DEVELOPMENT OF PROBIOTIC PALM FRUIT JUICE

A PROJECT REPORT

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BONAFIDE CERTIFICATE

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ABSTRACT

For the growth of probiotic microorganisms it has been suggested that fruit juices are an ideal media. The aim of our research work was to develop a probiotic juice with palm fruit. In order to develop the probiotic fruit juice the palm was clarified using pectinase. The parameters considered for clarification include time, pH, enzyme concentration and temperature. These parameters optimized were for the nine responses namely clarity ,turbidity ,total sugar, total solids ,total soluble solids(TSS), reducing sugars, pH, ascorbic acid and titratable acidity using RSM. The clarified palm juice was inoculated with 24hrs log phase culture of *L.plantarum* as probiotics between the concentration of 2-4%. The juice was further incorporated with prebiotic inulin in the concentration 1-3%. To this juice Stevia at a concentration of 1-3% was added as sweetener. All the three factors probiotic, prebiotic and Stevia were optimized using RSM, and analysed for every 24hrs time interval for the responses clarity, turbidity, total sugar, total solids, total soluble solids (TSS), reducing sugar, pH, ascorbic acid, titratable acidity, microbial viability.

Keywords: Probiotics, Palm fruit, Prebiotics, Sweeteners

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LIST OF ABBREVIATIONS

Abbreviation/Symbol	Description
LAB	Lactic Acid Bacteria
RPM	Revolutions Per Minute
GI	Gastro Intestinal
L.acidophillus	*Lactobacillus acidophilus*
L.plantarum	*Lactobacillus plantarum*
L.rhamnosus	*Lactobacillus rhamnosus*
L.delbruekii	*Lactobacillus delbruekii*
L.casei	*Lactobacillus casei*
L.lactis	*Lactobacillus lactis*
FAO	Food and Agricultural Organisation
WHO	World Health Organisation
CFU	Colonies forming Unit
TSS	Total Soluble Solids
g	Gram
mL	Milliliter
l	Litre
N	Normality
nm	Nanometer
µg	Microgram
HCL	Hydro chloric acid
DNS	Di Nitro Salicylic acid
M	Molarity
mm	Millimeter
Std	Standard
G	Guanine
C	Cytosine
RSM	Response Surface Methodology
ANOVA	Analysis of Variance
CCRD	Central Composite Rotatable Design
PAC	Physiologically Active compounds
MRS	De Mann Ragosa Sharpe

CHAPTER 1
INTRODUCTION

1.1 GENERAL

Diet is professed to be the most compelling and important factor contributing to health . Nowadays food industries' key researches are focussed towards the development of foods that are likely to promote health and well being of individuals. (Klaenhammer & Kullen, 1999). This recent trend has favoured consumption of foods containing physio- logically active components (PAC) such as prebiotics, probiotics, vitamins and minerals.

Beneficial micro-organisms like *Bifidobacteria* and *Lactobacilli* are also called probiotic strains that prove to be favourable to the life of the host organism. There are also other microfloras like *Pseudomonas*, *Clostridia* inhabiting our intestines that are harmful to the body. The favourable microflora prevents the growth of other harmful bacterias and imparts health benefits to the hosts' intestine (Saxelin,1996). The human gastrointestinal tract constitutes of a complex ecosystem of bacterial inhabitants. The bacterial flora is of varied variety along the length and cross section of the gastrointestinal tract. They perform activities involving nutritive, metabolic, protective and immunological functions of the hosts intestine. Bacterias constitute approximately 25-55%of the total of human colon. Many studies suggest that an inherent relationship exists between gut flora and the host. The dominant species found in all individuals are *Bifidobacteria*, *Eubacteria*, *Clostrdium*, *Peptococcus*. The various benefits exerted by

these microflora includes breaking down of complex food remains that are not digested properly, production of biotin, vitamin K, antagonizing of harmful bacteria, mediation of immune responses.

There are a wide variety of factors that influence the gut flora composition such as diet, age, lifestyle, stress, illness. The "friendly" bugs present are Gram-positive *Lactobacilli* and *Bifidobacteria* that dominate (> 85% of total bacteria).Also a concept of ‘super-organisms' has recently emerged to showcase the importance of mutually beneficial host-microbe interaction going on in the gut. When their concentration equilibrium is altered several gastrointestinal and extra intestinal diseases are likely to occur (Purchiaron *et al.*, 2013)

Probiotics are live microbial food ingredients that have a beneficial effect on human health or they can be defined as live a microbial feed supplement that improves the hosts intestinal microbial balance.(Salminen, S *et al.*,1999*)*.It was after the work carried out by Metchnikoff at Pasteur institute in Paris that, probiotics were known to the world. However initially the microbes in the gut were considered detrimental rather than beneficial.It was only after using them as substitutions for yoghurt and other dairy products the beneficial effects of the microbes were confirmed. Since then there has been an vast quest to use these indigenous microbes to favour the intestinal function to help improve the health status of humans. Quite a large number of definitions has been suggested by many researchers all over the world, one of the most appropriate one was given by (Hvenaar*et al.*,1992), according to which probiotics are defined as ”mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora ”. This definition was regarded appropriate for reasons like :-

- The probiotic activities are not restricted to the intestinal microflora; it is also extended to other sites of the body.
- The probiotics are from single species, rather spread out to more varied bacterial species
- Application of probiotics can be carried out in both man and animals.(Wilhelm *et al*., 1998)

After the series of researches that were carried out utilizing probiotics, it was only in the 1950s, a certain probiotic product was licensed to be used as a drug for the treatment of scour (*Escherichia coli*) infection among pigs by the United States Department of Agriculture. Since then in the last centuryor so, different micro-organisms have been used for their capability to prevent and cure diseases, which led to the coining of the common term probiotics. (Lee, Y.-K *et al.*,1999*)* .

It is very important to know the required adequate amounts of probiotic that must be supplied in order to trigger the targeted positive effects from the probiotic used on the host.Itis also known to depend on strain specificity, process and matrix. The accepted levels of probiotic that is to be used for a beneficial purpose is observed after ingestion of a concentration around 10^7 to 10^8 probiotics per gram, that involves servings of about 100 to 200 mg a day.

Probiotics are mostly added as supplementary to food products to render certain additional health benefits to the consumer, thus are named as "functional foods." Fruit juices are reported to potentially be good carriers for two different probiotic types. Certain strains of probiotics were found to be stable in a fruit juice, namely a mix of red-fruits, and they don’t affect the sensory score.

Currently most of the probiotic foods are dairy-based, but there is a wide growing interest toward non dairy probiotic products due to

lactose intolerance and cholesterol content. Fruit juices are mostly appealing because of their high content in beneficial nutrients like vitamins, minerals, dietary fibers, also antioxidants, and with increased additional research could become a good source of probiotics as well. (Martins *et al.*,2013*).* In the present years, there is a rising interest in the development of fruit-juice based probiotic products. The fruit juices are also known to contain beneficial nutrients that can be a very ideal medium for probiotics. Fruit juices have delightful taste profiles to all age groups and they are known to being refreshing and healthy. The fruits are full of many nutrients, and do not contain any dairy allergens that might prevent usage by certain segments of the population.Those characteristics allow the selection of appropriate strains of probiotics to manufacture enjoyable healthy fruit juice. The only downfall in this type of product is the sensory impact of probiotic cultures that would have different taste profiles compared to the conventional, non-functional products. The different or varied aroma and flavours have been reported when probiotic strain *L. plantarum* was added to orange juices which consumers do not prefer. But the information regarding health benefits of the juice if provided appropriately to the consumers, the preference increases over the conventional orange juices.Many different attempts have been made to decrease the sensations of unpleasant or displeasing aromas and flavours in probiotic fruit juice. Earlier studies reported that the perceived off flavours caused by probiotics that often contribute to consumer dissatisfaction may be masked by addition of 10% (v/v) of tropical fruit juices, mainly pineapple, and mango, passion fruit. (Peres CM *et al.*,2012*).*

PALM FRUIT

Palm trees are robust trees and they can live upto 100years.They are with good nutritional values and health benefits. They are found as a

huge population in the countries of South Asia. There are different traditional foods made with the palm fruits in different regions. They are used as alcoholic drinks. The palm fruits are said to have high level of carbohydrates and they are rich in vitamin B_1& B_2. They are also considered to be the ideal food that provides high range of nutritional benefits.(Al Shahib *et al.*,2003). They have also been proved to be good anti-inflammatory agents and have wide variety of uses in indigenous medicines.(Paschapur *et al.*,2009). Palm frits also been reported that they can suppress T-cells that can reduce delayed type hypersensitivity(DTH) response to sheep red blood cells(SRBC).

CLARIFICATION

The clarification process was mainly done to clarify the fruit juices and was done depending upon the enzyme concentration. The following Clarification studies were performed using the enzymatic treatment which was employed through the response surface methodology(RSM), this will analyse the effects of enzymatic treatment conditions. Here the fruit juices were treated up with the pectinase enzyme and the following conditions are performed in different incubation time period,enzyme concentration and temperature. From these three variables the effects on clarity,turbidity and colour can be evaluated (Abdullah, L.A.G *et al.*, 2007).There are two important characteristics for the clarified products like clarity and homogeneity for the complete removal of the suspended solids.The pectin was hydrolysed by pectinases to produce pectin-protein complexes to flocculate.Here some of the juices may have low amount of pectin and some may have low amount of viscosity, this will be advantageous for the clarification process. Enzymatic hydrolyses of the pectic substances

will depend upon the various physicochemical factors.From the various reactions and conditions it is found that enzyme concentration plays a most important factor. (Rahman, A *et al.,* 2006).Fruit juices are generally cloudy in nature specially due to the presence of polysaccharides i.e. pectin, during the processing of the clear fruit juices one of the main problem which constitutes is that high concentration of the pectin will leads to the colloid formation thus, the suspended particles can be removed only through the clarification process(Vaillant, F, *et al.*,2001).With the use of the pectinase enzyme, the pectin compound from the fruit can be degraded and thus resulting in the reduction of viscosity and cluster formation, this will separate as a result the juice will be presented with higher clarity as well as with more concentration and will have good flavour and colour (Abdullah L.A.G *et al*., 2007).

Response surface methodology (RSM) is seemed to be a statistical technique which is generally used for the optimization and development of various complex processes. RSM is also capable of providing an investigative approach towards optimization. It is also known as a collection of statistical and mathematical methods which are used in many affecting factors even in the existence of several composite interactions .The conventional methods of optimization or experimental method, use only one variable at a time. When compared to the conventional methods RSM is advantageous as it can use more than one variable at a time. RSM is generally a more economical approach because it is enough to perform small number of experiments in order to monitor the different variables on various responses. In contrast, the conventional method of optimization requires more number of experiments to be performed which leads to an increase in the expenses and time and also utilizes more reagents and materials for the

experiment. Many types of response surface designs are used for optimization such as Box-Behnken, Box-Wilson, CCRD (Complete Composite Rotatable Design).

