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AN IMPECCABLE STUDIES ON *Bacillus simplex* AND ITS *INVITRO* ANTIOXIDANT PRODUCTION, PRE AND POST EVALUATION OF ANTIOXIDANT INCORPORATION ON FOOD (IDLY)

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ABSTRACT

In this present investigation, the sand sample was collected from Pitchavaram and Boombuhar at Chidambaram District in Tamil Nadu. The sample was brought to the laboratory for microbial analysis. The bacteria Bacillus Simplex species were isolated from the two different sand samples. The isolated bacteria namely, B. simplex was used to antioxidant production using different medium at different temperature and pH. The maximum production was monitored in LB and NA medium the values are 77 % in LB and 68 % in NA. The effect of temperature and pH during antioxidant production the maximum was observed 78 % at 100ºC in LB medium, 71% in NA medium and the effect of pH during antioxidant production was resulted, 81% in basic level of pH respectively. The medium was used to identify the antioxidant molecule by TLC with reference compounds namely, vitamins and phenolic compounds. The isolated and identified compound was subjected for FT-IR and SEM to confirm the compound such as antioxidant molecule with standard reports. After the compound was allowed to purification and it was used to incorporate in IDLY to evaluate the nutrients by standard methods. The identified compound is one of the amino group and vitamin based compound to conform during this study and the antioxidant compound was incorporated in IDLY by two ways after and before fermentation to enhance the antioxidants on IDLY. At this study we conclude the high level of antioxidant enrichment was monitored in sample 1. To investigate the nutrients such as Protein carbohydrate cholesterol, minerals, vitamins and energy were high in sample1 then the sample 2 and control has low level of nutrients. The antioxidant compound has maximum level inhibitory activity against selected food associated microbes namely, E. coli, P. aeruginosa, Mucor and A. niger and the compound was to preserve the IDLY on room temperature without microbial contamination at 5 days after incorporation of antioxidant compound. Likewise in our present study, concluded that the isolated antioxidant from Bacillus simplex gave many antimicrobial activity and these the compound is suitable for antioxidant enrichment of food. So it is used for food industries to enhance the antioxidant rich food for naturally. It can be used regularly for our food preservation to avoid the contamination and spoilage.

KEYWORDS

Bacillus simplex, E. coli, P. aeruginosa, Mucor, A. niger, pH, FT-IR and SEM.

1. INTRODUCTION

Microbial food cultures are live bacteria, yeasts or moulds used in food production and preservation. Microbial food cultures carry out fermentation process in food stuffs. Starter cultures have mainly a technological function in the food manufacturing. They are used as food ingredients at one or more stages in the food manufacturing process, which develop the desired metabolic activity during the fermentation or ripening process. They contribute to the one or multiple unique properties of food stuff especially in regard to taste, flavour, colour, texture, safety, preservation, nutritional value, wholesomeness or health benefits (Wu *et al*., 2009).

Microbes can produce antioxidant, this antioxidant act as food preservatives. Food preservation is the process of treating and handling food to stop or slow down food spoilage, loss of quality, edibility or nutritional value and thus allow for longer food storage. *Bacillus simplex* strain with strong antioxidant activity was isolated from sand in coastal region. Bacteria containing significantly phenolics, including epicatechin, catechin, 3-O-methyl gallic acid, gallic acid and caffeic acid to generate aromatic metabolites dependent on bacterial species.

SCIENTIFIC CLASSIFICATION OF *Bacillus simplex*

Domain	: Bacteria
Division	: Firmicutes
Class	: Bacilli
Order	: Bacillales
Family	: Bacillaceae
Genus	: ***Bacillus***
Species	: ***simplex***

MORPHOLOGICAL CHARACTERISTICS OF *Bacillus simplex*

Strains of *Bacillus simplex* Gram-Positive rods shaped motile bacteria. The size ranges from 0.7-0.9 µm in diameter. Bacillus is a diverse bacterial genus characterized by cells growing aerobically and forming dormant endospores. Centrally or para centrally or sub terminally, ellipsoidal, occasionally spherical endospores in not obviously swollen sporangia. Colonies are cream, glossy, 3-6 mm in diameter after 2 days, with irregular margins, slightly raised and urbanite. Grow at pH 7 - 9 and at 20 and 30 ºC but are not able to grow at 45 ºC.

ANTIOXIDANT PRODUCING MICROBES

Bacillus simplex, Bacillus natto, Bacillus cereus, Lactobacillus dextranicum, Micrococcus freudenreichii, Sarcina lutea, Bacillus stearothermophilus Bacillus longum Lactobacillus acidophilus Thermothrix species, A*spergillus alternate, Penicillium, Deinococcus radiodurans* these Organisms are showed considerable antioxidant activity.

