

Production and Immobilization of *Lactobacillus Acidophilus* on Fruit Wastes Containing Media as Probiotics

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Abstract:

The exponentially increasing world population creates the challenge of providing adequate food sources. Specially, protein deficiency poses a problem since essential amino acids cannot be replaced by anything. The threat of rising protein deficiency diseases was becoming a disaster for humans. It is essential to do some efforts to explore alternative and unconventional sources proteins from human beings and animals. Recently, the attention had drawn to exploit microorganisms directly as new source of food and feed in the form of dried cells, terms as Single Cell Protein (SCP). On the other hand, there is a great monster of waste, engulfing the whole world at an over increasing rate. Organic wastes such as vegetable and fruit residues management are also a major threat area of research concern. It is imperative to use these waste cheap substrates for culturing microbes as a source of SCP. Present research focused on attempting, production of SCP/Probiotics by *Lactobacillus acidophilus*. They were grown in medium containing 5g/l (NH₂)₂SO₄, 1g/l MgSO₄.7H₂O, 0.5g/l NaCl, 0.1g/l CaCl₂ and fruit waste as a carbon source and incubated for 7 days. The fruit wastes of Orange and Pomegranate were used as an additional supplementary element in production medium. An immobilization approach was used to store the biomass produced.

Keywords:

Lactobacillus, Organic Waste, Population, Protein Deficiency, SCP

1. Introduction

Proteins consist of amino acid building blocks; these amino acids are essential for the existence of the living beings. Biological waste has been used now a day for the production unicellular microbial biomass, SCP grown in industry. Many raw materials were analyzed to produce single cell protein, which considered as the carbon and other energy sources. Due to high protein content SCP widely used for aquaculture and poultry forms as the cheap source of protein feed. It has been gaining a commercial interest. Many researches have been conducted to device the most suitable raw material for low-cost production of SCP.

One of such research illustrated SCP production and Biochemical Oxygen Demand (BOD) removal from whey by using mixed yeast culture with 11 yeast strains isolated from dairy products. These strains were tested for production of SCP from whey and testing their ability to reduce the BOD. Among these strains, *K. lactis* (M2) had the most SCP production from whey by giving the yield of 11.79 g/l. (Hassan Moeini et.al 2004). Jitendra Kumar Singh et.al 2011 studied that dairy waste water is particularly suitable to produce Single Cell Protein, using lactose utilizing microorganisms. Extracts of papaya fruit were also tried as substrate for single cell protein production by culturing *Saccharomyces cerevisiae*. Biochemical analysis of papaya extract and dry biomass were analyzed for nutrient contents (Ojokoh A.O. and R.E. Uzeh 2005). Another such investigation was done on the *Saccharomyces cerevisiae*, grown on different fruit wastes such as mango waste, banana skin, sweet orange peel, rind of the pomegranate and apple to produce SCP as shown by Mahnaaz Khan et.al in 2010. Cucumber and orange peels were also estimated to produce SCP using *Saccharomyces cerevisiae* by submerged fermentation technique.

Pineapple wastes from processing industries were utilized to produce culture medium for culture of probiotics *Lactobacillus* (Pyar et. al. 2014). Here results showed that fruit wastes might be susceptible to hydrolysis reaction as suggested by Amit Kumar Mondal et. al 2012. The protein obtained from such microbial cultures was cheap, nutritive and used as a food or feed additive. SCP production by bioconversion of fruit wastes has the immense potential to resolve the worldwide threat of protein deficiency. This piece of research also evaluates the performance of production medium for SCP/Probiotics production. Probiotic are microbial cell preparations that have a beneficial effect on health of its host. Lactic acid bacteria are the most commonly used as probiotics (Sumarno et. al. 2012). Probiotics helpful in defense against infection, cancer and had a role in stabilizing the physiological balance of the intestinal microbiota (Oelschlaeger 2010; Wohlgemuth et al., 2010).