1.2 MOTIVATION

Addition of probiotics to food productsto enhance health benefits have been investigated for many years. Probiotic drinks were largely based on dairy products due to the various health benefits they impart, but the negative effect of this health drink is that many people are allergic to dairy products. So thus the recent trends of addition of probiotics to fruit juices are carried out as a substitute for dairy based products which confers the same health benefits to lactose intolerant people. In this work palm fruit juice was taken as the probiotic drink, which is a very less explored and studied fruit. This drink is expected to impart cooling effect to the body and also render health benefits with addition of probiotics.

1.3 OBJECTIVES

- Todetermine optimum conditions for clarification of palm juice
- To standardize the concentration of *L.plantarum* for the production of probioticated palm juice
- To standardize the concentration of prebiotic required to enhance the growth of probiotics.
- To standardize the concentration of sweetener.

CHAPTER 2
LITERATURE REVIEW

The type and amount of food we consume in our daily life will determine our health. It is very much necessary to consume the right rations of food nutrients for maintaining a healthy body. A diet consisting of the appropriate amounts of fats, proteins, carbohydrates, vitamins, minerals is called a balanced diet. Food is the natural medicine that will keep us protected from various diseases. Development of such foods that will help us keep good health has remained the top priority for all food industries. (Klaenhammer and Kullen, 1999). Consumer interest in the relationship between diet and health has increased the demand for information about functional foods. Probiotics are live microbial food ingredients that have a beneficial effect on human health or they can be defined as live microbial feed supplements that improves the hosts intestinal microbial balance beneficially (Salminen , S *et al.,* 1999*).*

2.1 HISTORY

The early findings of probiotics dates back to the late 19th century, when microbiologists identified certain micro-flora in the gastrointestinal (GI) tracts of healthy individuals that differed or where not to be found present in diseased individuals, thus the term probiotics came into use to denote such beneficial micro-flora that were found in GI tract. Probiotics, literally meaning 'for life', are micro-organisms proven to render health promoting benefits in animals and humans. (Marteau, P *et al.,* 1995*).* It was in early 1900 that Nobel Prize-winning Elie Metchnikoff hypothesized that the long, disease free healthy lives

of Bulgarian peasants were the ardent results of their constant intake of fermented milk and its products and was later convinced that the organisms required to safeguard and protect the intestine from the ill effects of certain other harmful microorganisms were found to be present in yogurt. The very first clinical trial to be carried out on the effect of probiotics was performed in the 1930's for their effects on constipation. In the 1950's, a certain probiotic product was licensed to be used as a drug for the treatment of scour (*Escherichia coli*) infection among pigs by the United States Department of Agriculture. Over the last century or so, different micro-organisms have been utilized for their ability to ward off and cure diseases, which led to the coining of the common term probiotics. (Lee, Y.-K *et al.*, 1999*)*. Probiotic bacteria used for human nutrition usually belong to lactic acid bacteria or bifidobacteria groups.

2.2 SIGNIFICANCE

Most probiotics usually are known to fall into the class of organisms known as lactic acid-producing bacteria and are normally consumed in the form of yogurt, fermented products like milk, juices etc. Some of the advantageous effect of lactic acid bacteria consumption include: (i) improving the health of intestinal tract, (ii) enhancing the health of immune system, producing and improving the bioavailability of nutrients, (iii) reducing lactose intolerance symptoms, decreasing the occurrences of allergy in susceptible individuals; and (iv) reducing the development of risk of certain cancers. The mechanisms of action of probiotics by which they exert their beneficial effects are still largely unknown, but are expected to involve gut pH modification, antagonization of pathogens by antimicrobial compound production, competing for binding sites of pathogens and receptor sites also for the

availability of nutrients and growth factors, stimulating immunomodulatory cells, and producing lactase (O'Sullivan *et al.*, 1992).

2.2.1 MODE OF ACTION

Though already established that the mechanism of action of probiotics is not very well known, at the molecular level a probiotic can act in a wide variety of ways, such as the following 1) By direct interaction in the gut lumen with the complex ecosystem of the gut micro-biota . Also probiotics have a direct metabolic action in the gut by providing enzymatic activities, 2) by interaction with the epithelium and the gut mucus, providing barrier effects, in digestive processes, mucosal immune system, and enteric nervous system, 3) Through signalling to the host beyond the gut to the other potential organs such as liver, brain, and to the systemic immune system. Growth conditions such as biological and physiological conditions of the probiotics strains will help to modulate their metabolic ability in the human gut through their impact on the interaction with the host (van Baarlen P *et al.*, 2009*)*.

2.2.2 SAFETY

Though probiotics are considered as safe and healthy to use there are certain criteria's that must be accounted for. Probiotics must be proved safe for their required use. According to the FAO/WHO 2002 guidelines, it is recommended that, though bacteria may be Generally Recognized as Safe (GRAS), the safety of the potential probiotic should be characterised by the minimum required tests such as :-

- Determination of antibiotic resistance patterns of the probiotic
- Characterization of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation)

- Studies carried out for assessment of side-effects during human trials.
- Epidemiological assessment of adverse incidents in consumers (post-market studies).
- Toxin tests must be carried out for strain that is known to belong to a species that is a mammalian toxin producer. One required scheme for testing of toxin production has been recommended by the EU Scientific Committee on Animal Nutrition (SCAN, 2000)
- If the strain under consideration belongs to some species with known hemolytic potential, determination of hemolytic activity is required.

2.3 PROBIOTICS IN FRUIT JUICES

Probiotics are often added to certain food products in order to provide additional beneficial effects to the consumer, thus making them "functional foods." The common trend with probiotics has been with their addition to dairy based products, but recently there is a growing interest to incorporate them in fruit juices. This helps render the beneficial effects of the probiotic to lactose intolerant consumers also. (Martins, E.M.F.*et al.*, 2013). Although fruit juices are healthy when consumed, the organoleptic impact due the addition of probiotics would lead to a different taste profile which may be displeased by many. The variety in aroma and flavours has been reported many a times when *L. plantarum* was added to orange juices which consumers do not prefer. However when consumers are made aware of the wide health benefits of the drink, their conventional thinking would find a change. Previous studies reported that the perceptible off flavours caused by probiotics that often contribute to consumer dissatisfaction may be masked by adding 10% (v/v) of tropical fruit juices, mostly pineapple, and also mango or passion fruit (Peres, C.M. *et al.*, 2012*)*. Many studies where carried out with different fruit juices. Pomegranate juice was proved to

be a suitable probiotic drink as results have shown desirable microbial growth and viability for *L. plantarum* and *L. delbruekii*. Nine probiotic lactobacilli strains were evaluated for their ability to survive in a commercial fruit drink for 80 days when stored at 4 °C. The pH 4.2 was shown for the fruit drink, which enabled good stability of many cultures during storage. Also works were carried out with tomato juice for probiotication with four strains *Latobacillus acidophilus*, *Lactobacillus plantarum, Lactobacillus delbrueckii*, and *Lactobacillus casei*. Many effects were studied from them like fermentation times, storage periods, and microbiological, chemical analysis to determine the viable cell counts, acidity and pH. In all such studies pH seems to have decreased, thus increasing the acidity levels. (Kyung *et al.*, 2004).

2.4 FAMILY: Lactic acid bacteria

Lactic acid bacteria was first observed in sour milk by (Scheele N , 1789). Later on in the year 1857 Pasteur discovered that the source for souring of milk was lactic acid bacteria .The micro organisms belonging to the lactic acid bacteria family are said to be mostly Gram-positive. They are used in many fermentations as a starter culture (Zhang, Z. G., 2011). They are widely seen in food products such as milk, wine , cheese , curd etc. The members of this family lactic acid bacteria are capable of producing lactic acid as a by-product of their digestive function of carbohydrates(Nordqvist, 2004).The family lactic acid bacteria consists of the genus like *Lactobacillus , bifidobacterium, aerococcus, lactococcus, oenococcus, leuconostoc , pediococcus* etc. The majority of this family are non-pathogenous. But there are also pathogenic species in this family which mostly belong to the genus *streptococcus* (Stiles M & Holzapfel W, 1997). The most well known genera of lactic acid bacteria family is *lactobacillus*. Apart from

Lactobacillus strain the other important strain being used for probiotics are *bifidobacterium.* The bacteria exhibiting probiotic properties mostly belong to the family of lactic acid bacteria (Collins *et al.,* 1998). Lactic acid bacteria during lactic acid fermentation produce compounds such as organic acids , bacteriocins, diacetyl, hydrogen peroxide or bacterial proteins (Oyetayo, V. O *et al.,* 2003).

2.4.1 GENUS

The microorganisms belonging to the genus lactobacillus are gram-positive, facultative-anaerobic, acid tolerant and they are also known for their fermentative nature (Reiss, T, 2013). They belong to the phylum firmicutes. It is seen that some of the microorganisms belonging to this genus resides in the mucosal surfaces naturally, mostly seen in the walls of the intestinal tract , oral cavity and vaginal tract (Tannock G W , 2004).Among the *Lactobacillus* species some of them are specific and are found only in a limited number of niches, like the species *Lactobacillus delbreuckii* which is mostly adaptive to the dairy environment and are well employed in the production of yoghurt. *Lactobacillus acidophilus* is employed mostly in the production of probiotic products (Siezen, R J, 2010). In contrast, the species *Lactobacillus plantarum* is highly flexible to most of the environments such as dairy products, fish, vegetables, meat etc (Gardener N J, 2001) and they are also seen in the gastro-intestinal tract (Bringel F *et al*., 2005). *Lactobacillus plantarum* is known to be a facultative heterofermentative organism and it is also become a model organism in the *Lactobacillus* research studies (De Bruyne K *et al.,* 2009). In order to impart beneficial effects to the host a microbial viable count of 10^6 - 10^8 colony forming units(CFU/mL) is required generally (Sarkar S, 2013).