ANTIOXIDANTS IN FOOD

The beneficial influence of many food stuffs and beverages including fruits, vegetables, tea, red wine, coffee, and cacao on human health has been recently recognized to originate from the chain-breaking antioxidant activity (AOA) of natural polyphenols, a significant constituent of the above products. For this reason, the dietary value of such products is determined to a large extent by their AOA. The latter stimulated the development of effective and reliable methods for determining AOA. Although the kinetic approach provides the basis of the majority of these methods, only a few of them have been analyzed from the view point of chemical kinetics. The most popular methods for determining chain-breaking AOA of food are considered with the aim to estimate their reliability and limitations. The main requirements imposed on these methods have been suggested. The main attention has been paid to the repeatability of the data obtained. Along with the methods that are currently popular among researchers working in food

chemistry and biomedical sciences, perspectives of the application of the methods used to studying industrial antioxidants have also been considered (Vitaly Roginsky and Eduardo Lissi, 2005).

SCOPE OF USING ANTIOXIDANTS IN FOOD

The function of an antioxidant is to retard the oxidation of an organic substance, thus increasing the useful life or shelf life of that material. In fats and oils, antioxidants delay the onset of oxidation or slow the rate of oxidizing reactions. Oxidation of lipids chemically produces compounds with different odors and taste and continues to affect other molecules in the food. The main purpose of using an antioxidant as a food additive is to maintain the quality of that food and to extend its shelf life rather than improving the quality of the food. Illustrates how antioxidants can affect the quality maintenance of food in terms of oxidative rancidity.

2. MATERIALS AND METHODS

Sample Collection

In the present study, the sand sample was collected from Pitchavaram and Boombuhar at Chidambaram District in Tamil Nadu. The sample was brought to the laboratory for microbial analysis. The bacteria *Bacillus Simplex* species were isolated from the two different sand samples and it was allowed to the following studies.

Biochemical tests

Indole test

The test requires sterilized (121ºC for 15 min) tryptophan broth. The culture was inoculated in cooled sterilize broth. After 24 hrs of incubation, 0.3 ml of Kovac's reagent was added into the tubes for observe the result.

Methyl Red and Voges Proskauver's Test

The culture was inoculated into the tubes containing sterilized (121ºC, 15 min at 15 lbs) MR–VP broth; Tubes were incubated at 37ºC for 24 hrs; Add 0.5ml of MR reagent, 0.2ml of VP reagent A and B; After adding reagent to observe the result.

Citrate Utilization Test

Lightly inoculate a pure culture into a tube of sterilize (121ºC, 15 min at 15 lbs) Simmon's citrate medium, using needle to stab, then streak the medium; Be careful not to carry over any nutrient material; incubated at 37ºC for 24 hrs; After incubation were observe the results.

Urease Test

In this test 20% urea solution added and added on sterilized (121ºC, 15 min at 15 lbs/Inch2) media and it was transferred into the slant tubes for slanting position solidification. The culture was inoculated into the tubes and incubated at 37ºC for 24 hrs. After incubation observe the result.

Triple Sugar Ion Test

Inoculate pure culture by stabbing and streaking the triple sugar iron (TSI) slant tube; Incubate at 37ºC for 24 hrs in a incubator. Read and record reactions;

Catalase Test

A drop of culture broth was placed on clean microscope slide; two or three drops of hydrogen peroxide solution were added to culture broth on the slide for observe air bubble formation.

Oxidase Test

Oxidase disc coated with 1% N–N tetra methylparaphenylene diamine dihydrochloride was placed at the centre of the clean microscope slide. A drop of culture broth was placed over the surface of the oxidase disc for observe the colour change.

Microbial test

The bacterial and fungal species were isolated from the gill, muscles and intestine region of *C. punctatus.*

Serial dilutions of the sample

The nutrient agar medium were prepared, sterilized and poured in sterile Petri plates and allowed to solidify. The 10gm of the sample was added to 90ml of the distilled water in a flask. It was shaking vigorously and 1ml was transferred in test tube containing 9ml of distilled water. The content was mixed well and 1ml was transferred from 10-1 dilution to the next dilutions up to 10-9 dilution. After solidifying, the nutrient agar plates with dilution 10-6 and 10-7 were taken. 0.1ml sample was poured in Petri plates using spread plate technique. The Nutrient agar plates with dilution 10-4, 10-5and 10-6 were taken 0.1ml sample was poured in Petri plates using spread using spread plate technique. The plates were incubated for bacteria at 37ºC for 24 hrs.

