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Formulation of lacto-fermented orange juice with incorporation of dairy whey

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■ **Abstract :** Disposal of whey is a major problem in the dairy industry, but the whey can be one of the source for the lactic acid production. Production of lactic acid through the *Lactobacillus* sp. could be a processing route for whey lactose and various attempts have been made in this direction. The main objective of the study is the formulation of lacto-fermented orange juice using whey. The method which followed is the fermentation process using whey as a starter culture. Fully mature oranges were procured from the market and juice was extracted with juicer available in the laboratory. The blends of whey (used as a starter culture), fresh orange juice, salt and purified water were prepared. Theses blends were put in the tightly covered jars at ambient condition for 48 hours and then refrigerated for 15 days, after this transferred to the other jar for storage studies at regular interval of three months. The microbiological (TPC, Coliform, Yeast and mold and Salmonella), chemical (TSS, Acidity, Ascorbic Acid, Sodium and Potassium), heavy metals (Arsenic, Lead, Tin, Zinc and Copper) testing and organoleptic analysis were performed for judging the shelf-life of the blends. Small colonies of approximately 1mm diameter, lenticular, white or milky in color were observed on a solid medium. No pink color colonies confirmed the absence of coliform bacteria and also negative for the salmonella and yeast and mold colonies. The further microscopic examination reveals that the strains were gram positive with a cellular rod in pairs and chains which were further identified for the presence of lactic acid bacteria. The TSS, acidity, and ascorbic acid content of the blend was 7%, 2.5% and 250 ppm, respectively. The heavy metals such as tin, zinc, and copper in the blend were 50 ppm, 3 ppm and 2 ppm, respectively whereas heavy metals such as arsenic and lead were not found in the sample. During storage studies, each sample was also tested for color, texture, and taste. It shows that the fermented juice can be consumed within 15 days, whereas the juice kept in closed container can be consumed within 9 months.

■ **Key words :** Lacto-fermented orange juice, Dairy whey, Lactic acid

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Indigenous foods are very much popular for cooperation between the developing countries to increase their agricultural/ food production. Many fermented traditional foods also helps in improving the nutritional value at some extent. Microorganisms plays a very important role in making of fermented foods and beverages. Also the end product of the fermentation adds an income and helps to generate financial resources (Steinkraus, 1995). During fermentation, pyruvate get metabolized into various compounds by several process, one of them is lactic acid fermentation. Lactic acid fermentation occur when pyruvate converted into lactate

using enzyme lactate dehydrogenase and producing (NaD^+) in the process [-]. Food products which contains microorganisms, called probiotics for example, *lactobacillus acidophilus, L. casaei, L. bulgaricus* etc. Probiotics contain useful bacteria which have their own benefits (Shah, 2000).

Whey is one of the source for the production of lactic acid bacteria (Panesar *et al.*, 2007). Lactic acid bacteria considered to have various benefits like to strengthen the immune system, antimicrobial activity and antitumor genic activity (Kullisaar *et al.*, 2002).The dairy industries disposes a huge amount of waste liquid which generally called whey. Whey is the liquid which generates from the separation of milk fat and casein. Disposal of whey leads to the serious pollution and affects the aquatic life by depleting the dissolved oxygen (Gonzalez- Siso, 1996). Whey is low in acidity and produced from the coagulation of cheese. The total solid contents of sweet whey and acid whey is from 63 to 70% whereas lactose and lactate content varies from 46-52 % and 2-6.4 %. The difference between the two whey types are in the mineral content, acidity and composition of the whey protein fraction whereas the composition of whey also depends on the pre-treatment of milk (Jelen, 2003).

Lactic acid bacteria are the facultative anaerobic, catalase negative, non-motile and non-spore forming. Lactic acid bacteria are considered as "generally regarded as safe" (GRAS) bacteria which can be use in traditional foods and expand its range of novel food products. The Lactic acid bacteria have the nutritional benefits and other health enhancing benefits in the food products (Steinkraus, 1995). Lactic acid bacteria have been using as a starter culture in the fermented food industries because they have high metabolic activity also contributing to strong immune and digestibility system (Pescuma *et al.*, 2008). Many research made on the fermented whey beverages which based on the use of whey protein concentrate or de-proteinized whey which leads to losing the nutritional components (Shah, 2000). Some of the studies also done on the probiotic growth and fermentation with yogurt starters (Panesar *et al.*, 2007). The thermophilic lactic acid bacteria strains assayed in the work to be able to grow well in WPC after 12 hrs whereas in the previous studies, it showed that the lactic acid bacteria could grow and degrade whey proteins when incubated in reconstituted whey for 24 hours (Dragone *et al.*, 2009).

