CHALMERS

Optimization of Preservative in Wet BBQ Grill-Oil-Allround Product by Regulating pH and Oil Content

MASTER OF SCIENCE THESIS

WAHEDULLAH SHAMS

Department of Chemistry and Chemical Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden, 2016

Abstract

Many foods contain emulsions, typically the emulsions are oil- in -water or water-in-oil. Growth of microorganism bacteria, yeasts and moulds in emulsions may lead to spoilage of product. Preservatives are important in the prevention or control of microbial growth. Adverse effect of some preservatives on human have been disputed; for instance, sodium benzoate was recently linked to the hyperactivity in children. However, effectiveness of preservative have not only been controversial but also confusing for customers. In order to meet the emerging consumers' trend for more natural products, food companies are nowadays forced to limit the use of chemical preservatives. The aim of this study was to develop a mathematical model to predict probability of highly food resistant spoilage yeast (Saccharomyces cerevisiae) and bacteria (Lactobacillus plantarum) in different environmental condition, taking acidified cold-filled Grill Oil Allround (GOA) as target product. By applying this model, the stability of the product characteristics within investigated pH and oil content range can be evaluated. The effect and interaction of pH, oil content and preservative can be used in development of guidelines regarding formulation of new shelf stable GOA product hopefully without using any chemical preservatives.

The cubic Experimental design were preformed to manufacture the GOA product randomly because the order of manufacturing should not affect the spoilage of the product. The experiment design included three variables, each at two levels: pH (3.3and 3.7), Oil (20 and 50%), and potassium sorbate concentration (0 and 2000ppm). Duplicate sample included reference were inoculated with 7 log CFU/ml yeast (Saccharomyces cerevisiae) and bacteria (Lactobacillus plantarum) in order to show how resistant the GOA product is against spoilage microorganisms. Survival, growth and reduction were followed by analyzing the bacterial count by plate count at selected times during storage

The inoculated sample were plated on YPD (Yeast extract Peptone D-glucose) for yeast and MRS (De Man, Rogosa, and Sharp) for bacteria and the concentrations was measured after 120, 242 and 363 hours. Yeast showed direct reduction because this microorganism not tolerated 8% NaCl, which the product already contained. All experiment with L. planatarum showed a declining response. The estimate rate constant (K_d) for the reduction of the microorganism and the most appropriate experiment that followed the reference was with low pH (3.3), high oil content (50%) and no preservative. Logistic regression was used to create the predictive model. The pH and oil content effects were found to be significant factors controlling the (K_d) and probability of microorganism survival. At lower oil contents a low pH is important together with high concentrations of preservative.

At higher oil contents there is a need for more preservative when the pH increases, and in 50% Grill Oil Allround preservatives is of minor importance probably due to partitioning into the oil phase. The result of this thesis give possibility to minimize and also maybe eliminate it from GOA product. The results show that food structure is an important intrinsic factor when studying microbial spoilage since

the growth behaviour in liquid media and in viscous media differs significantly. It is of importance to consider ingredient interactions when formulating mayonnaise recipes stable against microbial spoilage.