Streak plate method

In this method a sterilized loop of transfer needle was dipped into a streak plate method offers in the most practical method of obtaining discrete colonies and pure cultures. Suitable diluted suspension of organisms, which is then streaked on the surface of an already solidified agar plate to make a serious of parallel. The aim of this method is to check whether the organisms are growing i.e., growth derived from a single or spore.

Isolation of bacteria by gram staining

Gram staining was done by the method of Hans Christian's gram.

SELECTION OF THE BEST MEDIUM FOR ANTIOXIDANT PRODUCTION

In order to select the best culture medium for optimal antioxidant activity production, several bacteria broth media were selected: Nutrient broth (NB) with glucose, Luria-Bertani broth (LB) with glucose, Tryptic Soy Broth (TSB) with glucose, and Tryptic yeast (TY) with glucose, pH 7.0, autoclaving (121°C, 20 min). After inoculation, the strain was incubated at 30°C with shaking at 150 rpm for 3 days. Cell growth was monitored by optical density measurement at 600 nm, and antioxidant activity was tested by Antioxidant assay.

EFFECT OF TEMPERATURE AND PH FOR ANTIOXIDANT PRODUCTION

Thermal stability of the antioxidant activity was evaluated by the incubation of microbial antioxidant production medium at different temperatures such as, 40^{o} C, 60^{o} C, 80^{o} C and 100^{o} C for 30 min or after autoclaving at 121°C for 20 min. After cooling at room temperature, antioxidant activity and Total antioxidant capacity was determined. The range of pH was 3, 5, 7 and 9 maintained at the antioxidant production. The antioxidant activity was analyzed by the production of antioxidant in different temperature and pH.

DETERMINATION OF ANTIOXIDANT CAPACITY

The total antioxidant capacity (TAOC) of sample was evaluated by the method of Prieto *et al*.

To determine the FE-EDTA scavenging activity of Sample by Koracevic *et al*., Method, 2001.

The sample was used to estimate the phenol with Folin-Ciocalteu reagent, according to the method of (Li *et al.,* 2008).

ISOLATION, IDENTIFICATION OF ANTIOXIDANT COMPOUND AND VITAMINS BY TLC METHOD

Vitamin – A, Vitamin – B2, Vitamin – C, Vitamin – E, Catechin, Proanthocynidin, Phenolic Acid and Antioxidant Molecule

FOURIER TRANSFORM INFRARED (FT-IR) SPECTRA OF ANTIOXIDANT COMPOUND

The presence of polyphenols (Antioxidant compound) in the investigated microbial production medium was studied by Fourier Transform Infrared (FT-IR) spectroscopy.

ANTIMICROBIAL SUSCEPTIBILITY TEST OF ISOLATED ANTIOXIDANT MOLECULE

The antimicrobial activity of sample against different pathogens was determined by Agar disc diffusion. The disc diffusion method for antibiotic susceptibility testing is the Kirby- Bauer method.

The agar used is Meuller-Hinton agar that is vigorously tested for composition and pH. Further the depth of the agar in the plate is a factor to be considered in the disc diffusion method. This method is well documented and standard zones of inhibition have been determined for susceptible and resistant values. There is also a zone of intermediate resistance indicating that some inhibition occurs using this antimicrobial but it may not be sufficient inhibition to eradicate the organism from the body (Boyan Bonev, 2008).

NUTRIENT ANALYSIS OF IDLY BEFORE AND AFTER INCORPORATION OF ANTIOXIDANT MOLECULE

Estimation of Protein by Lowry's Method (1951)

The amino acid like tryptophan, tyrosine, phenylamine present in the proteins react with phosphemolybdic acid phosphotungstic acid of folins phenol reagent top give intense blue colour which is read at 650nm.

Estimation of Lipid by Zak's Method (1954)

In this method, proteins in sample are precipitated with ferric chloride - acetic acid reagent. The protein- free filtrate containing cholesterol is treated with concentrated sulphuric acid. Cholesterol in presence of sulphuric acid undergoes dehydration to form 3, 5 cholestadiene this is in turn oxidized and sulphonated to form red color cholestapolyene sulphonic acid in the presence of Fe3+ ions. The intensity of red color formed is propotional to the amount of cholesterol present in the serum. The color intensity is measured by using a green filter (540nm).

Calcium Estimation Using EDTA Method

Calcium can be determined by EDTA titration in solution of 0.1 m sodium hydroxide (pH 12-13) against murexide. Just like during determination of magnesium all metals other than alkali metals can interfere and should be removed prior to titration. Magnesium in that high pH precipitates as Mg $(OH)_2$ and is not complexed by EDTA, thus its presence can be ignored. Note, that if the amount of magnesium is huge, calcium can co precipitate with Mg $(OH)_2$. Presence of ammonium salts is undesired, as they lower pH and make end point less sharp. To get rid of ammonia, solution can be heated after NaOH was added.