METHODOLOGY

Preparation of orange blend:

Ingredients:

Two 1-quart mason jars, 6 cups fresh squeezed orange juice, 4-6 tablespoons of prepared culture starter (WHEY), 2 tablespoon of orange extract, ½ tablespoon of salt, 1 cup purified water.

Procedure :

Take 1 quart Mason jar, add approximately 2½ cups fresh squeezed orange juice. Add ½ tsp veggie culture starter (or 2 tbsp. whey), fill with approximately 1 cup filtered water, leaving 1 inch or so of head space. Cover tightly, give a quick shake to mix and leave at ambient condition for 48 hours. Refrigerate and be sure to open carefully as gases do build up during fermentation.

Fig. A : Orange blend formulation

Microbial testing :

Media used :

CYGA [Chloramphenicol Yeast Glucose Agar]:

It is recommended for the selective enumeration of yeast and moulds.

Table A : CYGA media component

Composition	Concentration (g/L)
Yeast extract	5.00
Dextrose	20.00
Chloramphenicol	0.10
Agar	14.90
pH	6.6 (±0.2)

PCA [Plate Count Agar] :

It is for the determination of plate count of microorganisms present in food, water and waste water.

Table B : PCA media component	
Composition	Concentration (g/L)
Casein enzymatic hydrolyses	5.00
Yeast extract	2.50
Dextrose	1.00
Agar	15.00
pH	7.0(±0.2)

VRBA [Violet Red Bile Agar Media] :

Table C : VRBA media component	
Composition	Concentration (g/l)
Agar	12.0
Bile salts	1.5
Crystal Violet	0.002
Lactose	10.0
Neutral Red	0.03

Aim: Isolation and identification of coliform from juice sample by MPN method (Most probable number techniques) :

Apparatus :

Test tubes, Durham's tube. Incubator, Inoculating loop, Glass slab, Microscope.

Reagents required :

Mac Conkey broth

Procedure :

Shake the water sample thoroughly before making dilutions or before inoculations.

Test is performed in following steps:

Presumptive test :

Prepare five tubes of double strength, ten tubes of single strength of Mac Conkey broth with Durham's tube. Add 10 ml of the sample in to the five tubes of double strength. Add 1 ml of the sample into the first five tubes of single strength and add 0.1 ml of the sample in to the next five test tubes of single strength. Incubate all the test tubes at 37° C for 48 hrs.

Confirmatory test :

Add 0.1 ml of the inoculum from positive MC broth into the BGBB broth containing Durham's tube and incubate at 37° C for 24±2 hours.

Completed Test :

Streak the positive tubes on MacConkey agar plates. Incubate the colonies at 37° C for 24-48 hours.

Aim : Enumeration of TPC, yeast and mould :

TPC :

Apparatus :

Oven (Dry sterilization), Autoclave(Wet sterilization), Water bath(45°C±0.5°C), Incubator (30°C), BOD Incubator, Total delivery pipette(1 ml), Colony counter, pH meter, Petri dishes, test tubes, flasks and MRD Bottles.

Reagents :

Plate count agar, phosphate buffer or MRD (Maximum recover diluents).

Procedure :

Weigh 25 g or 10 gm sample in 225 ml or 90 ml of sterilized MRD using a sterile spatula. Serially dilute the sample and plate in Petri plate within 15 minutes. In case of liquid samples pipette out directly 1 ml of sample. Pour 10 to 15 ml of PCA agar (45°C±0.5°C), mix well by rotating clockwise and anti-clock-wise. Let the mixture stand till solidified (15 minutes) and then place inverted plates at 30°±1°C for 72 hours±3 hours in BOD incubator. Count the visible colonies with the help of a colony counter. Prepare maximum recovery dilution with the help of distilled water in a MRD bottle and wrap with aluminium foil. Prepare Plate count agar with the help of distilled water in a conical flask and apply cotton plug on flask and wrap with aluminium foil. Wrap spatula with aluminium foil. Place all items in Autoclave at 121° C at 15 psi for 15 min for sterilization. Then switch off Autoclave and release steam and remove all items and place PCA in incubator for cooling and place all other items in laminar air flow. Then add 10 ml of sample with 90 ml MRD using sterilized spatula. Then take 1 ml sample from MRD bottle and add to 9 ml MRD test tubes and 1 ml to a Petri plate named 10^{-1} and then serially upto 10^{-3} or 10^{-5}. Then add 10 to 15 ml of PCA agar and mix well by rotating clockwise and anti-clockwise and place till mixture solidified *i.e.* 15 minutes. Then place inverted plates at 35° C for 2 days in BOD

incubator.

