IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 5, Issue 1 (Jan. – Feb. 2019), PP 33-38
www.iosrjournals.org

Effective Microorganisms Based Fermentation for Antioxidant-Rich Health Drink from Medicinal Plants

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Abstract: *In the present work, production of a health drink through fermentation of medicinal plants-Asparagus racemosus (Shatavari) and Phyllanthus emblica (Amla) and along with Citrus sinensis (Orange) juice was carried out by using Effective Microorganisms (EM). EM is a combination of useful regenerative microorganisms predominantly containing lactic acid bacteria and yeasts. The fermentation of the health drink was carried out for 7 days under static conditions in two batches for each plant. Antioxidant and alcohol levels were measured during the fermentation period. The drink was then filtered using a commercial filter. The produced health drink showed high antioxidant levels and low alcohol content which can be useful for preventing chronic diseases and cancer by maintaining healthy gut biome and good overall health.*

Key Words: *Fermentation, Ayurveda, Effective Microorganisms, Antioxidants, Phytochemicals*

Date of Submission: 14-01-2019 Date of acceptance: 29-01-2019

I. Introduction

The ancient science of Ayurveda involves various plants, their extracts and decoctions. One of the most effective ways of making an Ayurvedic herbal preparation is fermentation.Fermentation is a metabolic process in which an organism converts a carbohydrate, such as starch or a sugar, into an alcohol or an acid.The present study employs the use of effective microorganisms (EM) for fermentation of medicinal plants. EM contains selected species of microorganisms including predominant populations of lactic acid bacteria and yeasts, and smaller numbers of photosynthetic bacteria, Actinomycetes and other types of organisms. All of these are mutually compatible with one another and can coexist in liquid culture.EM has found application in the many areas especially agriculture, production of health drink, waste water treatment, preparation waste biomass material for bioconversion into fuels such as bio-diesel and other etc. (Cóndor-Golec A,et al, 2006)

Fermentation of the plants brings out desirable changes in their properties(Caplice E. et al 1999). One of these is changes is the production of antioxidants. Studies have shown that antioxidants help slow ageing and prevents several diseases like cardiovascular diseases, cataract, brain dysfunction, oxidative stress, birth defects and also help enhance the immune system (Ames B.et al, 1993,Devasagayam T.et al,2004). Alcohol is the other product of fermentation which in moderate or low concentration can be beneficial to the body.

Increasing pollution and stress subjects our body to various oxidative damages which are brought about by free radicals. These free radicals adversely alter lipids, proteins, and DNA and trigger a number of human diseases. Hence, the inclusion of an external source of antioxidants can assist to cope with this oxidative stress (Lobo V. et al 2010). Consumption of fermented health drink helps regain the antioxidant balance in our body which will in turn help to counteract the effects of free radicals, thereby preventing ailments like cardiovascular diseases, carcinogenesis and ageing. (Chui C.H.et al,2006).

II. Materials And Methods

2.1 Preparation of effective microorganisms (EM stock):125gm of banana, papaya and pumpkin were collected and chopped into small pieces and transferred into air tight container and mixed with 500ml boiled water, subsequently 125gm of jaggery and one egg were added. The container was closed tightly and kept for fermentation for a week till a white layer appears on top. The fermented solution was then collected and used as the EM stock solution.

2.2 Isolation of effective microorganisms: The EM stock was isolated onto Nutrient agar and Tomato Juice Agar plates to study the morphological characteristics.

2.3 Detection of pathogens in EM stock:EM stock wastested for presence of pathogens using media like TCBS (ThiosulfateCitrate Bile saltsSucrose)agar, XLD(Xylose Lysine Deoxycholate) agar and SS (Salmonella Shigella) agar plates.

2.4 Activation of EM stock solution:20ml of EM stock solution and 20gm of jaggery were mixed with 400ml of boiled water in a 500ml conical flask. For the period of activation, the container was placed in shade at room temperature. This solution was then kept for a period of 3 weeks for activation. It was verified by a pH of 3.5 or lower.

2.5 Fermentation of health drink: The health drink was prepared in two batches. The first batch consisted of 250ml orange juice, 5ml EM stock solution and 5gm of dried leaves of Shatavari. The second batch was made using the same ingredients with 5ml Amla juice. The fermentation was carried out at room temperature for 7 days. The fermented product was then filtered and centrifuged at 500 rpm for 15 minutes and the supernatant was used to estimate the total anti- oxidation level and alcohol content.