Sammanfattning

Konsumenterna vill ha naturlig tillsatsfri, ”äkta” mat och livsmedelsindustrin tvingas finna nya lösningar för att tillgodose detta behov. Syftet med projektet var att undersöka effekten av konserveringsmedlen sorbinsyra i BBQ grillolja produkt. Hur mycket av konserveringsmedlet som är aktivt beror både på produktens pH och fetthalt, eftersom det påverkar hur mycket av syran som är i sin odissocierade aktiva form, eftersom detta konserveringsmedel alltid fördelas mellan fettfas och vattenfas. Det finns mycket få studier gjorda inom detta område, där både fetthalt, pH och konserveringsmedel ingår, och därför ville vi undersöka hur dessa faktorer tillsammans påverkar hämning av produktförstörande mikroorganismer i BBQ grillolja. Med mer kunskap om hur konserveringsmedel fördelas i en komplex struktur kan användandet av dessa tillsatser optimeras. I praktiken används idag den högsta tillåtna halten enligt EU-regler. Men när är det befogat att använda konserveringsmedel och hur mycket ska tillsättas? För att undersöka detta tillverkades BBQ grillolja med olika fetthalt, pH och koncentration konserveringsmedel. I dessa ympades det sedan in produktförstörande mikroorganismer som jäst Sacharromyces cervisiae och mjölksyrabakterien Lactobacillus plantarum. Överlevnad, tillväxt och avdödning följdes sedan genom att späda grilloljan och odla ut på agarplattor vid valda tillfällen. Fetthalt påverkar i hög grad mängden aktivt konserveringsmedel i vattenfasen i grilloljan. Tillsats av högsta tillåtna koncentration konserveringsmedel i fullfettsgrill olja ger nästintill obetydlig mängd aktivt konserveringsmedel. Saccharomyces cervisiae visade sig hämmas av hög salthalt (8 %) och konserveringsmedel i mycket låga halter var tillräcklig för att minska mängden jästen i grillolja. L. plantarum var däremot tålig mot varierande förhållanden av fetthalt, pH och konserveringsmedel, och olika försiktighetsåtgärder bör användas vid olika fetthalter. Vid låga fetthalter (20 %) är det viktigt med lågt pH tillsammans med en hög koncentration konserveringsmedel. Vid högre fetthalt (35 %) behövs stigande halter konserveringsmedel när pH ökar och i fullfettsgrill olja (50 %) har tillsats av konserveringsmedel ingen betydelse eftersom större delen går över i fettfasen. Resultaten visar att matens struktur är en viktig faktor att ta hänsyn till vid studier av produktförstörande mikroorganismer, eftersom deras beteende skiljer sig avsevärt i buljongsystem och i mera trögflytande media. Det är också viktigt att ta hänsyn till de faktorer så som olika ingrediensernas samverkan vid utformningen av GOA recept som är säkra och inte påverkar negativt.

TABLE OF CONTENT

1. Introduction

Many foods contain emulsions, typically the emulsions are oil- in -water or water-in-oil. Growth of microorganism in emulsions may lead to spoilage by bacteria, yeasts and moulds. Food structure may influence both rate of the growth and condition under which growth is initiated. The site of occupancy of microorganism is aqueous phase. Therefore the chemical composition of this phase is what has a direct influence on the survival and growth of microorganism. The organic acid components interact with the lipid phase, however and effect is a decrease in the preservation of the products [1]. Additional of preservatives influence food structure and its stability. Adverse effect of some preservatives on humans have been disputed; for instance, sodium benzoate was recently link to the hyperactivity in children [2]. However, effectiveness of preservative have not only been controversial but also confusing customers. In order to meet the emerging consumers' trend for more natural products, nowadays food companies are forced to limit the use of chemical preservatives [2]. Microbiology has assumed that, within the food matrix, it is the chemistry structure of the products that control the growth of microorganisms. Food stability can be found from combination of food technologies, which nowadays regains a lot of interest from the food industry. Several studies have been investigating the growth behaviour of spoilage microorganisms in broth systems mimicking the conditions of a salad dressing (Jenkins et al., 2000, Vermeulen et al., 2007, Vermeulen et al., 2008, Praphailong and Fleet, 1997, Dang et al., 2010). This is an easy, reproducible way of working. However, these studies do not take the emulsion structure into account, which can also have effect on microbial behaviour. These effects includes constrains on the mechanical distribution of water, the chemical distribution of organic acids and physical constrains on mobility of microorganisms. In gelled regions of the food, like emulsions with high oil content or in emulsion with added gums and thickeners, microorganisms are sometimes immobilized and constrained to grow as colonies (Wilson et al., 2002). This phenomenon has been shown in a study by (Brocklehurst et al., 1995). Immobilized cells results in a local accumulation of metabolic end-products which affect growth (Wilson et al., 2002).