Determination of Vitamin C by Titration Method

3. RESULTS AND OBSERVATIONS

The results and observations of bacteria in the sand, collected from the Coastal region, sand sample were inoculated in the nutrient agar media for microbial analysis. The bacteria *Bacillus simplex* was carried out of this study.

ISOLATION AND IDENTIFICATION OF TEST ORGANISMS

Bacillus simplex was the predominant organism isolated from the coastal sand. The biochemical test results for various tests like indole, methyl red test, voges Proskauver's test, catalase, urease, oxidase, citrate utilization, TSI were observed. All the isolates were characterized by their cultural characteristics and they were confirmed. The test organisms were confirmed by plating them in selective medium and the results were observed.

ANTIOXIDANT PRODUCTION USING *Bacillus simplex*

The *Bacillus simplex* produced significant quantities of Antioxidant when grown in different source of medium under submerged fermentation.

TEMPERATURE AND PH

The result shows the effect of temperature on the production of Antioxidant by using in carbon source, nitrogen source and ammonium source with peptone medium. The antioxidant production for the medium was maintained at

different temperature such as at 40°C, 60°C, 80°C, 100°C and pH ranges at 3, 7, 5, and 9 to evaluate the Antioxidant production.

During this study shows the high level of antioxidant production was monitored at 100 °C contains 78 % and the range of pH was 9 the production was 81 % respectively. The production level of antioxidant in different temperature and pH were noted and tabulated using various medium. Hence, the antioxidant was optimally active at its optimum growth temperature to fulfill nutritional requirements. The maintenance and favorable temperature and pH are essential for the production of antioxidant.

TOTAL PHENOLIC CONTENT

The total phenol content was present in the four different production medium namely, NA, TSB, TY and LB and the values were 29 %, 28 %, 21 % and 37 % respectively. The NA and LB medium have very high amount of phenolic content when compared with other selected medium.

ESTIMATION OF TOTAL ANTIOXIDANT ACTIVITY

The present study shows to evaluate the total antioxidant capacity of the production medium such as NA, TY, TSB and LB with different sources. The maximum level of Antioxidant capacity was observed in NA and LB broth such as 68 %, 77 % and the very minimum level of total antioxidant capacity was observed in TSB and TY such as 37 %, 27 % respectively. Based on the results the NA and LB medium were used to isolate the Antioxidant compound (Hydroxyl group) by TLC method.

ISOLATION OF ANTIOXIDANT MOLECULE BY TLC

The TLC profile of antioxidant molecule (Hydroxyl group) is isolated and identified. Among the chemical constituent the antioxidant compound has been determined from the sample of NA and LB medium that is amino based antioxidant molecule, were found to be the most abundant depicted by the Rf value of 0.84 and 0.93 respectively. The results showed. The present investigation has been undertaken to find out the effectiveness of the antioxidant compound isolated by TLC.

The isolated compound was compared to the standard vitamins namely vitamins A, vitamins B2, vitamins C and phenolic compound such as, catechin, proanthocynidin and phenolic acid were observed by TLC the results are denoted in table. Hence, we concluded that the isolated antioxidant compound was one of the Vitamin based amino group antioxidant molecule.

IDENTIFICATION OF ANTIOXIDANT COMPOUND BY FT-IR

The IR result shows the structure of reduced compound from fractions of antioxidant compound which is isolated from microbial production under *Invitro* method using different medium. All the findings are subjected to isolate with TLC, and FT-IR which is a hydroxyl group as the active component in fractions. The fractions showed in highest peak 1095.61 absorption in the IR spectra and the lowest peak 804.35 indicated that the compounds have hydroxyl (-O-H) and hydrogen bonds characteristic of phenols or alcohols. This study therefore confirms of Antioxidant compound as an active component in our present investigation.

SEM WITH EDAX ANALYSIS OF ANTIOXIDANT COMPOUND

The sample after bio reduction was centrifuged and the biomass was hot air dried and they are in the form of thin film cast was analyzed by means of SEM analysis (Scanning Electron Microscopy) for the confirmation of compound (Photo Plates). In plate plates was showed the biosynthesized compound by isolates *Bacillus sp*. The results of EDAX revealed the elemental analysis of antioxidant compound which have the minimum level of elements are present in the identified antioxidant compound.