Calculation :

$$[\{p\ C / (n_1 + 0.1\ n_2)\} \times d]$$

where, ΣC= sum of colonies on all dishes retained, n_1= no. of dishes used in first dilution, n_2 = no. of dishes used in 2nd dilution, d= dilution factor corresponding to first dilution.

Yeast and mould :

Reagents :

Chloramphenicol Yeast agar (CYGA), Phosphate buffer or MRD (Maximum recovery diluents).

Procedure :

Weigh 25 gm or 10 g sample in 225 ml or 90 ml of sterilized MRD using a sterile spatula. Serially dilute the sample and plate in Petri plate within 15 minutes. In case of liquid samples pipette out directly 1 ml of sample. Pour 10 to 15 ml of CYGA agar (45°C±0.5°C), mix well by rotating clockwise and anti-clockwise. Let the mixture stand till solidified (15 minutes) and then place inverted plates at 30°±1°C for 72 hours±3 hours in BOD incubator. Count the visible colonies with the help of a colony counter. Prepare maximum recovery dilution with the help of distilled water in a MRD bottle and wrap with aluminium foil. Prepare Chloramphenicol Yeast Glucose agar with the help of distilled water in a conical flask and apply cotton plug on flask and wrap with aluminium foil. Wrap spatula with aluminium foil. Place all items in Autoclave at 121° C at 15 psi for 15 mins. For sterilization. Then switch off Autoclave and release steam and remove all items and place CYGA in incubator for cooling and place all other items in laminar air flow. Then add 10 ml of sample with 90 ml MRD using sterilized spatula. Then take 1 ml sample from MRD bottle and add to 9 ml MRD test tubes and 1 ml to a Petri plate named 10^{-1} and then serially upto 10^{-3} or 10^{-5}. Then add 10 to 15 ml of CYGA agar and mix well by rotating clockwise and anti-clockwise and place till mixture solidified *i.e.* 15 minutes. Then place inverted plates at 35° C for 2 days in BOD incubator.

Calculation :

$$(p\ C / n_1 + 0.1\ n_2) \times d$$

where, ΣC= sum of colonies on all dishes retained, n_1= No. of dishes used in first dilution, n_2 = No. of dishes used in 2nd dilution, d= Dilution factor corresponding to first dilution.

Coli form :

Reagents :

Violet red bile agar (VRBA), Phosphate buffer or MRD (Maximum recovery diluents).

Procedure :

Weigh 25 g or 10 gm sample in 225 ml or 90 ml of sterilized MRD using a sterile spatula. Serially dilute the sample and plate in Petri plate within 15 minutes. In case of liquid samples pipette out directly 1 ml of sample. Pour 10 to 15 ml of VRBA agar (45°C±0.5°C), mix well by rotating clockwise and anti-clockwise. Let the mixture stand till solidified (15 minutes) and then place inverted plates at 30°±1°C for 72 hours±3 hours in BOD incubator. Count the visible colonies with the help of a colony counter. Prepare maximum recovery dilution with the help of distilled water in a MRD bottle and wrap with aluminium foil. Prepare Violet red bile agar with the help of distilled water in a conical flask and apply cotton plug on flask and wrap with aluminium foil. Wrap spatula with aluminium foil. Place all items in Autoclave at 121° C at 15 psi for 15 mins. for sterilization. Then switch off Autoclave and release steam and remove all items and place VRBA in incubator for cooling and place all other items in laminar air flow. Then add 10 ml of sample with 90 ml MRD using sterilized spatula. Then take 1 ml sample from MRD bottle and add to 9 ml MRD test tubes and 1 ml to a Petri plate named 10^{-1} And then serially upto 10^{-3} or 10^{-5}. Then add 10 to 15 ml of VRBA agar and mix well by rotating clockwise and anti-clockwise and place till mixture solidified *i.e.* 15 minutes. Then place inverted plates at 35° C for 2 days in BOD incubator.