This paper will describe the effect of organic acid used as preservatives oil-in-water emulsions as well as the influence of their structure on the food stability and optimize it by regulating the pH and oil content parameters. The control of microorganism is one of the most important

aspects of food safety. The effects of potassium sorbate, oil content and pH on the growth of L. plantarum CCUG 30503, Saccharomyces cerevisiae CCUG 38980 and mould populations will be studied. The experiment were arranged in a design that included the use of different levels of sorbic acid (0-2000 ppm), levels of Rapeseed oil (20, 35 and 50%) and pH from 3.3 to 3.7 in a grill oil system. In many cases bacterial destruction is not necessary for food preservation and the controlling of environmental factors that affect viability can be sufficient to inhibit microorganism growth [1]. In this case, the microorganisms will not be destroyed, but will not be able to grow, and the preservation techniques used which are much less intense, will affect food quality to a lesser extent.

1.1 Objective of this thesis

The objective of this master thesis was to evaluate how different combinations of preservative concentration, oil content and pH level will affect the growth of food spoilage microorganisms. The proposed theory is that there are certain combinations of preservative concentration, oil content and pH that do not support microbial growth. With more knowledge of these combinations, it is possible to define the boundaries for growth of the most significant spoilage microorganisms, and develop guidelines to optimize the use of preservatives or even to eliminate it from Santa Maria wet BBQ products. At the same time making these products shelf stable. Effect and interaction of the variables will be evaluated in terms of significance for growth of all microorganisms. By measuring the effect of each variable a regression model was developed.

2. Theoretical and Historical backgrounds

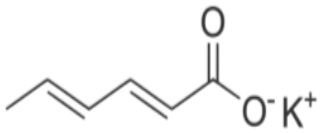
2.1 The role of lipids in controlling microbial growth

Grill oil is an oil in water emulsion made from oil and combination of different ingredients such as salt, sugar, vinegar, spices, etc. Emulsion are colloidal system of two immiscible liquids, one dispersed in the other, the continuous phase. Food emulsions can either be oil in water and water in oil. Emulsion are prepared by vigorous mixing of two immiscible liquids such that droplets of the disperse phase are formed. The droplet size is usually between 0.1-10 µm [2]. Emulsions stability is enhanced by the presence of substances such as phospholipid whose molecule contain both polar and non-polar region.

2.2 Definition and function of chemical food preservative

Preservatives are substances that are added to products such as food to prevent spoilage by microbial growth or by undesirable chemical changes. Preservative food additives reduce risks for nutritional losses due to microbiological, enzymatic or chemical changes of foods during its shelf life. Sorbic acid is a weak acid that have an effect on microbial growth both by reducing pH of the system and by the undissociate form of the acid being able to enter the microbial cell through its lipophilic membrane, dissociating in the cell's more neutral pH environment and thereupon disrupting the proton balance systems within the organism [3]. The use of sorbic acid as their salts is allowed by European legislation and its presence must be declared on the label [4]. The Extent to which weak acid is undissociate depends on the dissociation constant and pH of its environment. Handerson-Hassel-Balch equation give possibility to calculate the degree of the dissociation by pH measurement, and also calculate the pH by given concentration of weak acid in the water. Chemical preservation has become an important practice in modern food technology with increase in production of processed and convenience food. Benzoic acid and potassium sorbate are generally effective to control mould and inhibit yeast and bacteria growth.

Figure 1. Structural formula of potassium sorbate



2.3 Sorbic acid

Sorbic acid and its salt are well known since the 1940s [5]. Sorbic acid is a straight chain, trans-trans unsaturated fatty acid (Figure 1) that is used as a food preservative. The extensive use of sorbates as preservatives is based on their ability to inhibit or delay growth of numerous microorganisms, including yeasts, molds, and bacteria. Sorbic acid has a natural taste and odour and the acid and its salt are permitted widely in foods in USA, where they generally recognized as safe status. There is variation, however, in inhibition of microorganisms by sorbates, depending on differences in microbial types, species and strains, substrate properties. Under certain conditions, some microbial strains are resistant to inhibition by sorbate or even metabolize the compound. In general, however, sorbates are considered effective food preservatives when used under sanitary conditions and in products processed using good manufacturing practices. The mechanism of antimicrobial activity of sorbate are not fully defined [5, 6]. However, there is variation in inhibition of microbes by sorbates, depending on species, strains, substrate, pH, water activity, inoculum level, additives, storage temperature and more. According Swedish National Food Administration the allowed concentrations of sorbic acid in oil products are 1000 ppm when oil content is over 60 %, and 2000 ppm when under 60%.Sorbic acid dissociate in aqueous solution and release hydrogen ions.