2.6 Determination and Quantification of Lactic Acid Bacteria and Yeasts: During the course of fermentation,the viable count of Lactic acid bacteria and yeasts in the health drink was determined by plating onto Tomato Juice agar and Sabouraud's agar.

2.7Filtration of Health Drink: The prepared health drinkwas filtered using a muslin cloth to remove the larger debris. The cells were then separated out by first cold centrifuging it and then passing the drink through a commercial filter (Eureka Forbes AquaguardPersonal Purifier Bottle), to stop the fermentation. The efficiency of filtration was then be determined by taking viable count of organisms present in influent and effluent samples by standard pour plate method. The filtrate was then again filtered using a syringe filter to ensure that no living cells were left in the health drink.

2.8. Anti-oxidant activity assay by Phosphomolybdenum method: Ascorbic acid standards were pipetted out containing concentration varying from 4-20 µg into series of test tubes and made up to 2 ml with H_2O. The test tubes were then added with 2ml of Phosphomolybdenum complex reagent containing. 6M H_2SO_4, 28mM sodium phosphate, 4mM Ammonium molybdate and incubated in boiling water bath at 95^oC for 90 min. After the samples cooled at room temperature, the absorbance of the aqueous solution of each tube was measured at 695nm against blank.

2.9. Estimation of Ethanol by Potassium Dichromate method: To 30ml fermented juice, few drops of phenol red indicator was added and pH was adjusted between 7.0 and 8.0. To this 2ml of 25% $ZnSO_4$ solution was added and then 2ml of 1N NaOH was added for proper mixing. The mixture was adjusted to definite volume using distilled water and used for distillation. Alcohol standard solutions were prepared containing 1-5mg/ml. 10 ml $K_2Cr_2O_7$ was added and incubated at room temperature for 30 minutes. Then 100ml distilled water and 4ml KI solution were added. The I_2 liberated was then titrated against 0.1N $Na_2S_2O_3$using starch as indicator. End point was blue to colourless. Similarly, readings for blank samples were obtained.

2.10.Qualitative tests for Phytochemicals: The health drink was screened for presence of different phytochemicals like tannins, saponins, alkaloids, glycosides and flavonoids.

2.11. Determination of stability of antioxidants in the health drink: After filtration, the health drink was used daily for thePhosphomolybdenum assay to determine the daily antioxidant levels. The variation in the levels of the antioxidant indicates whether the antioxidants remain stable or were deactivated during the storage period due to environmental oxidation.

2.12.Preservation of the health drink: For increasing the shelf life of the health drink, the microbial growth in the health drink had to be prevented. For this purpose, chemical and natural preservatives like potassium meta sulphite and Clove oil, both were used for determination of their respective MIC against test organisms like *Escherichia coli* and *Staphylococcus aureus* preservatives using the agar cup method. Potassium meta sulphite was used in the concentrations of 50, 25 and12.5µg/ml. Whereas, the clove oil was diluted using 20%DMSO as the solvent to obtain two-fold dilutions up to 1:128. The minimum concentration which showed zone of inhibition was then used for preservation of the health drink.

2.13. Enhancement of the flavour of the health drink: For 250ml health drink, about 12.50 g sucrose was addedafter the preparation of the drink for sweetness. To the enhance flavor, few drops of orange essence was added to the health drink.

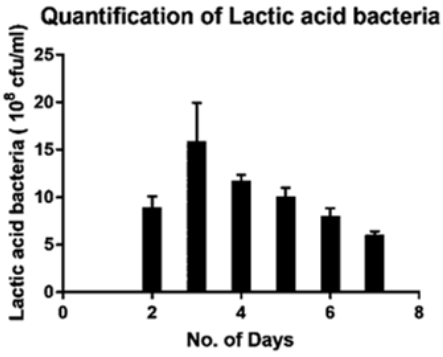
2.14. Statistical analysis-Statistical analysis was carried out using One Way Analysis of Variance (ANOVA). GraphPad Prism 7.03 software was used for all statistical analysis. P value(<0.0001)was considered significant.

III. Results

3.1 Preparation and isolation of EM : The EM showed different types of colonies on isolation on Nutrient agar and Tomato juice agar. Gram staining showed presence of Gram-positive oval cells and Gram-positive coccobacilli indicating presence of yeasts and lactobacilli.