Calculation :

$$p\ C = (n_1 + 0.1\ n_2) \times d$$

where, ΣC= sum of colonies on all dishes retained, n_1= No. of dishes used in first dilution, n_2 = No. of dishes used in 2nd dilution, d= dilution factor corresponding to first dilution.

Aim- To detect salmonella in a sample :

Apparatus :

Bottles, Test tubes, Spatula, Incubator, Laminar air

flow, Autoclave, Oven

Media :

Buffered peptone water (BPW), Brilliant green agar (BGA).

Procedure :

Primary enrichment :

Weigh 10 g sample in 90 ml buffered peptone water. Shake properly and incubate at 37° C for 24 hours. Observe the gas formation and turbidity.

Secondary enrichment :

Transfer 0.1 ml of pre enrichment culture to 10 ml RVM and 1 ml of pre enrichment to SCM. Incubate RVM at 42° C for 24 hours and SCM at 37°C for 24 hours. Observe the growth *i.e.* turbidity.

Selective isolation :

If colour of RVM fades and growth in SCM is observed then streak on XLDA, BGBA, BSA. Incubate at 37°C for 24 hrs. Then pinkish colony, pinkish yellow, brownish with blackish center colony on XLDA, BGBA, BSA, respectively indicates the presence of *salmonella.* Then perform gram staining and pink color indicates the presence of *salmonella* (Gram negative, rod shape).

Chemical testing :

Aim : Determination of acidity.

Principle :

The chemical method used for estimation of vitamin C is based on the reduction of 2, 6 –di chlorophenol indophenol by an acid solution of ascorbic acid.

Reagent required :

3% Meta phosphoric acid, dye solution and ascorbic acid solution.

Sample preparation :

10ml juice is make-up in 100ml.

Procedure :

Take 25ml sample. Add 2 drops phenolphthalein indicator. Titrate it with N/10 NaOH solution till pink colour. Note the titre value.

Formula :

Acidity (as citric acid) = factor × total volume × volume made up × Normality of NaOH × 100/ × weight of sample taken for estimation × 1000.

Aim: Determination of colour in food :

Apparatus required :

Separating funnel, Beaker, Glass beads, Burette, Spectrophotometer.

Reagent :

70% Acetone, Petroleum ether.

Procedure :

Take 5 g sample in separating funnel. Extract the colour with 70% acetone. Shake the acetone extract with petroleum ether (40^0C-60^0C) to remove carotenoids and other natural pigments. Continue extraction with petroleum ether until petroleum ether extract is colourless. Take acetone extract and concentrate the extract on water bath. Dissolve the conc. extract in 0.1 NHCl. Take the reading at specific wave length.

Aim- To determine the protein content :

Principle :

Destruction of organic matter by H_2SO_4 in the presence of a compound catalyst, alkalization of the reaction products, distillation of the liberated ammonia and collection in a H_2SO_4 solution, followed by titration with a standard volumetric sulphuric acid solution.

Apparatus :

Kjeldahl flask, Round bottom flask, Distillation apparatus, Beakers, Burette, Digestion flask.

Reagents required :

Copper sulphate, Sodium sulphate, Conc. H_2SO_4 (36%), NaOH, Methyl red Indicator, 0.1 H_2SO_4.

Procedure :

Weigh 0.01 to 10 g sample entrapped in paper so that it does not adheres to the wall of digestion flask. Add 10 g of sodium sulphate and 0.5 g of copper sulphate entrapped in the paper in to the digestion flask. Add 25 ml conc. H_2SO_4 in digestion flask and mix the contents so that the test Material becomes wet and then place digestion flask on digestion stand and heat for one hr. until the liquid becomes clear. Allow the flask to cool

and rinse with water and transfer the material to round bottom flask so that the Kjeldahl flask becomes free from any residue and fill the round bottom flask more than half and add some glass beads. Connect the RBF to distillation apparatus. Adjust the lower end of the condenser to a known volume of H_2SO_4 taken in a 200 ml beaker. Add 2-3 drops of phenolphthalein indicator and make the digestion liquid alkaline by adding NaOH by a separating funnel. Mix well and then start heating and turn on the condenser until the beaker containing 0.1 H_2SO_4 (50 ml) fill upto 200 ml in 20 to 30 minutes. Then turn off the heat and rinse the lower end of the condenser with water to remove any residue and collect in beaker. Then titrate the contents of the beaker with 0.1 N NaOH until the colour becomes yellow.