As mentioned in India Agricultural Research Data Book 2004, the total Fruit and vegetable waste generated was up to 50 million tons per annum. Fruit wastes rich in carbohydrate content and other basic nutrients could support microbial growth. Thus, fruit processing wastes are useful substrates for production of microbial proteins. Fruit wastes utilization for SCP will help in controlling pollution and in solving waste disposable problem to some extent in addition to satisfy the world shortage of protein rich food. Over the last few years, a lot of research has been done for reprocessing and reuse of different fruit wastes for the conversion of valuable and nutritive products. Here investigation was carried out to estimate the potential of different fruit wastes for cost effective yeast biomass production (Amit Kumar Mondal, et.al 2012).

In general, microalgae are rich in various phytochemicals like carotenoids, phycocyanine, phenolics, amino acids, polyunsaturated fatty acids, and sulphated polysaccharides. These compounds provide various antimicrobial, antiviral, antioxidants, antitumoral, anti-allergy effects and anti-inflammatory. Their healthy benefit seemed to be due to different biochemical mechanisms. However, some microalgae species such as *Chlorella*, *Spirulina* and species have been used in several areas in nutraceutical, pharmaceutical, cosmetics, nutrition and functional quality of foods. In 2006, World Health Organization has been described as one of the greatest super-foods on earth serving as an example of the potential of microalgae. This review provides background on current and future uses of microalgae as source of health promoting compounds (N. Vishnu and R. Sumathi 2014).

Deficiency of protein is becoming a major threat for world today. Some of the underdeveloped countries like Algeria, Botswana, Nigeria, Madagascar etc., are facing major food and nutrition deficiency problems. India, although a developed nation, its major population is facing nutrition deficiency and food scarcity problems. In the face of such worldwide issues, single cell proteins derived from the waste organic products had been proved a very useful technology. Dried cells of microorganisms, rich in proteins and can be used as dietary supplements, are called Single Cell Proteins (SCP) (Gour Suman, Mathur et.al 2015).

From ancient ages, milk and milk products have been utilized by human being and known to be a source of lactobacilli. The present study is directed towards the study of prevalence, isolation and identification of *Lactobacillus* species in milk. Some samples were collected from cow and buffalo sheds of a local dairy in Aarey Milk Colony, major supplier in the city of Mumbai. A total of 40 milk samples were obtained and 163 colonies were isolated from them. These colonies were subjected to characterization by standard microbiological methods. By physiological testing and sugar utilization pattern, 163 isolates were confirmed that of the genus *Lactobacillus*: *L. fermentum* (48%), *L. acidophilus* (34%), *L. viridescens* (8%), *L. brevis* (5%), *L. gasseri* (4%) whereas two isolates could not be identified upto the species level. The results indicate that *L. fermentum* is predominant in the milk obtained from this sector (Sarangdhar mithun et.al 2015)

The aim of this study was to determine *Lactobacillus bulgaricus* and *Lactobacillus casei* isolated from yoghurt, different kinds of cheese and a traditional food named 'tarhana' (a fermented food made of a mixture of cereal, yoghurt and thyme), and to determine the antimicrobial activity and antibiotic resistance of these isolates. The identity of the culture was based on characteristics of the strains of *Lactobacillus* spp. as presented in Bergey's Manual of Determinative Bacteriology, carrying out microscopy (morphology), Gram staining, growth at 15 and 45 °C, and fermentation of different carbon sources and growth in 7.5% NaCl. Based on all the identification tests, one strain was isolated from the cheese and identified as *Lactobacillus casei*, and the other strain isolated from the probiotic dairy product was identified as *L. bulgaricus*. The *L. casei* isolate was resistant to all the antibiotic discs used in this study. Culture supernatants obtained from the 2 isolates of *Lactobacillus* spp. Showed inhibitory activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Enterobacter cloacae* (Özlem ERDÖRUL et.al 2006).

During the last decades, it became clear that the human body lives in close harmony with a complex ecosystem that is composed of more than 1,000 different bacterial species inhabiting the oral cavity, upper respiratory tract, gastrointestinal tract (GIT), vagina, and skin. This collection, known as the microbiota, is acquired soon after birth and persists throughout life. Together, these microbes play an important role in the physiology of their host, including the digestion and assimilation of nutrients, protection against pathogen colonization, modulation of immune responses, regulation of fat storage, and stimulation of intestinal angiogenesis. However, understanding how these different species contribute to human health remains a major challenge (Sarah Lebeer et.al 2008).