2.4.2 HEALTH BENEFITS

It is been proven that *Lactobacillus* has the capability of preventing diarrhea by the effects of the organisms on the immune system. These probiotic microorganisms are also known for preventing infection as they compete with harmful or pathogenic viruses or bacteria for the various binding sites on the epithelial cells (Perdigon G *et al.*, 1995). Some of them produce bacteriocins like nisins which prohibit pathogenic bacteria from growing(Jack R.W. *et al.,* 1995). It has been studied that *Lactobacillus acidophilus* has cholesterol lowering properties. *Lactobacillus caseii* if taken on a daily basis has the capability of reducing bladder tumours and also prevents certain carcinogens (Spanhaak S *et al*., 1998). Many probiotic strains are used in sectors such as pharmaceuticals in the form of tablets or drops in order to treat or prevent various intestinal diseases by exploiting the anti-microbial activity of these organisms (Gao X W *et al*., 2010). They are also known to reduce to reduce inflammatory bowel disease which is a gastro-intestinal disorder (Singh Y *et al*., 2013). It was proved that yoghurt with the right amount of viable probiotic bacteria imparts various health benefits to the humans (Ciorba M A, 2012). In some cases they also help to improve the efficiency of vaccine by their adjuvant effects (Thomas L V, 2012). They are also capable of modifying the gut microbiota by the nutritional and metabolic functions of the probiotic bacteria (Ciorba M A , 2012).

2.4.3 MECHANISM OF ACTION

The bacterial biotransformations enzyme activities (β-glucosidase,β-glucuronidase,nitratereductase) were determined in the content of human faeces over the pH range of 6-8. These three enzymes

activity varies the mode of action in human and rat (Mallett A.K *et al*., 1989). In human large intestine colonizing the bacterial population involved in the metabolism with the huge range of foreign compounds with the formation of subsequent products exert toxicological or pharmacological activity. These metabolic activities of the expression may be modified by certain dietary components (Rowland, 1981). Another potential mechanism is that the gut flora metabolism which was modulated by the diet can shift through in luminal pH. From the microbial fermentation of proteins and carbohydrates catabolism can result with the release of short chain fatty acids or ammonia respectively. Therefore due to this mechanism of action there will changes in the gut environment, directly affect the bacterial enzyme activities, long period of time determine the different types of bacteria and these bacterial enzymes are found in the intestine (MacFarlane *et al*., 1986). The investigation towards the mode of action of vanillin regard to the antimicrobial activity against the *Lactobacillus plantarum.* Vanillin is generally regarded as safe and its mainly used for the flavours and aroma in fruit juices and foods (Walton *et al*., 2003). Moreover, vanillin exhibits antimicrobial activity against yeast,fruit-based agar and in fruit juices (Lopez-Malo *et al*., 1995). The mechanism of action of antimicrobial activity can be classed into the following groups (a) cell membrane reaction, (b) essential enzymes are inactivated, (c)genetic material are destructed (Davidson, 1993). In the fermented milk industry lactobacilli are used which have active β-galactosidase this will decrease lactose concentration in the dairy products which may affect the severity of osmotic diarrhea due to organisms as rotavirus (Mcfarlange *et al*.,1986)

2.4.4 APPLICATIONS

Lactic acid bacteria are used as starter cultures in many food processing industries. The starter cultures which are used maybe only one pure strain or a mixture of a strains (Ross *et al.,* 2005). *L.casei, L.plantarum, L.lactis* etc are used in the products like cheese, fermented milk etc. They are especially used in the case of yoghurt as they have beneficial effects in the gastro-intestinal tract. They are also used in wine industry for the maturation of fruit wines (Liu *et al.,* 2003). They are also used in the contemporary pharmaceutical industry for the production of enzymes. These bacteria produce bacteriocins which are compounds or substances which possess anti-microbial activity. These bacteriocins are widely used in preservation of food like dairy industry, meat products etc. It is also studied that the species *L.rhamnosus* are effective in preventing and treating atopic dermatitis which is a type of eczema. It is also noted that these microorganism are useful for general digestive health (Goldin and Gorbach , 2008).

2.5 CLARIFICATION

Clarification is mainly done to clarify the fruit juices, generally the fruit juices contain cloudy in nature with suspended particles and this can be removed only through clarification process. Mainly the fruit will be pectin-rich juices which contain high concentration of pectin and other carbohydrates, proteins, etc. This will constituents to highly viscous of the juice which will lead to the difficulty in subsequent clarification process. Periodically enzymatic treatment is to be carried out by using pectinase enzyme. Pectinases are to be used in juices for the clarification purpose and done at the constant temperature. The enzymatic treatment mainly depend upon the reaction time, enzyme concentration, temperature of incubation time and pH. From these three

conditions the varying different forms of the parameters are to be calculated such as turbidity, clarity, titratable acidity, reducing sugars , viscosity, total sugars, total solids etc., were studied and employed in second order central composite design. From this it is noted that the enzyme concentration act as the most important factor in the juice and affecting the characteristics of the juice which exert as highly significant on all dependent variables. As there is a increase in the time and concentration of the enzyme there will be increase in the filterability and clarity of the juice and as well decrease in the turbidity and viscosity (Lee, W.C *et al*., 2007). From the study of the two factor central composite design the optimum conditions for the enzymatic treatment of the clarification of the juice were calculated and established with the following different parameters. When the pectinase enzyme were added at the different significant regression models there it forms changes in clarity,turbidity,color and viscosity with respect to the independent variables were established with coefficient determination R^2 which is allowed to be greater than 0.70. Thus from the results it is recommended that the enzyme concentration in the juice plays a major role in following conditions (Abdullah *et al*., 2007).

2.6 PREBIOTIC

The common delivary route for probiotics to the human body is through foods. They act as carriers for transport of probiotics through the gastrointestinal tract, and also the bioactive components in them interact with the probiotic to change their functionality and efficacy. The longevity or survival of the probiotic during their passage via the gastric depends on the physio-chemical properties of the food carriers. The food ingredients interacts with the micro-organism and alters their adherence to gastrointestinal epithelium and also their acid production thus

making them differ from their original characteristics, whereas prebiotics are known to encourage the growth of probiotics. Their appropriate combinations could render very beneficial effects. (Ranadheera *et al*., 2010). Examples of such naturally present prebiotics are inulin and oligofructose that are dietary fibres present in the chicory root. They are also naturally present in edible fruits and vegetables. (Van Loo *et al.*, 1995). They bypass metabolism from the small intestine and help in beneficial alteration of the human colonic micro-flora to increase the composition of favourable bifidobacteria and lactic acid bacteria. Their various benefits apart from providing fibre effects of improved bowel movements, they also contribute to improved mineral absorption, increased host defences and intestinal protection.(Alexiou ,H and Franck, A, 2008) .

2.7 SYNBIOTICS

The nutritional supplements combining both probiotics and prebiotics have risen to the concept of synbiotics. The main reason for using synbiotic is that due to the true probiotic because without the use of the prebiotic food in the digestive system it does not survive. Without the food source necessary for the probiotic, this will have a greater intolerance for temperature, oxygen and low pH. Many benefits of probiotics and prebiotics have studied into the synergy,where for the betterment of our health the number of good bacteria have increased in the digestive system. Generally the synbiotic contains one to ten billion active cells and it works for in two ways 1) the viability of the probiotics were been improved and 2) the specific health benefits were delivering (Sekhon, B.S *et al*., 2010). The synbiotics products provide the potential to develop prebiotics at specific sites for the probiotic strains to figure out the health benefits. In the last few years, the main studies of

the synbiotics is being against the applications of disease (Kolida S *et al.*, 2011). The gut metabolic reaction were modulated when there is an intake of the synbiotic food and also it leads to the maintenance of gut biostructure. Later this will enhance the health benefits of the synbiotic food where there is a significant increase in the ketones,short chain fatty acids etc (Vitali B *et al.*, 2010). Probiotics is generally used as the beneficial microorganisms that are microbial food supplements which will improve the intestinal balance and as well as there will be a change in the composition of colonie microbiota.In contrast, intake of prebiotics in turn are nondigestible food ingredients which beneficially provide by stimulating the growth or activity of some bacterial species which is already existing in the colon of the host and thus it will improve the host health. While intake of the prebiotics there will be specific changes in increasing the number of bacteria and also there is a modulation in the colonie microbiota and thus it changes the composition of microbiota (Gibson *et al.*, 1994).

2.8 SWEETENER

Stevia is the sweetener used in the probioticated palm juice. It is a natural sweetener and are 30-40 times sweeter than sugar. Sweetness in Stevia is due to the presence of glycosides known as stevioside and rebaudioside. Stevia can be suitable for diabetic and phenyl ketonuria patients. (Genus, J.M.C., 2000). They are also considered to be vadilator agent in normo and hypersensitive animals. (Melis, M.S, 1996).

CHAPTER 3
MATERIALS AND METHODS

3.1 Palm fruit

Ripened palm fruit *(Borassus flabellifer*) with 80-90% maturity were purchased from local vendors of perur region of Coimbatore.

3.2 Enzyme

Commercial enzyme (pectinase) from the source organism *Aspergillus niger*, was obtained from HIMEDIA with activity of 8000-12000 U/g proteins was used.

3.3Palm juice preparation

The raw palm fruit procured was washed and its outer cover was removed. The inner fleshy part was macerated manually using a morter and pestle to get a smooth water like liquid which was then pasteurized (fruit : water ratio, 1:1) at 80°C for 3 minutes (Sarkar , B C *et al*.,2009).

3.4 Experimental Design

The design adopted was Response surface methodology (RSM) to study experimental combinations. The software employed for this purpose was Design-Expert version 9 Stat-Ease Inc 2015(Trial version). The main advantage of RSM, is that the number of experimental runs required to provide sufficient information will be greatly reduced. Under RSM, a Central Composite Rotatable Design (CCRD) was employed to study the combained effect of independent variables used.

In the development of the probiotic palm juice 4 independent variables namely Clarification of palm juice, addition of probiotics

(2%,3%,4%), addition of prebiotics (1%,2%,3%) and sweetener (1%,2%,3%) were studied in detail and the concentration of each was optimized using individual trials. These variables, parameters and their levels were chosen based on the limited literature available (Chauhan A.S *et al.*,2004). For the different factors evaluated the responses studied were pH, absorbance, titratable acidity, ascorbic acid, TSS, total sugars, reducing sugar, total solids, yield, turbidity. ANOVA analysis and F-test were used as significant criteria for the fitted models.RSM graphs generated was used for reference of data. The optimized condition for each variable was derived from desirability ramp generated.

3.5 Physiochemical and microbiological studies

3.5.1 Determination of pH

pH of the samples were determined using pH meter.

3.5.2 Determination of Turbidity

The turbidity was measured as formazinnephlometric units (FNU) using a Nephelometry.

3.5.3 Determination of clarity

Test for clarity was measured using Visible spectrophotometer at 660 nm.

