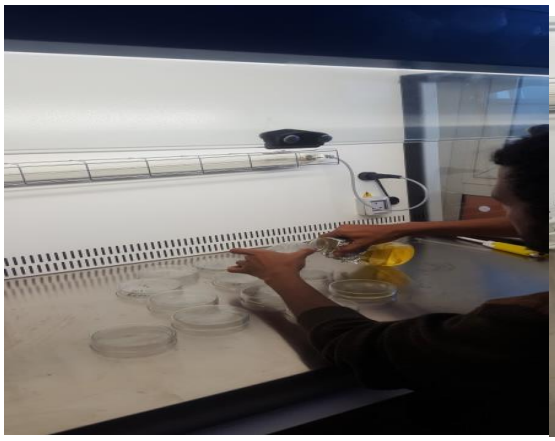
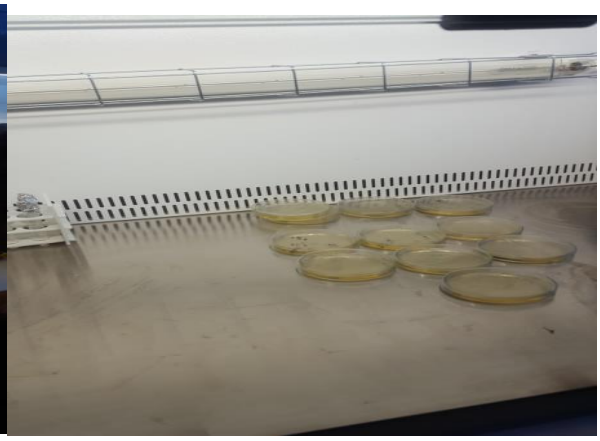
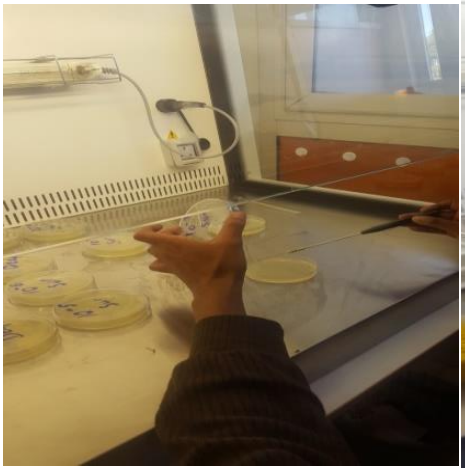
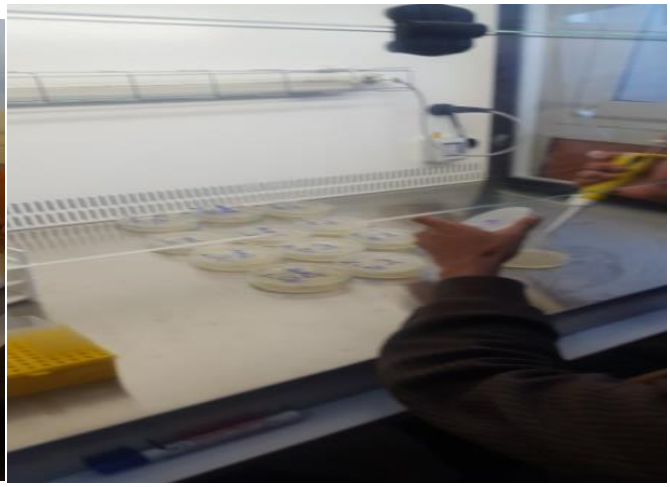
Appendix B

Photograph captured during the laboratory

Plating


Dilution of sample

Streaking of microbes on plate

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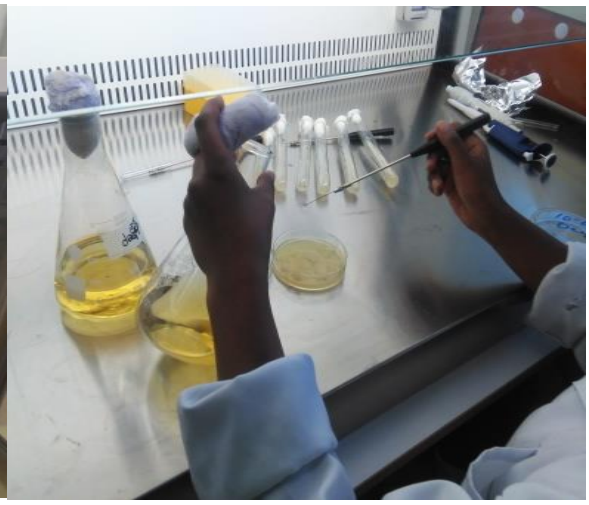
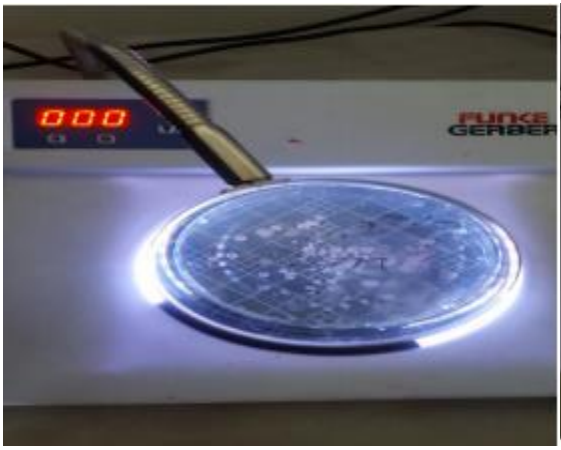
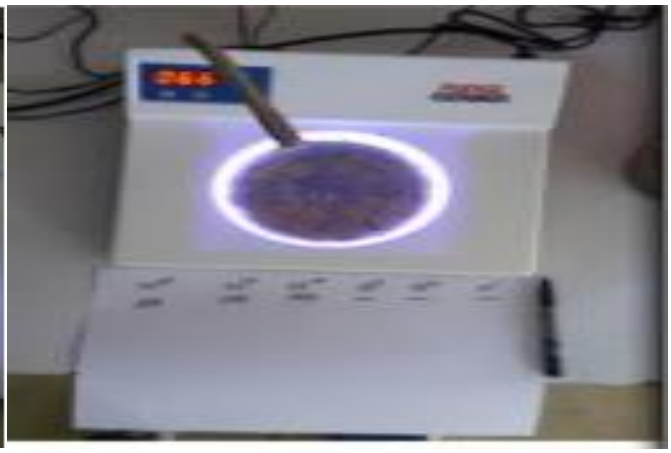
Inoculation of microbes and incubation