Chemistry

The carboxyl group of sorbic acid is highly reactive and result in formation of various salts and esters. Double bond of the sorbic acid influence the antimicrobial activity as well as on the quality and safety of food product. The salt which is commonly formed is Potassium sorbate. The salts are to prefer when using this preservative since it has a greater solubility in water. The effectiveness of sorbic acid as a preservative depends on the amount of preservative in the aqueous phase, where it is needed for microbial control, as well as how much of the acid that is in the undissociated form, since that is the active form of the preservative[7]. The amount of

sorbic acid in the aqueous phase is reduced when the oil content of the food increases, this is due to the approximately three times higher solubility of sorbic acid in oil than in water [8]. At higher pH the acid dissociates, resulting in accumulation of anions and protons and inhibition of key metabolic functions leading to inhibited cell growth. Enzymes that has been shown to be inhibited by sorbic acid is α-ketoglutarate, succinate dehydrogenases and phosphofructokinase [9].

The value of Kp for sorbic acid is 3.1 [10], but is dependent on the sugar and salt concentrations. Higher concentrations of salt and sugars reduce the concentration of sorbic acid in the aqueous phase [9]. The amount of the undissociated form is dependent on pH, when the pH of the product is at the pKa of the acid, 50% of the preservative is in its undissociated form. Although the activity of sorbic acid is greater at lower pH values, sorbic acid is effective at a pH as high as 6.5. This is an advantage since the maximum pH for antimicrobial activity by most other common food preservatives is lower, e.g. 4.0-4.5 for benzoic acid. Studies have indicated that the dissociated sorbic acid has antimicrobial activity to, but it is 10-600 times less effective than the undissociated form [8].

Antimicrobial activity

Yeasts inhibited by sorbates include species of the genera *Zygosaccharomyces*, *Candida* and *Saccharomyces*, among others, and the use of this preservative for inhibition of yeast is especially important in products with low pH and/or intermediate water activity, such as carbonated beverages, ketchup, and salad dressings [11]. Also, numerous studies have documented effect of sorbates against various species of moulds, sometimes by inhibiting the formation of mycotoxins [8]. Bacteria inhibited by sorbates include species of the genera *Lactobacillus, Escherichia, Salmonella, Clostridium and Yersinia,* among others. The action of sorbates against bacteria is very extensive; it can inhibit Gram-positive and Gram-negative, catalase-positive and catalase-negative, aerobic and anaerobic, mesophilic and psychotropic, as well as spoilage and pathogenic bacteria. Inhibition of bacteria by this preservative causes an extension of the lag phase, and do not influence the rate and extent of growth. Sorbic acid is considered a more effective preservative against yeasts and moulds than bacteria, and the inhibitory action against lactic acid bacteria (LAB) is less than that towards yeast [8].

The combination of sorbic acid and benzoic acid inhibit several bacterial strains better than either preservative alone [9]. However, benzoic acid has a narrow pH range in which it is effective, 2.5-4.0 for sodium benzoate compared with 6.5 or less for potassium sorbate [12].

Microbial growth requires an aqueous medium and in that case it is important to know the distribution of the preservatives between the lipophilic and aqueous phases [13].