3.5.4 Determination of Reducing sugars by Dinitro Salicylic acid (DNS)method

Tests the presence of free carbonyl group (C=O),the so called reducing sugars. Oxidation of aldehyde functional group will take place and also simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced under alkaline conditions to 3-amino,5-nitrosalicylic acid.

Reagents used:

1.Stock standard Glucose solution: 100 mg of Dglucose was dissolved in distilled water and made up to 100 ml.

2.Working Standard Glucose solution (100 µg/ml):10 ml of the stock standard was made up to 100 ml with distilled water.

3. Potassium sodium tartaratesolution , 40%

4. DNS reagent: 1 g of 3,5-DNS reagent, 50mg sodium sulphite, 200 mg crystalline phenol all dissolved in 1% NaOH solution.

Procedure

1.0.2, 0.4, 0.6, 0.8, 1 ml of working standard solutions were taken in different test tubes, and were made upto 1 ml with distilled water.

2. 1 ml of water was taken as blank.

3. 3 ml of DNS reagent was added to all the test tubes and were kept in boiling water bath for 5 minutes.

4. 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color.

5. The absorbance value was taken at 540nm. (Miller, G.L, 1959).

6.The concentration of reducing sugars in the sample was obtained by standard graphs and calculations were carried out as follows:

Amount of reducing sugar (mg/100mL) = [(Concentration of working standard (µg)) / (Amount of the unknown sample taken in mL)] × 1000]

3.5.5 Determination of Titratable acidity

Materials

1. Conical flask

2. Burette

3.Micropipette

4.Phenolphthalein indicator

5.0.1 N NaOH

Procedure

1.10 ml of juice was added in a conical flask and diluted with 40ml of distilled water.

2.Two drops of phenolphthalein were added to the mixture.

3.Then this mixture was titrated against 0.1N NaOH in burette.

4.The appearance of pale pink colour indicated the end point.

5.Volume of 0.1 N NaOH required for the appearance of colour was noted as V1.The total titratable acidity was calculated as percent of citric acid.1ml of 0.1N NAOH represents 0.0070g citric acid.

$$\text{TTA in \% is} = \frac{V \times 0.0070g \times 100 \times N}{1000 \times V_s}$$

V- Mole of 0.1N NAOH used

N- Normality

V_s -Volume of juice sample used.

3.5.6 Determination of Total soluble solids(TSS) in probiotic juice

Materials

1.Hand refractometer-(0-32◦brix)-ERMA with ATC-built in Automatic Temperature Compensation system

2.Distilled water

3.Soft cotton

Procedure

1.First a droplet of distilled water was placed on the front end of the refractometer to wipe it clearly , which was then adjusted to the direction of a bright light source.

2.To the daylight plate one or two drops of distilled water was added and closed and calibration screw was adjusted to make the white and blue line to coincide with the null line.

3.The daylight plate was opened and cleaned with soft cotton cloth.Then one to two drops of sample was placed on the prism surface.

4.The sample was allowed to remain on the prism foe 30 seconds and the corresponding reading was taken in ◦Brix.

3.5.7 Determination of viability

Materials

1.Sterilised petri plates

2.Sterilised test tubes

3.Sterilised conical flasks

4.Sterilised MRS (De Man,Rogosa and Sharpe) broth and agar

5.Ethanol(70%)

6.Laminar air flow chamber

7.Micropipette with sterilised tips

8.Cooling centrifuge

9.Sterilised centrifuged tubes

10.24 hour log phase bacterial culture Lactobacillus plantarum

11.L rod and rotating table

12.Incubator

13 .Paraffin film

Procedure:

1.24 hour log phase culture of Lactobacillus plantarum at concentrations 2%,3%,4% was prepared using MRS broth and centrifuged at 5300 rpm for 15 minutes.

2.The pellets after the centrifugation of the cultures were taken and inoculated into the respective clarified palm juice samples.

3.The samples were incubated at 37◦C in the rotating shaker and subjected to different time intervals.

4.The samples after 24^{th} hr ,48^{th} hr and 72^{nd} hr incubation were collected and subjected to serial dilution upto 10^9 CFU /mL (Mousavi Z.E. *et al*.,2011)

5.0.1 ml of the serially diluted samples [10^5 to 10^8(CFU/mL)] was innoculated into the plates using spread plate technique(Pereira *et al*.,2010) and the petri plates were sealed by paraffin.

6.The plates were incubated at 37◦C for 24 h and the contable colonies (30 -300) were counted .

7.Viability of the probiotic culture was determined in (CFU/mL) by using the following formula.

(Number of colonies)/(Dilution factor × Amount of sample)

3.5.8 Determination of Total Sugars by Phenol Sulphuric Acid method

Materials

1. Phenol(5%): 50 gms of reagent grade phenol is diluted with 1L of distilled water.
2. Concentrated Sulphuric acid(96%)
3. Boiling tubes

Procedure

1. 0.5ml of samples were taken in boiling tubes and were made upto 1 ml with distilled water.

2. 1 ml of phenol and 5ml of sulphuric acid(96%) were added to the sample.

3. This was incubated at room temperature for 20 mins and the green color developed was measured at 490nm using calorimeter.

4. From the standard graph total amount of carbohydrates present in the sample were determined (Michel *et al*.,1956).

3.5.9 Determination of Ascorbic acid

Juice sample was determined for ascorbic acid by redox titration using iodine solution

Materials

1.Burette

2.Iodine solution

3.Distilled water

4.Starch

Titration

1. Pipette a 20 mL aliquot of the sample solution into a 250 mL conical flask and add about 150 mL of distilled water and 1 mL of starch indicator solution.

2. Titrate the sample with 0.005 mol L^{-1}iodine solution. The endpoint of the titration is identified as the first permanent trace of a dark blue-black colour due to the starch-iodine complex.

3. Repeat the titration with further aliquots of sample solution until you obtain concordant results (titresagreeing within 0.1 mL).Calculations

1. Calculate the average volume of iodine solution used from your concordant titres.

2. Calculate the moles of iodine reacting.

3. Using the equation of the titration (below) determine the number of moles of ascorbic acid reacting.

ascorbic acid + $I_2 \rightarrow 2\ I^-$ + dehydroascorbic acid

4.Calculate the concentration in mol L^{-1} of ascorbic acid in thesolution obtained from fruit juice. Also, calculate the concentration, in mg/100mL or mg/100g of ascorbic acid, in the sample of fruit juice.

3.5.10 Total solids

Total solids were determined by the method used by (Osborne and Vougt, 1979).An empty petri dish was washed, oven dried for 15 min and cooled in a desicator for 20 min.The dried and cooled petri-dish was weighed empty and then reweighed after 10mL of juice was put into it through a pipette.The petri dish with its contents was oven dried at 100^0C for 6h, cooled in the desicator and weighed.The procedure was repeated until a constant weight was obtained.The total solids, S_T in %, were calculated as follows:

$$S_T = \frac{100W_2}{W_1}$$

3.5.11 Yield (%)

The juice yield was estimated as a percentage of weight of the juice obtained to the initial puree. The formula is:

Yield= Weigh of juice – Weight of added water/(Weight of palm)×100%

3.5.12 Clarity (%)

Clarity was determined by measuring the absorbance at 660nm using a spectrophotometer. Distilled water was used as a reference

CHAPTER 4
RESULTS AND DISCUSSIONS

The palm juice was clarified using pectinase enzyme after standarisation of various factors . The independent variables involved in the development of probioticated palm juice include sweetener.

Probioticated palm juice was prepared by standardizing the variables using RSM, CCRD method. The software used is design expert trial 9. Palm fruits were clarified using the enzyme pectinase and they included 30 trials for conditions temperature 30°C-60°C, incubation time 30-120 minutes, pH 3-6 and enzyme concentration 0.3-0.8U.(Chauhan, A.S, *et al.*,2004) Various responses were studied and from that the optimum conditions were obtained from the desirability ramp generated. They were temperature 30°C, time 120 minutes, enzyme concentration 0.8U and pH 3. From the obtained optimum condition, the clarified juice was taken for the addition of probiotic, species *Lactobacillus plantarum* of concentration 2% - 4% and incubation time 24-72 hours were taken. Accordingly 13 trials were conducted and their responses were studied to select a suitable desirability ramp. The standarized result obtained was innoculum concentration 4% and time 48 hours. Further the probioticated juice was incorporated with prebiotics and sweetener under the same procedure, 13 trails for the concentrations 1% - 3% and time 24-72 h was carried out and the optimum condition was 3% , prebiotic 3% and time was 48 hours and sweetener concentration was 2% and 48 h. Thus the standardised probioticated palm fruit juice was prepared.

4.1 Clarification of palm fruit juice under different conditions

TABLE 4.1 : Design table CCRD for Clarification of palm juice

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Response 7	Response 8	Response 9
Std	Run	A: temp	B:ph	C: time	D: conc	turbidity	TSS	yield	ascorbic acid	Red sugar	total sugar	clarity	titratable acidity	total solids
15	1	30	5	120	0.8	43.9	4.2	83	47.1	145.1	554.1	0.43	0.216	3.32
18	2	60	4	90	0.55	44	4.2	78	55.09	145	554.1	0.4	0.234	3.33
16	3	50	5	120	0.8	42.1	4.1	80	53.55	144	553	0.42	0.234	3.54
23	4	40	4	90	0.05	44.9	4	82	47.3	147	555	0.42	0.288	3.23
29	5	40	4	90	0.55	44.6	4.2	79	56.09	147	553.09	0.4	0.216	3.33
24	6	40	4	90	1.05	43.9	4	75	52.93	147	554.89	0.4	0.2	3.23
17	7	20	4	90	0.55	44.2	4	77	41	146.7	555.6	0.41	0.216	3.24
6	8	50	3	120	0.3	42.1	4.1	80	47.2	144	553	0.42	0.252	3.54
25	9	40	4	90	0.55	44.9	4	82	55.99	147	555.09	0.41	0.216	3.23
20	10	40	6	90	0.55	43.8	4.3	79	47.3	147	555	0.42	0.2	3.37
9	11	30	3	60	0.8	43.9	4.1	81	45.6	145.9	554.98	0.44	0.216	3.36
2	12	50	3	60	0.3	42.9	4	83	52.39	145	554	0.4	0.288	3.23
5	13	30	3	120	0.3	42.1	4.2	74	44.6	144	553.09	0.43	0.27	3.5