ANTIMICROBIAL SUSCEPTIBILITY TEST

The antioxidant compound was isolated and it was allowed to observe the Antimicrobial activity against selected bacteria and fungus. The bacteria namely, *E. coli, P. aeruginosa*, fungus such as *A. niger* and *Mucor*. The antibiotic which is used as a standard for this test namely tetracycline for fungus and erythromycin for bacteria. These organisms are normally associated to food microbes and also intestinal pathogens. Hence, this study shows the compound has valuable inhibitory property against the selected pathogens namely, bacteria and fungus. So the compound is used for the further study. The results are recorded.

INCORPORATION OF ANTIOXIDANT MOLECULE ON IDLY

In order to quantify the difference in anti-oxidant level, the isolated Antioxidant compound (Hydroxyl group) was purified and induced in different stages in the idly flour namely before and after fermentation and the results were tabulated.

The purified antioxidant compound was used to enhance the antioxidant activity in the food. In this present study, the food IDLY was taken to investigate the nutrients, antimicrobial activity and antioxidant activity between compound incorporated idly and normal idly. The purified compound was mixed with the flour in two ways namely, before fermentation, and allowed it for overnight, and after fermentation, mixed with flour at the time of usage. The following methods were used in the identification of nutrients and antioxidants.

NUTRIENT ANALYSIS OF IDLY BEFORE AND AFTER INCORPORATION OF ANTIOXIDANT COMPOUND

Protein

The protein was the most important nutrient for maintaining the structure of the body. In animals, amino acids are obtained through the consumption of foods containing protein. The amount of protein was present in normal and incorporated food was estimated, 6 mg of protein was present in control sample and 9 mg of protein in sample 1, 10.3 mg in Sample 2.

Carbohydrate

The amount of carbohydrate was present in normal and incorporated food was estimated. 60 mg of carbohydrate was present in control sample and 80 mg of carbohydrate in sample 1, 50 mg in Sample 2.

Cholesterol

Cholesterol is required to build and maintain membranes. It modulates membrane fluidity over the range of physiological temperatures. All animal cells manufacture cholesterol with relative production rates varying by cell type and organ function. The control sample contains 5 mg and the sample 1 has contains 6 mg, 3 mg in Sample 2 respectively.

Energy

The energy level of the control sample has contains 309 Kcal, Sample 1 has 410 Kcal and sample 2 has 267 Kcal respectively. The sample 1 contains high amount of energy when compare with sample 2 and control.

Calcium

Calcium is a mineral that plays an important role in the development and maintenance of the bones. The high level of calcium is present in the sample 1 26 mg and the sample 2 has 18 mg respectively. The control sample contains 22 mg of calcium.

Vitamin C

In our present investigation the vitamin C was estimated and recorded. 10.2 mg of vitamin C is present in the control sample and the sample 1 contains 59 mg when compare with sample 2 has 22 mg of vitamin C respectively. Hence, thispresent study shows to conclude this result the sample 1 (before fermentation) has contains high amount of nutrients namely, protein,

carbohydrate, cholesterol, mineral such as calcium and vitamin C when compare with Sample 2 (after fermentation) and control. The nutrients level has gradually increased after incorporation of antioxidant molecule.

ANTIOXIDANT ACTIVITY

Free radical scavenging potential of IDLY sample has allowed evaluating the Antioxidant activity before and after incorporation of antioxidant molecule. Reactive oxygen species generated in the human body cause oxidative damage and responsible for many degenerative diseases such as coronary heart diseases, atherosclerosis, diabetes, ageing and cancer. The degree of discoloration indicates scavenging potentials of the antioxidant were observed and tabulated. The antioxidant activity was observed by following methods.

TOTAL ANTIOXIDANT CAPACITY

The sample 1 was having a maximum level of antioxidant capacity 25 % respectively. The sample 2 contains minimum level of antioxidant capacity 22 % and the control sample contains very low level of antioxidant capacity 4 % respectively.

FE- EDTA SCAVENGING ACTIVITY

The reducing ability of a compound generally depends on the presence of reluctant which have been exhibited ant oxidative potentially breaking the free radical chain, donating a hydrogen atom. The presences of resultants (i.e. antioxidants) in the sample 1, sample 2 and control cause the reduction of Fe^{3+}/ ferricyanide complex to the ferrous form. In this present study find out the antioxidant molecule and its uses.