Mechanism of action

The proposed mechanisms of inhibition include alterations in the morphology and function of cell membranes, inhibition of transport functions and metabolic activity [8]. The disturbed metabolism may be the result of inhibition of enzymes, nutrient uptake or various transport systems. Studies have shown that sorbic acid inhibits the activity of several enzymes, including key enzymes in glycolysis and the citric acid cycle like enolase, α-ketoglutarate dehydrogenase, succinic dehydrogenase, malate dehydrogenase and fumarase as well as alcohol dehydrogenase, aspartase and catalase [8]. Sorbic acid is lipophilic and it may interfere with substrate and electron transport mechanisms. Studies have been documented that sorbic acid inhibit the uptake of glucose and amino acids, as well as the electron transport system. Inhibition of nutrient uptake may be the result of incorporation of sorbic acid into the cell membrane, where it may cause steric disorganization of active membrane transport proteins [9]. Undissociated sorbic acid is able to move into the cell by diffusion. In acidified products like ‘’grill oil’’, the intracellular pH is much higher than outside the cell and when the acid enters the cytoplasm it will dissociate, decreasing the intracellular pH. Inhibition of microbial growth by sorbic acid has been associated with depletion of ATP [14]. A proposed mechanism to explain this is the hydrolysis of ATP by the sodium/hydrogen pump and the H+-ATPase, which attempt to maintain ion balance in the cell. According to this, the inhibition action of sorbic acid could be due to the stress response that attempts to maintain intracellular pH and therefore results in less energy available for growth and cell division [14, 15]. The accumulation of protons inside the cell also acts as an inhibitor by interfering with metabolic processes and may cause neutralization of the proton motive force (PMF) that exists across cell membranes. This may lead to cell starvation since some compounds, like amino acids, are transported actively by the PMF [9].

2.4 Microorganisms

Lactobacillus plantarum

The genus *Lactobacillus* belongs to the lactic acid bacteria (LAB) since they produce lactic acid as an exclusive or major end product [16]. Lactobacilli are Gram positive rods, non-spore-forming and facultative anaerobes. Since the genus is quite diverse no general limits for pH, water activity and temperature exists for the LAB. The growth-limiting circumstances vary depending on the type of species. Many *lactobacilli* tolerate low pH conditions, and strains of *L. plantarum* have been reported to grow at pH as low as 3.2. They are generally mesophilic, with optimum growth temperature of 20-40 Celsius degree, but LAB comprise many psychrotrophic species able to grow at refrigerated temperatures [1]. The maximum acceptable concentration of lactobacilli in a product is 3 log CFU/g [17]. If the microbial load is higher there are usually sensory implications with spoilage, e.g. formation of aftertastes, smells, souring and sometimes production of gas, and the product becomes inedible [17].

L. plantarum species are not only classified as spoilage bacteria, as in grill oil , but are in some cases wanted in products since they have proven health effects, so called probiotics. Two examples are L. plantarum 299v in the fruit drink ProViva and L. plantarum in fruit juice Bravo Friscus [18, 19]. Members of the Lactobacillus genus are found in a number of fermented food products where they contribute to the preservation, nutrition availability and flavour. Lactobacilli are added as deliberate starters to take part in the fermentation in food products like vegetables and sausages [15]. Lactic acid bacteria have been shown to be responsible for about 25% of the spoilage of oil products. Lactobacillus fructivorans is a common cause of spoilage in these products.

Saccharomyces Cerevisiae

It's well known that 40% of all food that is thrown away during human consumption is caused by microbial spoilage. *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* growth is a major factor in the spoilage of foods and beverages that are characterized by a high sugar content, low pH, and low water activity, and it is a significant economic problem. These yeasts can usually be retarded by weak organic. There are many variety of food products that yeast can grow. These products are such as pickle, cheese, butter, salted dried and fresh meats. Growth of yeast on food is regarded as cause of spoilage rather than a safety issues. The symptom of the yeast spoilage is formation of clouds, white colonies and also alternation of

flavour and odour. Hurdles such as pH, sugar content, and chemical preservatives prevent the growth of most organisms in ready-to-drink beverages [20]. Spoilage yeasts, such as *Saccharomyces cerevisiae*, *Candida lipolytica,* and *Zygosaccharomyces bailii,* are sometimes able to overcome these hurdles. These organisms tolerate acidic environments and are resistant to chemical preservatives, such as potassium sorbate and sodium benzoate [21].