Calculation :

$$\text{Protein (N x 6.25) \% by wt.} = \frac{\text{(B - S) x 6.25 x 0.001401 x } N_{NaOH} \text{ x 100}}{\text{(S. wt x 0.1)}}$$

Presence of protein Content in juice sample : Sample weight = 0.3071, Blank =52.75

Aim- Determination of total soluble solids :

Apparatus :

Shallow flat Bottomed dishes of aluminium alloy, nickel, stainless steel, porcelain or silica, 7 to 8 cm diameter, about 1.5 cm in height and provided with easily removable but closely fitting lids.

Procedure :

Weigh accurately the clean, dry empty dish with the lid. Pipette into the dish about 5 ml of the prepared sample of milk and weigh quickly, with the lid on the dish. Place the dish, uncovered, on a boiling water-bath. Keep the base of the dish horizontal to promote uniform drying and protect it from direct contact with the metal of the water-bath. After at least 30 minutes, remove the dish, wipe the bottom and transfer to a well-ventilated oven at 98 and 100°C, placing the lid by the dish. The bulb of the oven control thermometer shall be immediately above the shelf carrying the dish. The dish shall not be placed near the walls of the oven, and shall be insulated from the shelf, for example, by a silica or glass triangle. The shelf used shall not be placed near the walls of the oven, and shall be insulated from the shelf, for example, by a silica or glass triangle. The shelf used shall be near the middle of the oven. After three hours, cover the dish and immediately transfer it to a desiccators. Allow to cool for about 30 minutes and weigh. Return the dish, uncovered, and the lid to the oven and heat for one hour. Remove to the desiccators, cool and weigh, as before. Repeat if necessary, until the loss of difference between successive weights does not exceed 0.5 mg. Note the lowest weight.

Calculation :

Total solids, per cent by weight = (100 x w)/W)

where, w = Weight in g of the residue after drying, W=Weight in g of the prepared sample taken for the test.

Aim- Determination of alkalinity :

Principle :

Alkalinity of water is the capacity of that water to accept protons. It may be defined as the quantitative capacity of an aqueous medium to react with hydrogen ions to pH 8.3 and then 3.7. This equation is

$CO_3^{--} + H^+ \rightarrow HCO_3^-$ (pH 8.3), from 8.3 to 3.7. The following reaction may occur $-HCO_3^- + H^+ \rightarrow H_2CO_3$.

Apparatus :

pH meter, Burette- 50 mL, Magnetic stirrer assembly.

Reagents :

Distilled water, Sulphuric acid, standard solution of sulphuric acid phenolphthalein indicator, mixed indicator solution.

Procedure :

Pipette 20 ml or a suitable aliquot of sample into 100 ml beaker. If pH is over 8.3 then add 2-3 drops of phenolphthalein indicator. Titrate with standard sulphuric acid solution till the pink colour observed by indicator just disappears. Record the volume of standard sulphuric acid solution used. Add 2-3 drops of mixed or to the solution in which the phenolphthalein alkalinity has been determined. Titrate with standard acid to light pink colour. Record the volume of standard acid after phenolphthalein alkalinity.

Calculation :

Alkalinity (as mg/l $CaCO_3$) = (A x N x 50000)/ V

A= volume of standard sulphuric acid used to titrate to pH 8.3in (ml), B= volume of standard sulphuric acid

used to titrate from 8.3-3.7(ml).

Instrumentation testing :

Aim- Determination of lead, arsenic, copper and zinc in iuice I.E. by ICP. :

Principle :

Inductively coupled plasma atomic emission spectroscopy (ICP-AES), also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES) is an analytical technique used for the detection of trace metals. The ICP-AES is composed of two parts: the ICP and the optical spectrometer. The ICP torch consists of 3 concentric quartz glass tubes. The output or "work" coil of the radio frequency (RF) generator surrounds part of this quartz torch. Argon gas is typically used to create the plasma.

Procedure:

Weigh 1g sample in a 50 ml volumetric flask. Add 10 ml of conc. HNO_3. Keep it on hot plate till brown fumes ceases. Cool it to room temperature. Make up the volume with distilled water till 50 m and Run in the ICP.