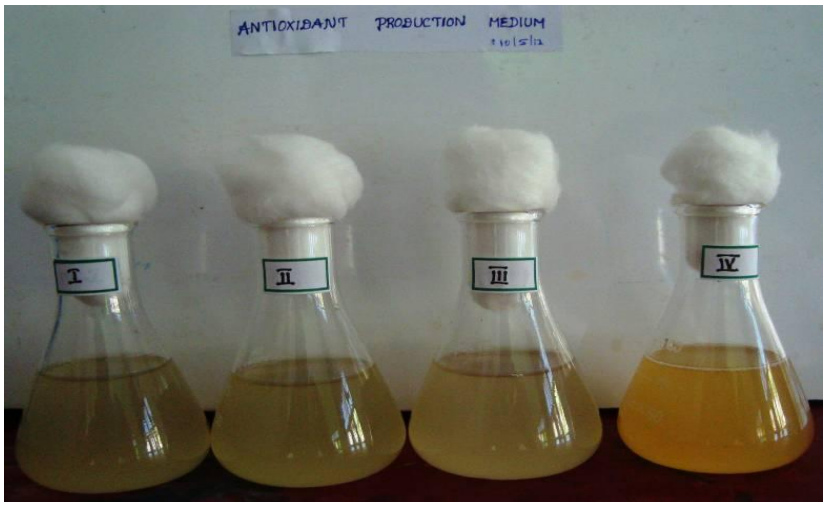
4. SUMMARY AND CONCLUSION

In the present study shows, the bacteria *Bacillus simplex* was isolated from sand which is used to antioxidant production and it was investigate the isolation, identification, evaluation purification and incorporation of antioxidant on IDLY. The summarized data reveals the following information.

So we conclude, that this study the compound was enrich the antioxidants activity on IDLY. The present study shows the microbial species are not only produce harmful effect such as food poisoning and diseases, but most of the microorganisms and its products are beneficially used for many commercial preparations such as food preservatives, and compounds like antioxidants.

Likewise in our present study, concluded that the isolated antioxidant from *Bacillus simplex* gave many antimicrobial activities and these compounds are suitable for antioxidant enrichment of food. So it is used for food industries to enhance the antioxidant rich food for naturally. It can be used regularly for our food preservation to avoid the contamination and spoilage.

Plate I: Antioxidant Production by using *B. simplex* on Different broth



I - NA Broth
II - TSB Broth
III - TY Broth
IV - LB Broth

Table: 1 Effect of temperature for Antioxidant production at different medium

Name of Medium	Various ranges of Temperature / Values in %				
	Control 30°C	40°C	60°C	80°C	100°C
NA	68	61	64.5	69	71
TSB	37	33	34	32.6	33
TY	27	22	23.4	21	22
LB	77	73	74	74.5	78

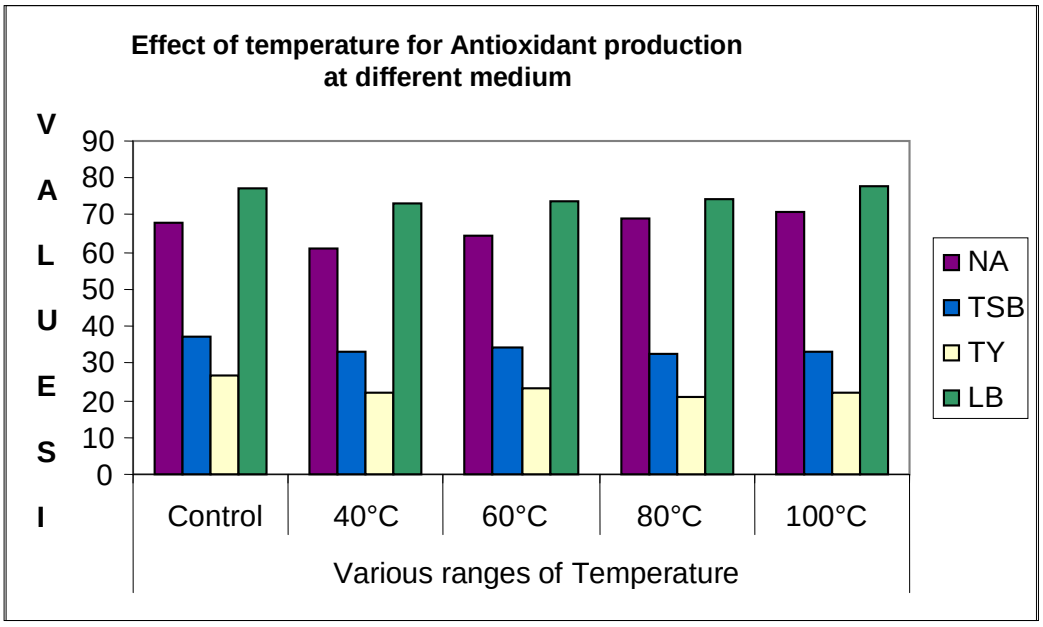


Table: 2- Shows Comparative Nutrient Analysis of Idly Before and After Incorporation of Antioxidant Compound