S. cerevisiae is inhibited by the lower concentration of these preservatives but tolerate much better acidic environment. The inhibitory response is stronger in the pH value less than 5 where a greater proportion of weak acid is present in undissociated form. It is usually necessary to use preservatives at milimolar rather than micromolar levels in order to prevent yeast spoilage of low pH foods and beverages. There are studies that summarize the current knowledge of the mechanism of weak acid resistance in *S. cerevisiae* and *Zygosaccharomyces bailii,* as two important food spoilage yeasts. *S. cerevisiae* is able to maintain lower intracellular levels of weak acid than would be expected on the basis of a free equilibration across the cell membrane. Saccharomyces cerevisiae expends considerable energy in actively extruding acid from the cell, high levels of a specific ATP binding cassette (ABC) transporter (Pdr12) being induced in its plasma membrane in order to catalyze this efflux[22].

Moulds

It is well known that food commodities are frequently contaminated by moulds, and have adverse effect on significant losses in quality, quantity, nutrient composition, and thereby reduce market value. According to the food and agriculture organization (FAO), millions of food is spoiled globally each year due to mycotoxin produced by storage moulds [23]. There are moulds and mycotoxins that are reported with different food items, including cereals, grains, nuts, fruits, vegetables and spices. Microbial contamination in account, the (FAO) also subjected to oxidative deterioration during process and storage and it is serious problem because it reducing the shelf life of the food product. There is a positive correlation oxidative stress and stimulation of aflatoxin biosynthesis [24], which makes the gravity of the food spoilage problem even more severe. Plant essential oils (EOs) have been reported to possess strong antimicrobial, antimycotoxigenic and antioxidant activity. Rosemary is known to be shelf life enhancer of food items and are kept in Generally Recognized as Safe (GRAS) list by US Food and Drug Administration [23]. Efficacy of Rosmarinus officinalis essential oils have been reported [24], its aflatoxin inhibitory potency, the practical efficacy in food system and

its antifungal mode of action are lacking. This antioxidant agent and its compound made it possible to find out the synergism and antagonism effect of compounds.

2. 5 Effect of pH

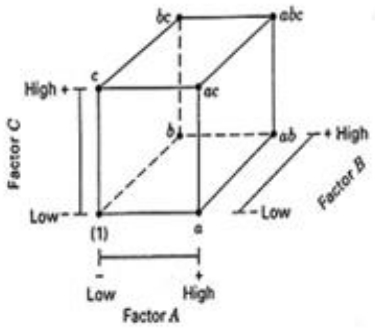
Solutions with a pH less than 7 are acidic and greater than 7 are considered basic. pH is the main parameter in this project for inhibition and spoilage microorganism. The effectiveness of preservatives dependent on the pH of the product, as the antimicrobial effect of the undissociated acid is much stronger than the dissociated acid. The pKa values of the benzoic acid and sorbic acid are 4.18 and 4.76 respectively [25]. Below the reaction describe that when the pH of the food is equivalent the pKa, the proportion of the acid and its salt are equal. If the pH of the food is decreased, then the concentration of the undissociated form is increase. If the pH of the food increases, however, the concentration of the undissocited form declines. Acetic acid acts mainly by lowering the pH of the environment the point where microorganisms can no longer grow. Although the undissociated form of acetic acid has antimicrobial action, it is primarily the hydrogen ions and the resulting decrease pH that inhibit microorganisms. The amount of molecule in the dissociated form is determined by pH. The antimicrobial effect of sorbate increase as the pH value approaches its dissociation constant pKa, which is 4,76. At this pH value, 50% of sorbic acid is in the effective undissociated form.