"Tarkhineh" is a traditional fermented food produced from a mixture of spontaneously fermented butter milk and wheat flour in Iran. 9 samples of were collected from different rural areas of Kermanshah, Iran. The isolates were grouped

and identified using a combination of phenotypic and genotypic methods including repetitive extragenic palindromic polymerase chain reaction (REP-PCR) fingerprinting, biochemical methods and carbohydrate profiling and then evaluation the probiotic properties of them. These 54 isolates belonged to group *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus pentosus*, *Lactobacillus brevis* and *Lactobacillus diolivorans* that profile bonding from rep-PCR showed *Lactobacillus plantarum* have a high intra-species diversity. Media of pH = 2.0–7.0 and bile salt concentrations of 0.0-0.5% were used as stress conditions (Vasiee et.al 2014).

Nowadays, a new trend is the development of novel fermented milks, which contain microorganisms, called probiotics such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and others. Probiotic lactic acid bacteria act beneficially in human health, and therefore, a wide variety of lactic acid bacteria strains are available to consumers in both traditional fermented foods and in supplement form. Generally, the production of probiotic foods that will contain specific probiotic strains at suitable levels of viable cells during their shelf life is a technological challenge. The shelf life of probiotics should be controlled to manufacture products with adequate live bacteria (at least 10⁷ CFU/g) to obtain the health promoting benefits of probiotic cultures. Lactic acid is also an important chemical used in a wide variety of applications, being used primarily in the food industry as an acidulant, preservative, and to produce emulsifying agents. *Lactobacillus casei* cells have been immobilized in some supports for lactic acid production. Agar was more effective than polyacrylamide for *L. casei* entrapment for lactic acid production from whey. Also, calcium pectate gel and chemically modified chitosan beads were used as supports for *L. casei* cell immobilization (Y. Kourkoutas et.al 2005).

The increasing demand for food production leads to a wide gap in-between demand and supply. To bridge this gap the microbial biomass i.e. single cell protein (SCP) can be considered as an alternative to conventional source of food or feed. In this present work we have attempted for production of SCP by using the probiotics such as *Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Bacillus coagulans* as they don't cause any harm if ingested. These probiotics were grown in medium containing 5g/l (NH₂)₂SO₄, 1g/l MgSO₄.7H₂O, 0.5g/l NaCl, 0.1g/l CaCl₂ and fruit waste as a carbon source and incubated for 7 days. The fruit wastes of Custard apple, Watermelon and Sweet lime were used in this study (Shweta S. Potnis et.al., 2016).

Certain valuable products can be made by bioconversion of fruit wastes such as single cell protein (SCP) can solve the worldwide food protein deficiency by obtaining an economical product for food and feed. However, using wastes as substrate to produce high nutritious product may also alleviate environmental pollutant up to some extent. In the light of this, an attempt was made in this study by selecting *Aspergillus niger* and *Saccharomyces cerevisiae* to produce SCP. The orange peels were used as sole carbon source for preparation of fermentation media on which the two selected fungal strains were grow (Sadiq Azam 2014).

Fermented dairy products usually consist of lactic acid bacteria. *Lactobacillus* is a genus of lactic acid bacteria and described as heterogeneous group of regular, non-spore forming, gram positive, rod shaped, non-motile bacteria and absence of catalase enzyme. This study focused on isolation of *Lactobacillus* from curd samples. A total of 14 curd samples were collected from the local areas of Gurgaon (Haryana) and Lakshmangarh (Rajasthan). 28 isolates were isolated by growing on MRS agar

medium and characterized by their phenotypic characteristics. The *Lactobacillus* isolates also possess homofermentative and heterofermentative characteristics (Renuka Goyal et.al 2012).