Nutrients	Control	Incorporated food	
		Sample - 1	Sample - 2
Protein (mg)	6.0	9.0	10.3
Carbohydrate (mg)	60	80	50
Cholesterol (mg)	5.0	6.0	3.0
Energy K calories	309	410	267
Calcium (mg)	22	26	18
Vitamin – C (mg)	10.2	59	22
Total Antioxidant Capacity (%)	4.0	25	22

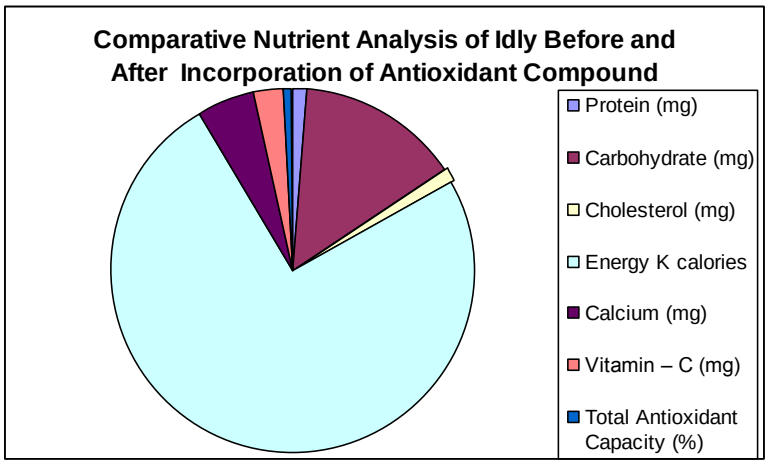


Plate II: FT – IR result for Antioxidant Compound

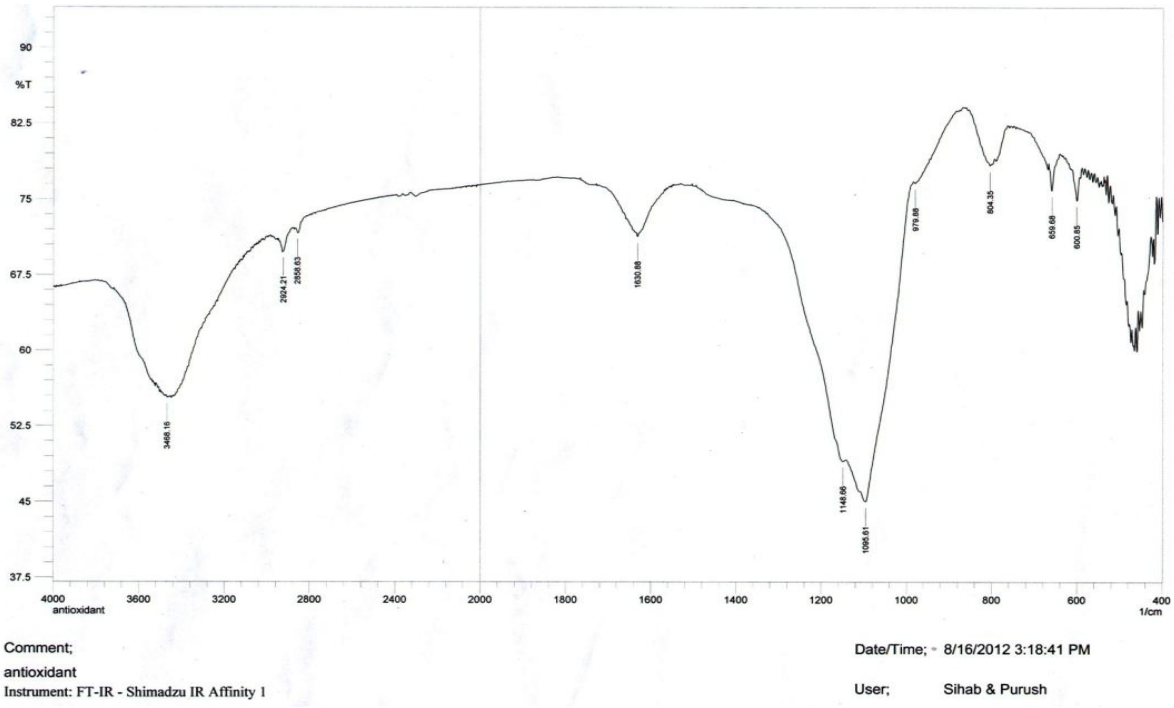