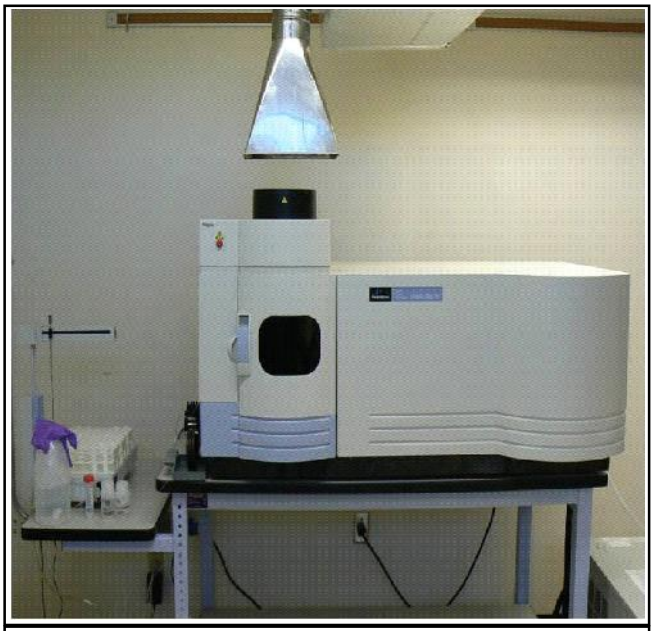

Fig. B : ICP

■ RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Characterization of the isolates:

The strains retained give small colonies of approximately 1mm of diameter, lenticular with a white or milky colour, smooth surface and a regular circular circumference were observed on solid medium. The microscopic examination reveals that the tested strains were gram positive, with a cellular rod form associated in pairs or in chains.

Result of determination of coliform in sample:

The presence of pink colonies indicates the colonies of coli forms. Perform Gram staining. Coli form appears as gram negative rods. NO PINK coloured colony is observed.

Observations of total plate count in the sample:

Fig. 1 : Total Plate Count on PCA media

Observations of yeast and mould in sample:

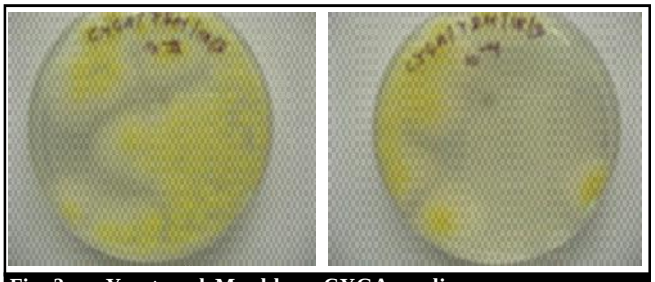

Fig. 2 : Yeast and Mould on CYGA media

Result of determination of salmonella in the sample:

The gas formation and turbidity in primary enrichment indicates the presence of Salmonella. The presence of turbidity in secondary enrichment indicates the presence of Salmonella. Then pinkish colony, pinkish yellow, brownish with blackish center colony on XLDA,

Table 1 : Calculated results

Sr. No.	Parameter	Result
1.	Total plate count, cru/ml	45 cfu/ml
2.	Coliform Count, cfu/ml	ND (≤10 cfu/g)
3.	Yeast andMould ,cfu/ml	ND (≤10 cfu/g)
4.	Salmonella/25ml	Absent
5.	Total soluble Solid	7%
6.	Acidity (as citric Acid)	2.5%
7.	Ascorbic acid and its sodium, potassium	250 ppm
8.	Sulphur dioxide	50 ppm
9.	Arsenic	ND
10.	Lead	ND
11.	Tin	50 ppm
12.	Zinc	3 ppm
13.	Copper	2 ppm

BGBA, BSA, respectively indicates the presence of salmonella. During gram staining pink color indicates the presence of salmonella. NO PINK COLORED colony is observed.

Conclusion :

The lacto fermented orange juice is a valuable functional food through the microorganisms involved, the changes in composition that occur during fermentation, and most importantly the effect of these foods and their active ingredients on human health. The lacto fermented orange juice represents an important source of vitamin C that exercise an antioxidant action in the human body. The amino acids content is changing slowly, reaching at the final of lactic acid fermentation. Shelf life of lacto fermented orange juice is extended upto 15 days whereas the juice kept in closed container can be consumed within 9 months. The energetic value of the lacto fermented orange juice is small, because the reducing sugars are transformed under the lactic acid bacteria action, the lacto fermented juice having a content of reducing sugars. The lacto fermentation is a biochemical process which can be described by a polynomial mathematic trend model for the evolution of vitamin C, reducing sugars, lactic acid.

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