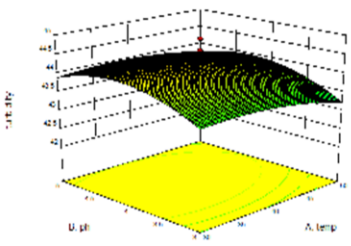
27	14	40	4	90	0.55	44	4	77	56	147	555	0.41	0.216	3.23
4	15	50	5	60	0.3	43.7	4.3	75	46.3	145.48	554.37	0.42	0.288	3.5
30	16	40	4	90	0.55	44	4	77	56	147	555	0.41	0.216	3.23
14	17	50	3	120	0.8	43.4	4	85	53.55	145.9	554.98	0.43	0.234	3.23
26	18	40	4	90	0.55	44	4	77	56	147	555	0.41	0.216	3.23
28	19	40	4	90	0.55	44	4	77	56	147	555	0.41	0.216	3.23
21	20	40	4	30	0.55	44.2	4.5	80	43.2	147	555	0.4	0.216	3.24
1	21	30	3	60	0.3	43.9	4.1	78	41.7	144.09	553.09	0.42	0.288	3.54
22	22	40	4	150	0.55	44.6	4	74	57.91	147.9	554.98	0.41	0.216	3.33
12	23	50	5	60	0.8	42.9	4.5	79	46.49	144.9	553.98	0.4	0.27	3.55
13	24	30	3	120	0.8	43.1	4.3	80	36	145.67	554.56	0.43	0.252	3.23
8	25	50	5	120	0.3	43.7	4	80	46.3	146.34	555.23	0.42	0.234	3.23
19	26	40	2	90	0.55	42.1	4.2	78	44.5	143.1	552.09	0.41	0.252	3.54
3	27	30	5	60	0.3	43.1	4.3	79	41	143.9	552.98	0.44	0.27	3.5
10	28	50	3	60	0.8	42.9	4.2	83	52.3	145	554	0.4	0.2	3.23
7	29	30	5	120	0.3	43.1	4.2	80	36	145.67	554.56	0.43	0.234	3.23
11	30	30	5	60	0.8	43.2	4.5	82	47.2	145.49	554.38	0.4	0.27	3.23

TABLE 4.2 -ANOVA table for TSS- clarification

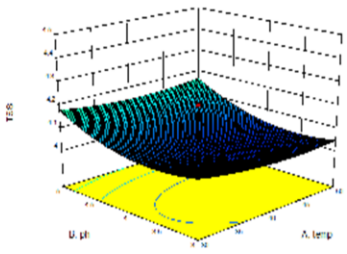
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	0.60	14	0.043	4.01	0.0057	significant
A-temp	3.750E-003	1	3.750E-003	0.35	0.5611	
B-ph	0.070	1	0.070	6.64	0.0211	
C-time	0.15	1	0.15	14.18	0.0019	
D-conc	0.020	1	0.020	1.92	0.1857	
AB	6.250E-004	1	6.250E-004	0.059	0.8115	
AC	0.031	1	0.031	2.89	0.1100	
AD	6.250E-004	1	6.250E-004	0.059	0.8115	
BC	0.11	1	0.11	9.95	0.0065	
BD	5.625E-003	1	5.625E-003	0.53	0.4778	
CD	0.016	1	0.016	1.47	0.2437	
A^2	0.016	1	0.016	1.48	0.2420	
B^2	0.10	1	0.10	9.76	0.0070	
C^2	0.10	1	0.10	9.76	0.0070	
D^2	2.976E-005	1	2.976E-005	2.805E-003	0.9585	
Residual	0.16	15	0.011			
Lack of Fit	0.13	10	0.013	1.89	0.2505	not significant
Pure Error	0.033	5	6.667E-003			
Cor Total	0.76	29				

From the above Table 4.2 , the F-value of 4.01 implies that the model is significant. there is only a 0.57% chance that F-value this large could occur due to noise. There is a25.05% chance that a" Lack of fit F-value" this large could occur due to noise.

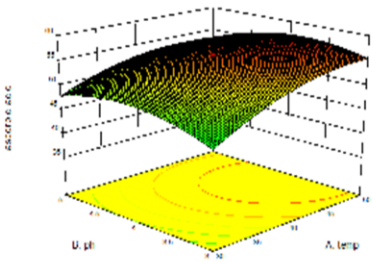
The below Fig 4.1 shows the optimum conditions for development of clarified palm juice by testing the various factors. As clarification is carried out to reduce viscosity and increase clarity of juice without significantly affecting the sugars and essential solids present in the juice. Each graph represents the effect of temperature, pH on the responses studies. After analysis of each graph , the condition that favoured increase in clarity, titratable acidity , with no much change in original charecteristics of the juice was selected. In previous studies conducted for clarification of kabab date fruit , it was proved that that clarity of the sampe after clarification is highly influenced by the pH , rather than any other factors.(Bahramian, S. *et al.*,2011)



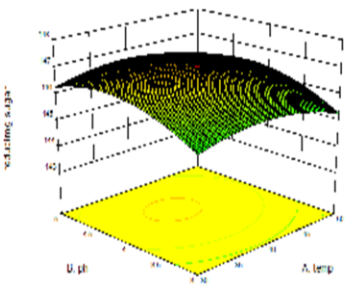
Effect of pH and Temperature on Turbidity



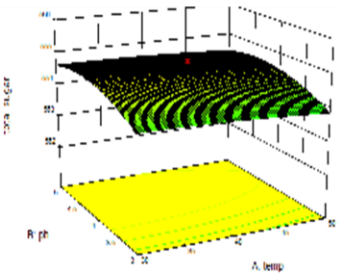
Effect of Temperature and pH on TSS



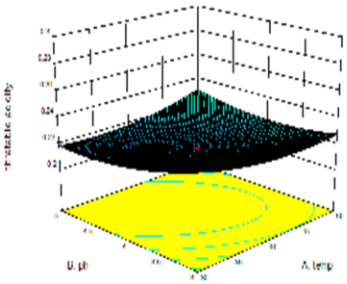
Effect of pH and Temperature on Ascorbic acid



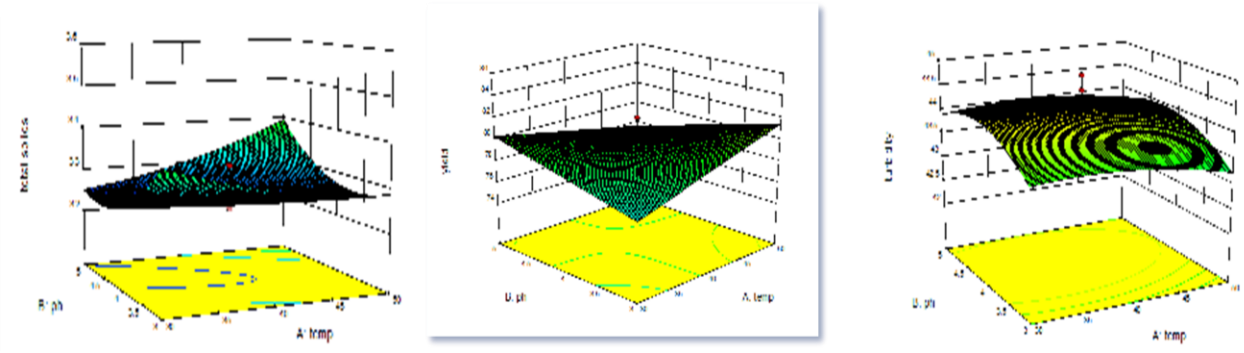
Effect of Temperature and pH on Reducing sugar



Effect of pH and Temperature on Total sugar



Effect of pH and Temperature on Titratable acidity



Effect of Temperature and pH on Total solids

Effect of Temperature and pH on Yield (%)

Effect of Temperature and pH on Clarity

Fig 4.1: Response surface plots of physiochemical properties of palm juice after clarification.

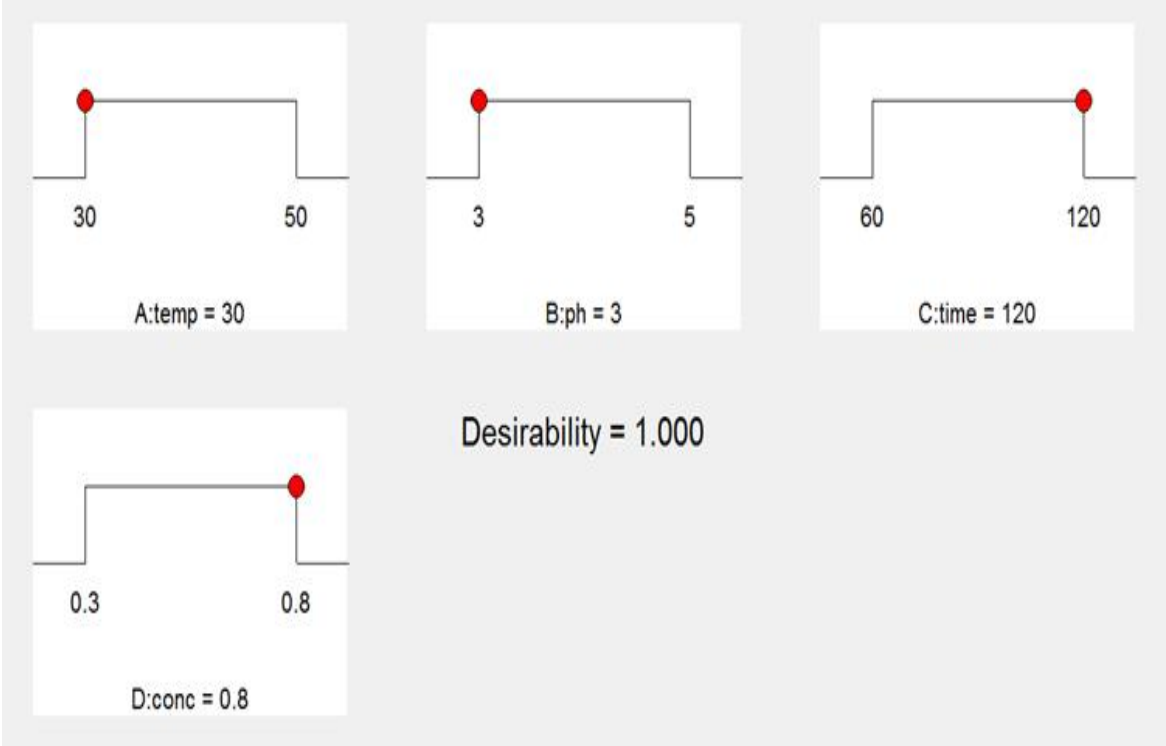


Fig 4.2 : Desirability ramp for the factors tested

The analysis predicted that the optimum conditions were 0.8 U enzyme concentration, 30°C temperature, pH 3 and 120 min incubation time.