Lactobacillus involves heterogeneous group of gram-positive, rod shaped, non-spore forming and nonmotile bacteria. A total of 30 dairy samples were collected from the local areas of Solan in Himachal Pradesh. 30 bacterial strains were isolated on MRS agar medium and its pure culture was obtained by sub-culturing. Purity of each culture was confirmed by morphological investigation, Gram's staining and further identification by *Lactobacillus* specific biochemical tests. Finally, 12 bacterial isolates were identified as *Lactobacillus* spp. after morphological, cultural and biochemical characterization (Ashwani Kumar et. al 2014).

2. Materials and Methods

Among the other dairy products such as milk, buttermilk, curd is the best source for *Lactobacillus* sp. Curd is taken in sterilized flask. Under the aseptic conditions curd was serially diluted from 10⁻¹ to 10⁻¹⁴ from these 14 dilutions 10⁻⁵ 10⁻⁷ 10⁻⁹ are selected. For this spread plate technique and further streak plate technique is done on MRS medium. They are incubated in the incubator at 37°C, which is optimum temperature for *Lactobacillus* species for 24 hours. After the period of incubation, isolated colonies were grown, and characterization is done. It was found to be *Lactobacillus* species. 1-2 colony shows total resemblance with *Lactobacillus acidophilus*.

The isolated colony formed on the MRS agar plates was identified using the Gram stain, biochemical tests. The identification was performed according to Bergey's manual of determinative of bacteriology. The culture was stored at 4 °C.

2.1. Characterization of *Lactobacillus Acidophilus* (Biochemical Tests)

Characterization of *Lactobacillus acidophilus* carried out by different tests as described: The isolated bacteria were examined using the Gram staining kit and observed under light microscope with a magnification of 100x. Hanging-drop wet method was performed to detect motility of the bacteria. A single isolated colony was streaked on a glass slide and one drop of 3 % hydrogen peroxide was added on to it. The positive response of the bacteria to catalase test was indicated by the effervescence of oxygen. For carbohydrate fermentation test phenol red broth base medium was used as a medium. Different sugar substrates namely, arabinose, sucrose, maltose, lactose, sorbitol and glucose were used. 0.1 g (0.1 % w/v) of each sugar substrate was added to 100 ml of the medium. 5 ml of each mixture were transferred into each tube. For gas detection, the Durham tube was inserted into the test tube containing glucose. All the tubes were sterilized for 15 min at 121 °C and were inoculated with a bacterial colony under study. The positive reaction of the bacteria was indicated by the changes in the colour of the medium.

2.2. Collection and Analysis of Fruit Wastes

By the choice of availability, the wastes of Orange and Pomegranate were obtained from local vendors from Satna city. The wastes were cleaned with distilled water, shade dried and grinded to fine powder as shown in Figure 1 and 2. The dried powdered wastes were then analyzed for protein content by Biuret method and carbohydrate content by Benedict's method.



Figure 1. Dry Pomegranate and Orange Fruit Waste.



Figure 2. Fine Powder of Orange and Pomegranate.

2.3. Harvesting of SCP

SCP was harvested from broth through filtration. The biomass (Bacterial biomass + residual waste substrates) was filtered through Whatman filter paper No. 1. The biomasses were washed repeatedly with distilled water to remove any adherence, and then dried in an oven at 90 °C till constant weight. The dried filter papers with their content were weighted and the biomass (mg/100 ml broth) was estimated.

2.4. Media Optimization for SCP Production

The production media for SCP was further optimized for carbon source, pH and temperature to obtain maximum yield of biomass as SCP. Carbon source was optimized with fruit waste and then its different concentrations such as 1%, 2%, 3%, 4%, 5%. The second parameter was selected as pH such as 4.5, 5.5, 6.2 and 7.5. Also, different temperatures for incubation such as 25 °C, 28 °C, 35 °C and 45 °C were selected for optimization.

2.5. Immobilization of *Lactobacillus* cells

By Sodium alginate method

- In a 50 ml centrifuge tube, 20 ml of the yeast culture was taken and to 20 ml of the 4% Sodium alginate solution was added.
- Mix the culture with Sodium alginate solution properly.