Shaker incubator





Counting colony of microbes

Coffee husk

caffeine

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Rotary evaporator


Autoclave and Media ready to sterilize

Oven

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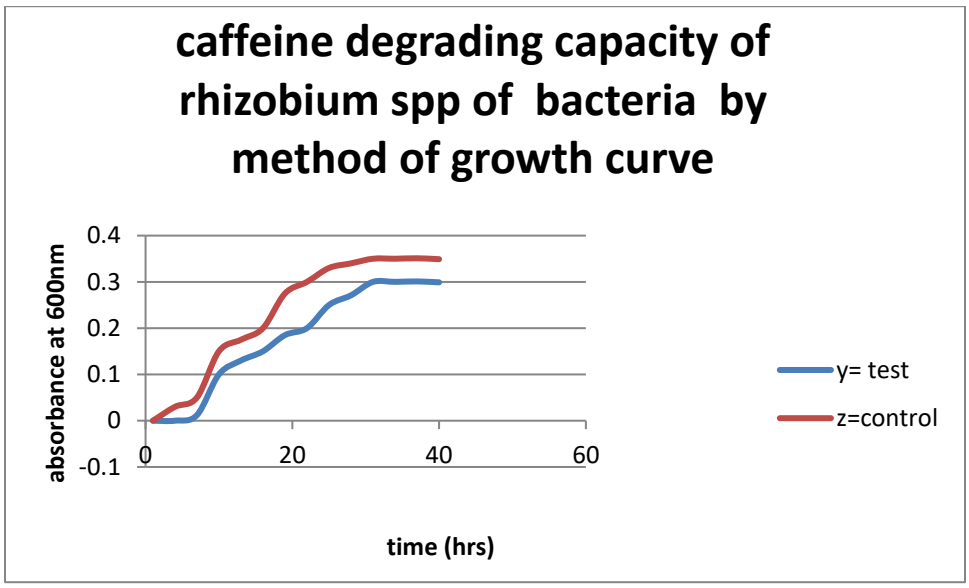
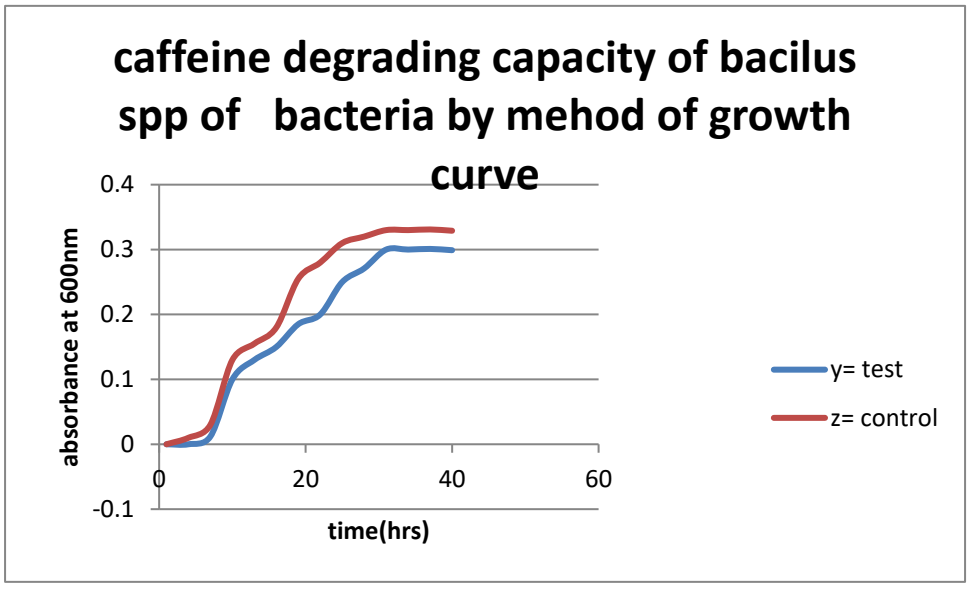
Appendix C

Caffeine degrading capacity of identified microbes with growth curve experiment

For 1C (rhizobium spp)



For 2C (bacillus spp)



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Appendix D

Interaction of Individual factor effect