4.2 Addition of probiotics

The probiotic *L.plantarum* was added to the clarified palm juice. To optimize the concentration of probiotics added the responses considered were- pH, absorbance, titratable acidity, total sugar, TSS, ascorbic acid, reducing sugar, microbial viability.

TABLE 4.3: Design table for addition of probiotics to palm juice

		Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Response 7	Response 8
Std	Run	A:Inn conn	B:time	pH	OD @660	acidity	ascorbic acid	TSS	microbial viability	total sugar	reducing sugar
11	1	3	48	2.83	1.71	0.162	0.00185	1.45	2.4	0.6	1.2
13	2	3	48	2.83	1.71	0.162	0.00185	1.45	2.4	0.6	1.2
8	3	3	72	3.08	1.57	0.163	0.0021	1.35	3.5	0.7	1
1	4	2	24	2.93	1.55	0.162	0.0021	1.3	3.8	0.72	1.1
6	5	4	48	2.97	1.75	0.162	0.00205	1.3	2.4	0.7	1
2	6	4	24	2.9	1.37	0.265	0.00205	1.3	3.5	0.72	1
5	7	2	48	3.02	1.47	0.265	0.00205	1.3	3.5	0.6	1.24
4	8	4	72	3.2	1.57	0.263	0.0021	1.3	3.5	0.7	1
1	9	3	48	2.83	1.71	0.162	0.001	1.45	2.4	0.6	1.2

2							85				
10	10	3	48	2.83	1.71	0.162	0.00185	1.45	2.4	0.6	1.2
7	11	3	24	2.93	1.31	0.163	0.0021	1.45	3.5	0.72	1.2
3	12	2	72	2.88	1.54	0.162	0.0021	1.3	3.8	0.7	1
9	13	3	48	2.83	1.71	0.162	0.00185	1.45	2.4	0.6	1.2

The above table 4.3 depicts the design table for addition of probiotics *Lactobacillus plantarum* to the clarified palm juice .The two factors considered in the trial were inoculum concentration and incubation time and following eight responses were studied. The eight responses were pH, absorbance, titratable acidity, ascorbic acid content, microbial viability, TSS , total sugar, reducing sugar.

TABLE 4.4 -ANOVA Table for pH

ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	2.70	5	0.54	350.62	< 0.0001	significant
A-Inn con	0.029	1	0.029	19.06	0.0033	
B-time	0.000	1	0.000	0.000	1.0000	
AB	0.000	1	0.000	0.000	1.0000	
A^2	0.000	1	0.000	0.000	< 0.0001	
B^2	2.29	1	2.29	1482.40	< 0.0001	
Residual	0.011	7	1.543E-003		< 0.0001	
Lack of Fit	0.011	3	3.600E-003	1.11	0.5069	not significant
Pure Error	0.000	4	0.000			
Cor Total	2.72	12				

In the table 4.4, the F-value of 350.62 implies that the model is significant. There is only 0.01% chance that F-value could occur due to noise. There is 50.69% chance that a" Lack of fit F-value" this large could occur due to noise. Such lack of fit values aregood for the model.

The fig 4.3 represents various responses generated from the trials conducted. pH of the sample seems to have decreased with time causing an increase in acidity value of the sample. This is due to increase in fermentation of the sample by the probiotics innoculated which has grown in number as the absorbance values show an increase. The total sugar and the solids present in the sample is increasingly used up by the growing organisms. With increase in time, organisms also shows an increase in viability. *L.plantarum* grew rapidly in palm juice and reached about 2.4×10^{11} CFU/mL after 48 h of fermentation and the viability remained stable even after that. Previous studied shows that tomato juice fermented for 48hrs with *L.plantarum* reached viable population greater than 1.0×10^{8} CFU/mL and shows stable and not much significant difference after 48h (Yoon *et al.*,2004) and the same with cabbage juice at similar conditions shows cell counts of nearly 10×10^{8}CFU/mL (Yoon *et al.*,2006).

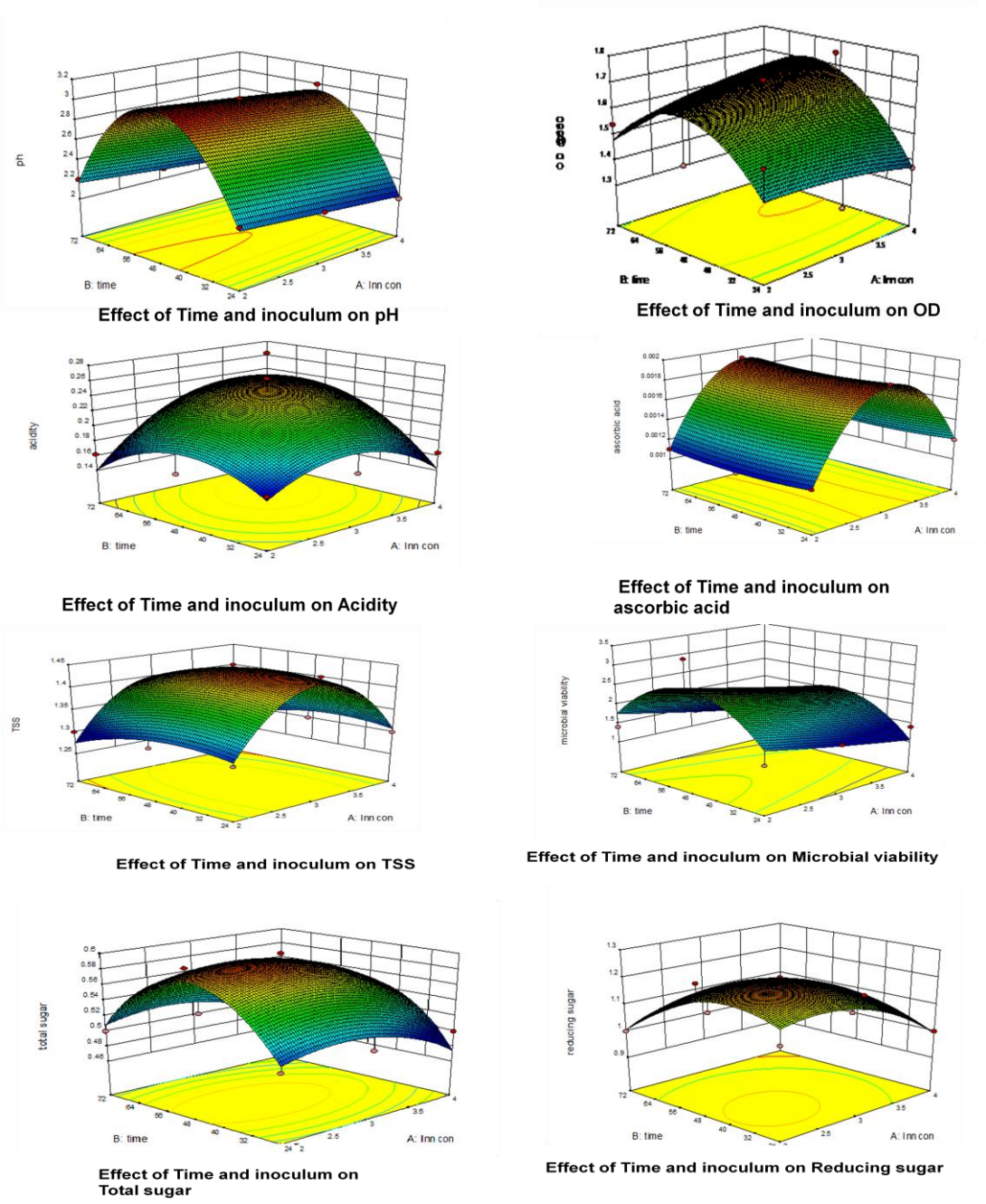


Fig 4.3: Response surface plots for factors studied and responses yielded after addition of probiotics

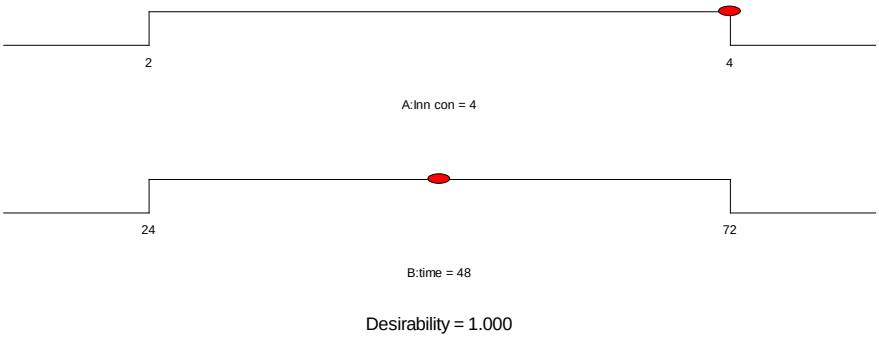


Fig 4.4: Desiribility ramp for the probiotic factors

Thus for suitable growth and optimisation with the right pH and acidity, an innoculum concentration of 4% and at time 48 h is selected from the numerical solutions generated from the design software.

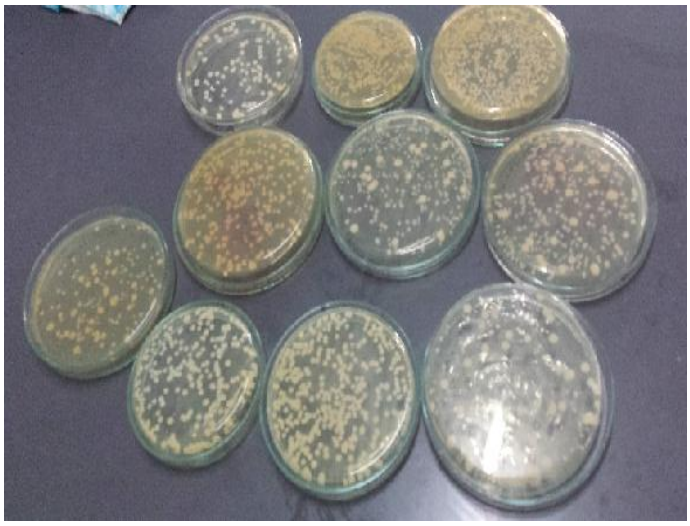


Fig.4.5 Microbial viability test for different dilutions with samples

Fig.4.6 . 48th hour L.plantarum 4%

4.3 Addition of prebiotics

The probioticated palm juice is then optimized with prebiotic inulin considering the factors- innoculum concentration 1-3% and time 24-72 h. The various respones yeilded from this trials were studied and reported in the design table

TABLE 4.5 : Design table for addition of prebiotics to probioticated palm juice

		Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Response 7	Response 8
Std	Run	A:prebiotic conc	B:time	pH	OD @660	Vit C	acidity	TSS	Micro-viability	total sugar	reducing sugar
12	1	2	48	2.92	1.92	0.00219	0.108	4	8	0.243	0.563