- Take 50 ml of Calcium chloride solution in a conical flask. Crush the ice and keep the flask on ice.
- With a 1 ml pipette, take the alginate and yeast culture mix and add drop wise to the Calcium chloride solution. Gentle swirling was done while addition.
- Leave the immobilized yeast cell beads to harden in the Calcium chloride solution for 5–10 minutes. The alginate will be ionically cross-linked by the calcium ions.

Isolate the beads after discarding the solution.

3. Observations and Results

The preliminary investigations included macroscopic analysis, microscopic analysis (Gram-positive bacilli as shown in Figure 3), lactic acid biosynthesis, endospore test, milk coagulation activities and the negative catalase reaction permitted the classification of the working bacterium into the *Lactobacillus* genus. In characterization they were Gram-positive, rod shaped, non- motile, catalase activity absents and no endospore. The isolates were tolerant to a range of salt concentrations (1-9%) and had the ability to coagulate milk. The main task of Carbohydrate fermentation test is to investigate the ability of bacteria to ferment different types of carbohydrate. Phenol red broth base medium was used to analyze the bacteria based on their patterns of carbohydrate utilization. The isolated bacteria could ferment maltose, lactose, sucrose and glucose, but not sorbitol and arabinose. No bubbling was detected in Durham tube suggesting that there was no gas produced from growth of bacteria. Thus, the results obtained coincided with *L. acidophilus* strain characteristic.



Figure 3. Gram Staining and Streak Plate of Lactobacillus.

3.1. Analysis of Fruit Wastes

After collecting the fruit wastes they were dried and powdered to fine. The powder was analyzed for total protein contents by Biuret method and carbohydrate contents by Benedict's method. The results were shown in Table 1.

Table 1. Analysis of fruit waste: Total protein and carbohydrate analysis of dried fruit waste of Orange and Pomegranate.

Fruit Waste	Carbohydrate	Protein
Orange fruit powder	1.890mg	5.483mg
Pomegranate fruit powder	5.512mg	7.223mg

3.2. SCP Production

The SCP was produced by *L. acidophilus*. After incubation of 7 days, at 28°C the SCP was harvested by filtration method. The obtained SCP content was further weighed, and biomass content was determined. The obtained results were shown in Table 2. The biomass content of SCP on Orange and Pomegranate fruit powder containing media obtained was as follows.

Table 2. Analysis of Biomass of *L. acidophilus* in fruit waste containing media.

Fruit waste containing media	<i>Lactobacillus acidophilus</i> biomass content
Orange waste + MRS media	325mg
Pomegranate waste + MRS media	540mg

3.3. Immobilization of *Lactobacillus acidophilus*

Immobilization using alginate has the advantage of being economical because of its low cost and simplicity: adding an alginate solution to a solution containing a mixture of calcium chloride can result in the formation of alginate beads with pore sizes of less than 17 nm. Alginates, which are natural anionic polysaccharides composed of D-mannuronic and L-guluronic acid residues joined linearly by (1-4) glycosidic linkages, are also an accepted food additive and can be used safely in foods (Chávarri *et al.*, 2010; Chan and Zhang 2005). Immobilized cells of *L. acidophilus* can be easily stored and handled with maximum activity.

4. Conclusions

Current shortage of protein especially in the developing countries, due to the rapid rise of population was overcome completely with the use of SCP as an alternative source of protein. Lactic acid bacteria are the natural microflora in food and dairy products. In present research, *Lactobacillus acidophilus* was isolated from curd samples. All the isolates were characterized based on microscopic investigation and biochemical test.

For cost-effective production, growth of *L. acidophilus* was obtained successfully on Orange and Pomegranate fruit waste containing production media. Quantitative analysis of carbohydrate and protein of fruit wastes and biomass of SCP was obtained. Present study had shown that the carbohydrate and protein content of Pomegranate waste was better than Orange waste, thus Pomegranate fruit waste was better to use for probiotics production. Probiotics production on Fruit waste was a better option for low-cost production and immobilization of cells can be done for better handling and storage.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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