Plate III: SEM Analysis of Antioxidant Compound

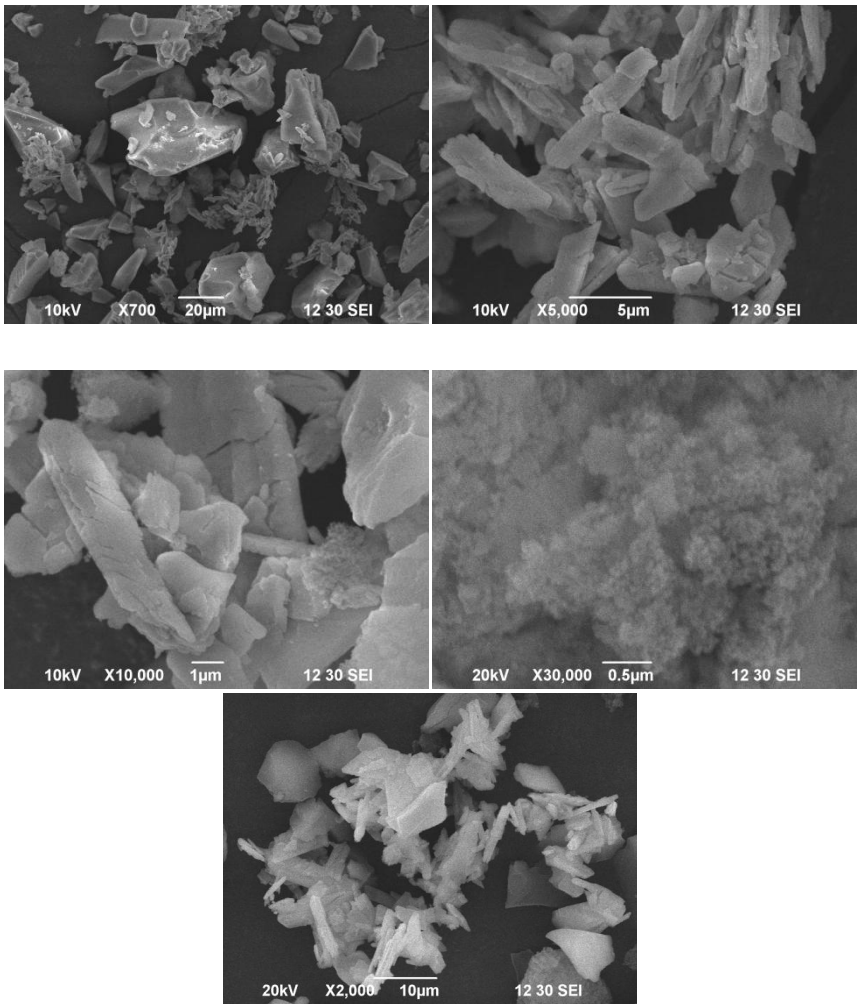


Plate IV: SEM with EDAX Analysis of Antioxidant Compound

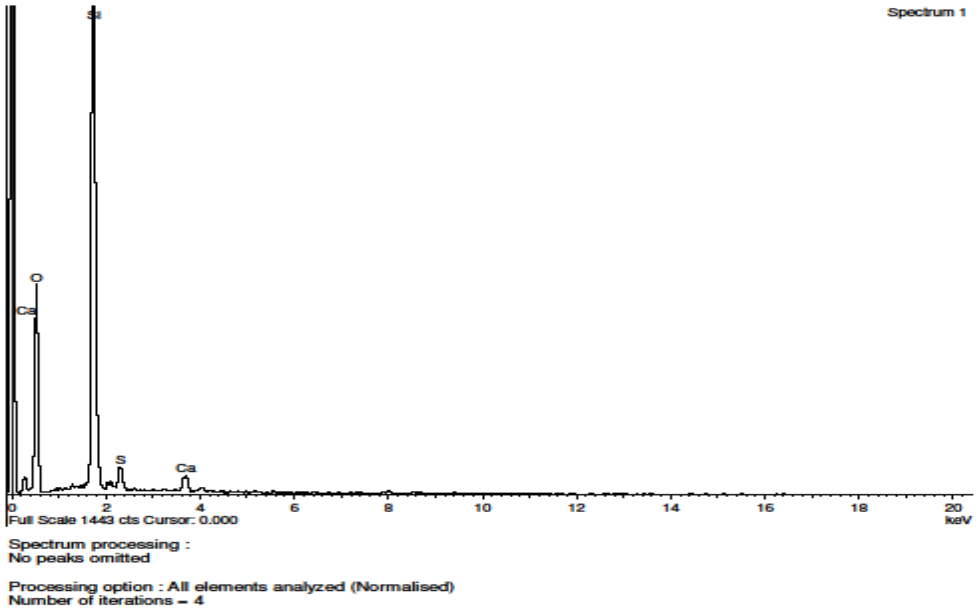


Plate V: Incorporation of Antioxidant compound on IDLY

A1-Antioxidant incorporates before fermentation.
A2- Antioxidant incorporates before fermentation.

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