3.2Determination and Quantification of Lactic acid Bacteria and Yeasts:

To check whether the provided conditions are favourable for the growth of the Effective microorganisms, the lactic acid bacteria and yeasts were quantified



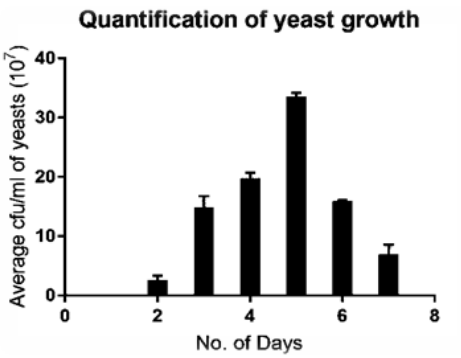


Figure 1: Growth of lactic acid bacteria (cfu/ml)Figure 2: Growth of yeasts (cfu/ml)

3.3. Centrifugation and Filtration of the health drink:

The health drink was centrifuged and filtered so as to remove all the fruit pulp and leaf particles from the juice and separate out the living cells of yeasts and lactic acid bacteria which if allowed, would continue the fermentation and eventually adversely affect the drink.

The filtration efficiency was determined by plating out serial dilutions of the influent (juice after centrifugation) and the effluent (juice after filtration using the commercial filter).The juice was then further filtered using syringe filters of pore size 0.1μm to filter out the remaining microbial cells. The juice thus obtained was then refrigerated and used for further analysis.

	Dilutions used	cfu/0.1ml	Average cfu/ml
Influent	10^5	146	1.74×10^8
	10^6	98	
	10^7	42	
Effluent	10^1	59	8.4×10^3
	10^2	11	
	10^3	-	

Figure 3: Filtration efficiency of commercial filter

3.4. Quantification of Antioxidant and Alcohol content:

For health drink containing orange juice and leaves of Shatavari (Batch1) antioxidant content of 701.76 μg/ml equivalent of Ascorbic acid was detected. For the health drink containing orange juice and Amla (Batch 2), an antioxidant level of 737.25 μg/ml equivalent of Ascorbic acid was detected.The alcohol content in both the batched were found to be low with Batch 1 (containing Shatavari) showing 0.145gm% and Batch 2 (containing Amla juice) showing 0.04gm% alcohol respectively.

3.5 Determination of Antioxidant stability:

The antioxidant content in the health drink may undergo changes due to environmental conditions leading to oxidation. Hence, the stability of the antioxidants in the health drink was determined.

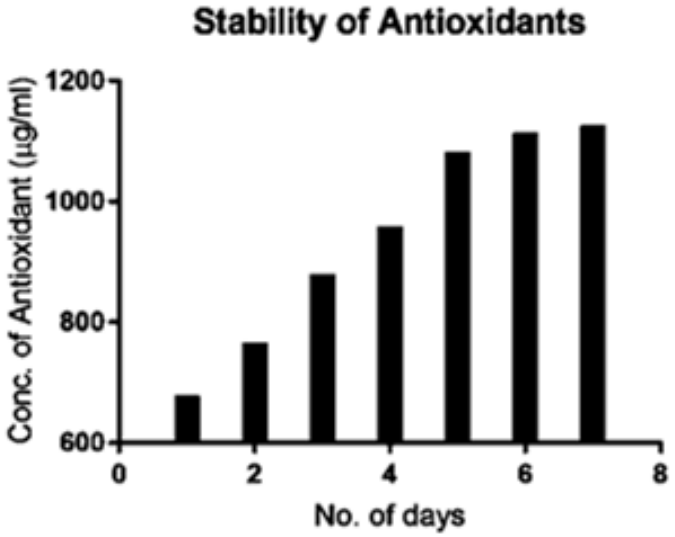


Figure 4: Determination of antioxidant stability with time

3.6Qualitative testsfor Phytochemicals:

The filtered health drink was used to detect phytochemicals. The methanolic and chloroform extracts were used to perform several tests. Phytochemicals like Carbohydrates, Flavonoids and Saponins were detected,

3.7 Preservation of the health drink:

The MIC of natural and chemical preservatives like Clove oil and Potassium meta bisulphite were carried out to determine the ideal concentration which would help prevent microbial growth in the health drink. Organisms like *Eschericha coli* and *Staphylococcus aureus* were used in this study.