4	2	3	72	2.4	1.7	0.001	0.06	1	2	0.15	0.48
5	3	1	48	3.2	1.96	0.0018	0.162	4	8	0.236	0.516
1	4	1	24	2.4	1.7	0.001	0.05	4	2	0.15	0.52
6	5	3	48	2.35	1.7	0.001	0.05	1	2	0.15	0.48
10	6	2	48	2.92	1.92	0.0021	0.108	4	8	0.243	0.563
11	7	2	48	2.92	1.92	0.0021	0.108	4	8	0.243	0.563
3	8	1	72	2.4	1.7	0.0014	0.06	1.6	2	0.15	0.52
8	9	2	72	2.4	1.7	0.0014	0.06	2	2	0.15	0.47
7	10	2	24	2.4	1.7	0.0020	0.05	4.8	8	0.15	0.513
9	11	2	48	2.92	1.92	0.0021	0.108	4	8	0.243	0.563
13	12	2	48	2.92	1.92	0.0021	0.108	4	8	0.243	0.563
2	13	3	24	2.4	1.7	0.001	0.05	1	2	0.15	0.48

TABLE 4.6 . ANOVA table for Ascorbic acid

ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	3.087E-006	5	6.175E-007	10.51	< 0.0001	significant
A-prebiotic conc	2.563E-007	1	2.563E-007	4.36	0.0752	
B-time	1.402E-008	1	1.402E-008	0.24	0.0642	
AB	4.000E-008	1	4.000E-008	0.68	0.0001	
A^2	1.391E-006	1	1.391E-006	23.67	0.0018	
B^2	4.087E-007	1	4.087E-007	6.95	0.0336	
Residual	4.113E-007	7	5.876E-008			
Lack of Fit	4.113E-007	3	1.371E-007	2.54	0.1246	not significant
Pure Error	0.000	4	0.000			
Cor Total	3.499E-006	12				

For the table 4.6 the F-value of 10.51 indicates that the model is significant. There is only a 0.01% chance that F-value could occur due to noise. There is a 12.46 % chance that a" Lack of fit F-value" this large could occur due to noise

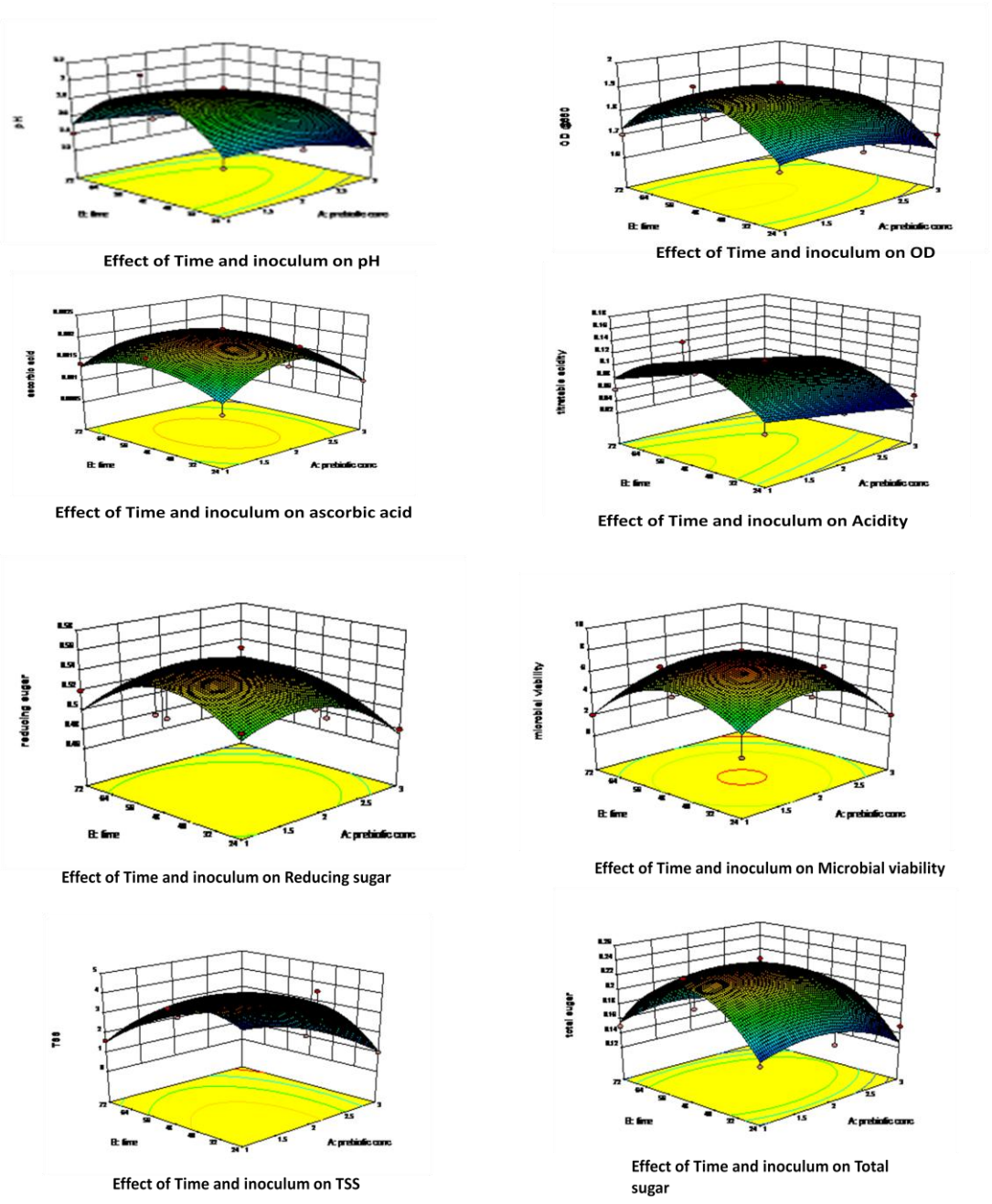


Fig 4.7: Response surface plots for factors studied and responses yielded after the addition of prebiotics

The Fig 4.7 represents graphs generated after conducting trials with the above mentioned factors- the addition of prebiotic inulin has not affected the pH of the sample significantly . The sample still portrays the same decline in pH with increase in acidity due to lactic acid produced by the growing microorganism. In a similar study conducted on carrot juice with *L.rhamnosus,* the addition of prebiotic displayed very less or no evident changes in the juice after 48hours of incubation at 37 ◦C .The viable count of cells containing prebiotic and probiotic were reported similar to the values attained in probiotic samples.(Nazzaro, F. *et al.*,2008). The ascorbic acid content also shows similar characteristics as shown with just probiotic addition**.** The probiotic also has shown a much increase in growth due to enhanced utilization of sugars and solids present in the sample. Due to increased consumption of carbohydrates in the palm juice, there is a significant decrease in total and reducing sugars and solids present in the sample, which indicates a good growth of the probiotic. The prebiotic has enhanced the growth of *L.plantarum,* as the microbial viability shows an increase in colonies. The time for optimum charecteristics is 48h, but even increase in time shows no significant variation.

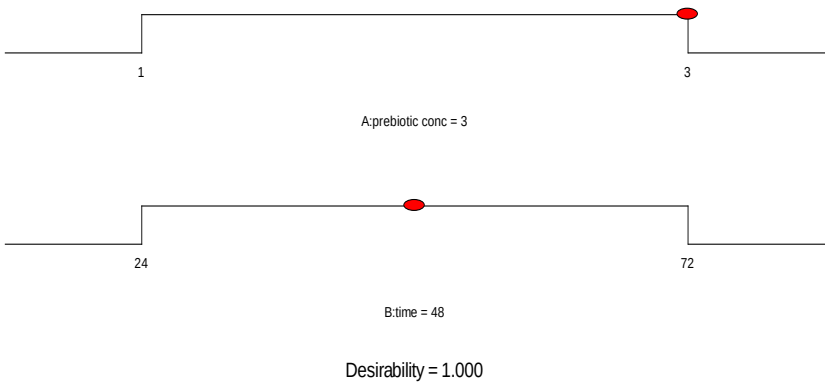
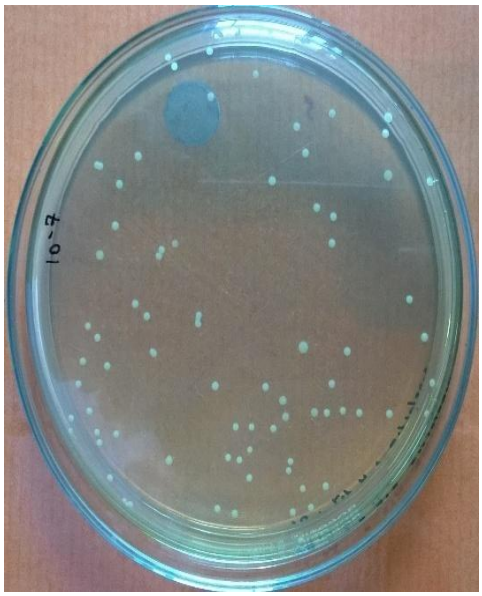
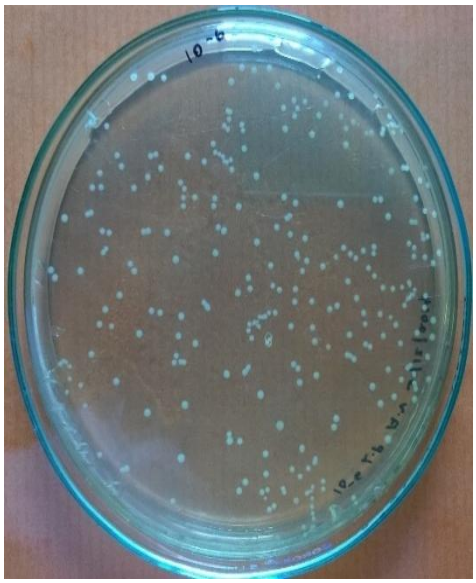


Fig 4.8: Desiribility ramp for the prebiotic factors

For optimum addition of prebiotic that contributes the growth of probiotic, without causing much changes in the palm juice charecteristics was chosen. The numerical solution derived was prebiotic concentration 3% and time 48 h.



10^6 CFU/mL



10^7 CFU/mL

Fig. 4.9: Microbial viability of *L.plantarum* after addition of prebiotics

4.4 Addition of sweeteners

The probioticated palm juice with the added prebiotic is then supplemented with sweetener to enhance the properties of fruit juice. To standardize the concentration of the sweetener added, it is subjected to trials with factors like sweetener concentration 1-3% and time 24-72 hrs. The various respones yeilded from this trials were studied and reported in the design table.