A) MIC of Potassium metabisulphite-

According to food safety standards, the permissible limit for Potassium meta bisulphite is 100ppm (100µg/ml).

Conc. Of potassium meta bisulphite (µg/ml)	Zones of inhibition (mm)	
	Eschericha coli	***Staphylococcus aureus***
100	24	31
50	17	23
25	11	15

Figure 5: Zones of inhibition observed using Potassium meta Bisulphite against *E.coli* and *S.aureus*

B) MIC of Clove oil

Dilution of Clove Oil	Zones of inhibition (mm)	
	Eschericha coli	***Staphylococcus aureus***
1:2	26	40
1:4	18	35
1:8	13	31
1:16	11	26
1:32	-	18
1:64	-	11

Figure 5: Zones of inhibition observed using Clove Oil against *E.coli* and *S.aureus*

IV. Discussion

Food fermentation is one of the earliest technologies developed by humans. Although yeasts are the principle organisms involved, filamentous fungi, lactic acid bacteria, acetic acid bacteria and other bacterial groups all play a role in the production of alcoholic fruit products (Guleria A. et al 2012). Studies conducted on health drink preparation using EM have demonstrated high antioxidant, antibacterial, tyrosinase inhibition activities, and biofilm inhibition activity of pathogenic bacteria, modulates the oxidative damages against kidney and liver ((Li Z.et al, 2010, Aruoma O.et al, 2002).

Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. Overproduction of oxidants can cause an imbalance, leading to oxidative stress,

especially in chronic bacterial, viral, and parasitic infections (Liu R. et al, 1995). Oxidative stress can cause oxidative damage to large biomolecules such as proteins, DNA, and lipids, resulting in an increased risk for cancer and cardiovascular disease (Ames B.et al, 1991). Thecurrent study shows the production of a health drink with high concentration of antioxidants i.e. 700µg/ml after 7 days of fermentation. Studies have shown that antioxidants help slow ageing and prevent several diseases like cardiovascular diseases, cataract, brain dysfunction, oxidative stress, birth defects and also help enhance the immune system (Ames B.et al, 1993).

Experimental as well as epidemiological data indicate that a variety of nutritional factors can act as antioxidants and inhibit the process of cancer development and reduce cancer risk.(Lobo V.et al,2010)

The medicinal plants used here are very beneficial from the health point of view. Shatavari plant possesses aphrodisiac, demulcent, general tonic, diuretic, anti-inflammatory, antiseptic, anti-oxidant and antispasmodic properties (Lee Y.et al 2009). Experimental studies conducted with the fruits of Amla indicate that they have significant cytoprotective effect against isoprenaline-induced myocardial injury, radiation induced chromosomal damage and heavy metal induced hepatotoxicity and nephrotoxicity. Clinical studies also suggest that the fruit has anabolic activity (Bhattacharya A.et al, 1998).

The alcohol content in the health drink was very low which makes it safe for consumption for all age groups.Also, the presence of mild alcohol concentration makes it more beneficial since the idea that moderate to low alcohol use is beneficial in the context of preventive medicine is not new. It has been associated with lower mortality, fewer cardiovascular events, and less dementia and diabetes(Reid M.et al,2002; Baum C.et al,1985). Certain Ayurvedic preparations called *Arishtas* are herbal wines which have very low concentrations of alcohol. Also, the drink showed the presence of several phytochemicals through qualitative assay thereby notably increasing its health benefits since studies have demonstrated that phytochemicals have significant effects on detoxificationenzymes, scavenging of oxidative agents, stimulation of the immune system, regulation of geneexpression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviraleffects (Dragsted L.et al,1993). Phytochemicals also play an important role in the development ofcancers (Waladkhani A.et al, 1998).

V. Conclusion

Fermentation of the plants increasesthe levels of the antioxidants and makes the phytochemicals more bioavailable for absorption intothe body. Ayurveda, which is one of the most primitive fields of medicine, has been using the science of fermentation for this very purpose since ages. The fermented health drink prepared in thepresent study, being high in antioxidants and showing presence of phytochemicals can therefore beconsidered as a versatile health drink which also shows minimal alcohol concentration, thus beingsafe for consumption by the young and old alike.

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IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) is UGC approved Journal with Sl. No. 4033, Journal no. 44202.

Mohanan M. "Effective Microorganisms Based Fermentation for Antioxidant-Rich Health Drink from Medicinal Plants." IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) 5.1 (2019): 33-38.