TABLE 4.7 : Design table for addition of sweeteners to the palm juice

		Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6	Response 7	Response 8
Std	Run	A:sweeteener con	B:time	ph	TSS	titratabile acidity	ascorbic acid	clarity	reducing sugar	total sugars	microbial viability
10	1	2	48	3.37	1.2	0.48	0.0025	1.86	6	0.68	8
9	2	2	48	3.37	1.2	0.48	0.0025	1.86	6	0.68	8
11	3	2	48	3.37	1.2	0.48	0.0025	1.86	6	0.68	8
8	4	2	72	3.85	0.8	0.3	0.0031	1.9	4.67	0.4	6
5	5	1	48	3.73	1	0.27	0.0027	1.92	5	0.336	8
3	6	1	72	3.28	0.8	0.3	0.0027	1.66	5	0.4	6
2	7	3	24	3.7	0.8	0.3	0.00192	0.83	5	0.4	6
12	8	2	48	3.37	1.2	0.48	0.0025	1.86	6	0.68	8
4	9	3	72	3.51	0.8	0.3	0.0023	1.7	5	0.4	6
1	10	1	24	2.82	0.8	0.3	0.00184	0.59	5	0.4	6
13	11	2	48	3.37	1.2	0.48	0.0025	1.86	6	0.68	8
7	12	2	24	3.55	0.8	0.3	0.00177	1.27	5.94	0.4	6
6	13	3	48	3.72	0.8	0.3	0.0027	1.92	5	0.4	8

TABLE 4.8 : ANOVA table for TSS

ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	0.39	5	0.078	30515.80	0.0107	significant
A-sweetener con	6.667E-003	1	6.667E-003	4066.94	0.4573	
B-time	5.551E-017	1	5.551E-017	5.20	1.0000	
AB	5.551E-017	1	5.551E-017	6700.52	1.0000	
A^2	0.067	1	0.067	679524.47	0.0419	
B^2	0.18	1	0.18	16.70	0.0047	
Residual	0.075	7	0.011			
Lack of Fit	0.075	3	0.025	2.33	0.3141	not significant
Pure Error	0.000	4	1.000E-006			
Cor Total	0.47	12				

The F-value of 30515.80 implies that the model is significant. There is only a 0.01% chance that F-value this large could occur due to noise. There is a 31.41% chance that a" Lack of fit F-value" this large could occur due to noise.

The Fig 4.10 represents the responses generated by conducting the trials. After the addition of sweetener, No significant changes was brought to the juice developed. The probiotics and prebiotics added are not disturbed and the viability still shows similar results as before with addition of just probiotics. The increase in acidity and decrease in pH is due to fermentation of palm juice resulting in production of lactic acid. The consumption of total and reducing sugars in the sample implies a

good growth of probiotic. The sweetener added has contributed to the improvement in organolyptic properties of the fruit juice.

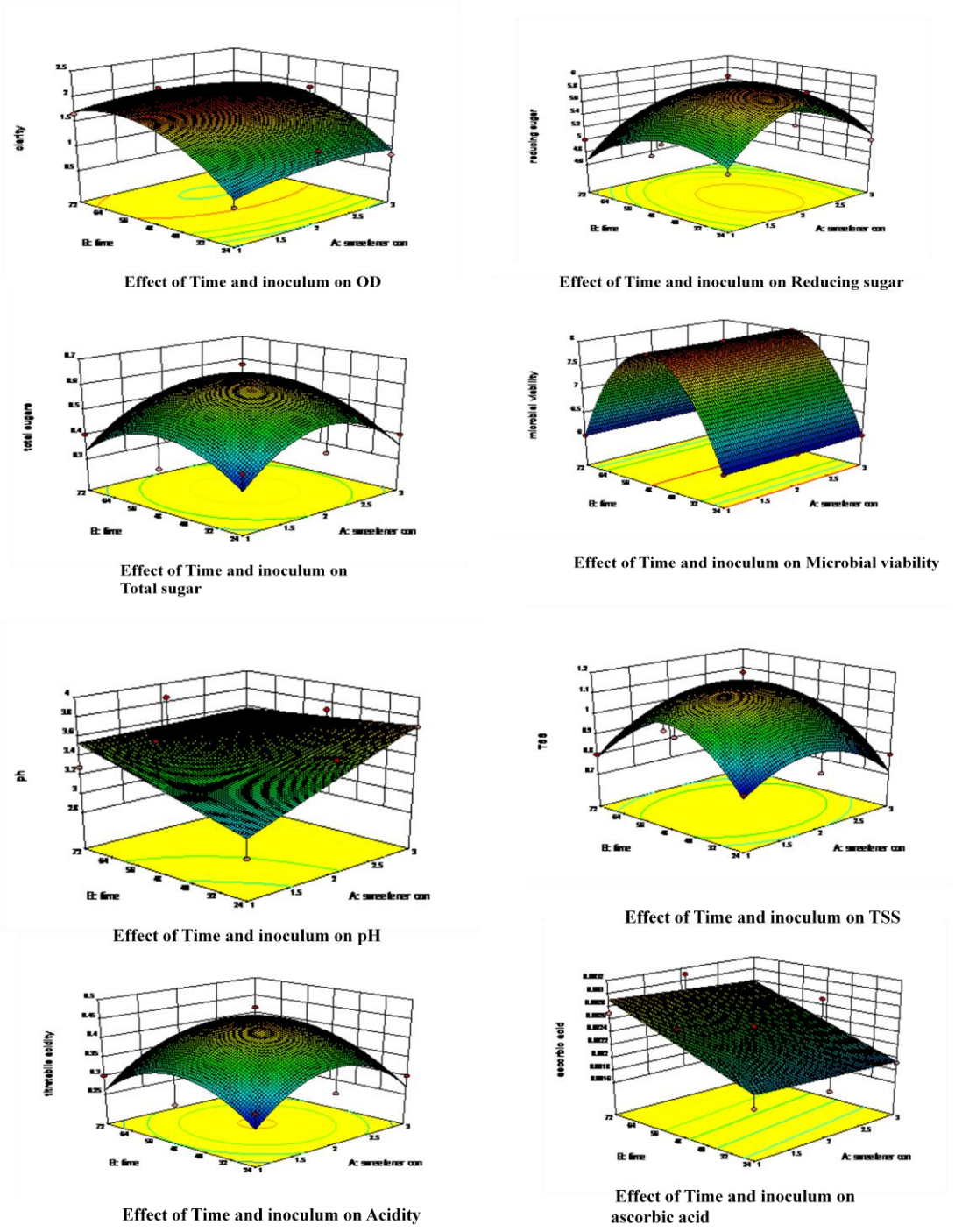


Fig 4.10: Response surface plots for factors studied and responses yielded for sweeteners

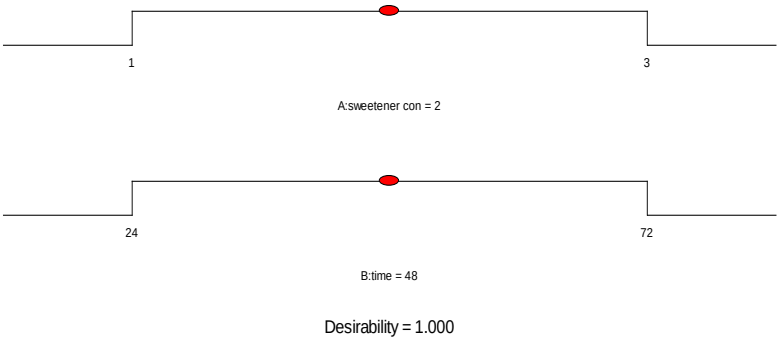


Fig 4.11: Desirability ramp for the sweetener factors

The addition of sweetener was standardized from the desirability ramp generated with sweetener concentration 2% and time 24hrs. At these conditions the characteristics of the juice remains unaffected and the organolyptic characteristics of the fruit juice seems to have improved.

2% sweetener at 24h

Fig.4.12: Microbial viability of *L.plantarum* after addition of sweetener

Probiotic cultures are most commonly used in the addition to dairy products and some other fruit and vegetable juices as they impart very good health properties, but a few products such as lactic acid, diacetyl, acetaldehyde produced during its fermentation is the reason for the loss of viability of the added probiotic (Post, R.C, 1996). The main causes for loss of viability of probiotic bacteria's have been reported due to the decrease in the pH of the medium and accumulation of acids and other products during the growth and fermentation of the culture. (Shah and Jelen, 1990). In this study the added *L.plantarum* culture survived in the palm juice at various time intervals with increase in acidity and at low pH. The prebiotic inulin added showed similar results to the values attained in probiotic samples.(Nazzaro, F. *et al.*,2008). The addition of sweetener similarly resulted in no significant changes to the characteristics of the probioticated palm juice sample. Thus a healthy probiotic drink with standardized concentrations of prebiotic and sweetener has been developed.

CHAPTER 5
CONCLUSION

Palm juice was found to be a good medium for the growth of lactic acid bacteria like *Lactobacillus plantarum*. It was observed that *Lactobacillus plantarum* produces lactic acid rapidly by utilizing the available palm juice. As a result of production of lactic acid there is a decrease in pH and increase in acidity .Clarification procedures were carried out with four parameters and nine responses .The optimized parameters were time -120 min , pH -3 ,temp -30º C and enzyme conc -0.8 U.With these parameters the next step of addition of probiotics was carried out. For this two parameters were considered probiotic concentration and time.The optimized values of both are 4% and 48 h. Then prebiotics was carried out in order to optimize the same two parameters as in probiotics. The optimized parameters were prebiotic concentration 3% and time 48 h. The addition of inulin as a prebiotic has seemed to increase the probiotic growth.In the next step sweetner (Stevia) was added and the optimized parameters were 2% sweetener concentration and time 48 h. It was also observed that the addition of sweeteners did not affect the growth of probiotic micro-organisms.

So from these results we conclude that palm juice is a suitable media for the growth of *Lactobacillus plantarum* and this in turn can act as a good health drink for lactose intolerant people.

APPENDICES

5.1. *Lactobacillus* de Mann, Rogosa and Sharpe broth (g/1000mL)

Protease peptone	10g
Beef extract	10g
Yeast extract	5g
Dextrose	20g
Polysorbate 80	1g
Ammonium citrate	5g
Sodium acetate	5g
Magnesium sulphate	0.1g
Dipotassium phosphate	2g
Manganese sulphate	0.05g
pH	6.5±0.2

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