3 Material and method

3.1 Meth ology and design of experiment

When many factors and interactions affect desirable responses, response surface methodology is an effective tool for optimizing the process. Collection of statistical and mathematical techniques will be used to determine the effects of several variables to optimize processes [26]. The main advantage of experiment design is to reduce number of experimental trials needed to evaluate multiple variables and their interactions. Therefore, it is less laborious and time-consuming than other approaches required optimizing a process. Design of experiment is a systematic way of changing process input and analyzing the resulting process output in order to quantify the cause and effect relationship between them as well as the random variability of the process while using a minimum number of runs. The fig 2 is a full factorial design with three variables and 8 unique points. Each corner of cubic represent each run, Instance of defect in every corner. The defect shows up when A is in the high level and C is in the high level. In the actual project the variables A= pH, B= Oil and C= preservative. To understand the effect of these three different variables the following model was constructed which shows in the figure 2. The experiment was repeated in each experimental point and four repeated in the middle. The experiment was done forward and reverse because it must be sure that the order of the experiment doesn't matter. Figure 2 demonstrate also geometric view, design matrix and algebraic signs for calculating effects in the 2^3 design.

Figure 2. The minimal experiment plan of the test variable, A=pH, B= Oil content and C= Preservatives content.



Run	Factor A	B	C
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

Treatment Combination	Factorial Effect I	A	B	AB	C	AC	BC	ABC
(1)	+	-	-	+	-	+	+	-
a	+	+	-	-	-	-	+	+
b	+	-	+	-	-	+	-	+
ab	+	+	+	+	-	-	-	-
c	+	-	-	+	+	-	-	+
ac	+	+	-	-	+	+	-	-
bc	+	-	+	-	+	-	+	-
abc	+	+	+	+	+	+	+	+

Variables	Low level (-)	High level (+)	(0) = center
A=pH	3.3	3.7	3.5
B=Oil	20%	50%	35%
C=preservative (ppm)	0	2000	1000

Experiment design

3.2 Grill Oil Allround Preparation

The Grill oil Allround comprised: Spice mix, malic acid, acetic acid Raps oil and pasteurized apple concentrate. The Grill oil Allround was manufactured according to original recipe with some deviation from the oil, preservatives, pH (Acetic acid 12%). The manufacturing process was cold mix and also cold filled in the room temperature. The batches were prepared by suspending spice mix, premix, raps oil, malic acid, pasteurized apple concentrate and water according to process chart. Extraction of spice mix preformed under mixing with an electric whisk finally, the acetic acid solution was added to give the desired pH and it was in the range of 3.3 and 3.7 .Some of ingredients are shows in the table 1. The preservatives potassium sorbate (E202) were added to give 0-2000 ppm sorbic acid in total system. Preliminary test was preformed to estimate the boundary condition and its objective was to investigate boundary condition for pH. Investigation required further acetic acid because we wanted to decide the boundary condition. The addition of acetic acid 12% was in the range of 5-25g and gave the lowest pH to 3.3. Acetic acid acts mainly by lowering the pH of the environment, the point where microorganisms can no longer grow. Although the undissociated form of acetic acid has antimicrobial action, it is primarily the hydrogen ions and the resulting decrease pH that inhibit microorganisms and it is the main parameter in this project for inhibition and spoilage microorganism. The effectiveness of preservatives dependent on the pH of the product, as the antimicrobial effect of the undissociated acid is much stronger than the dissociated acid. The intrinsic factors investigated in this study were oil content, pH and preservative concentration. The water activity of the GOA and temperature is not a controlled parameter, however, the water activity was examined and was < 0, 86. To determine interaction of the test variable to GOA the minimal experiment plan was suggested and show in the figure 2. The data from different experiment will be used to fit and evaluate the growth/no growth interface model.

Table 1. Concentrations of Raps oil, water, acetic acid malic acid in Grill Oil Allround

Oil (g/kg)	Water (g/kg)	Acetic acid 12 %(g/kg)	Malic acid (g/kg)
200	571	12, 5	3, 9
500	230	52, 5	3, 9
200	530	53, 5	3, 9
500	272	12, 5	3, 9
200	570	12, 5	3, 9
500	232	52, 5	3, 9
200	532	52, 5	3, 9
500	270	12, 5	3, 9
350	401	32, 5	3, 9

3.3 Aseptic techniques

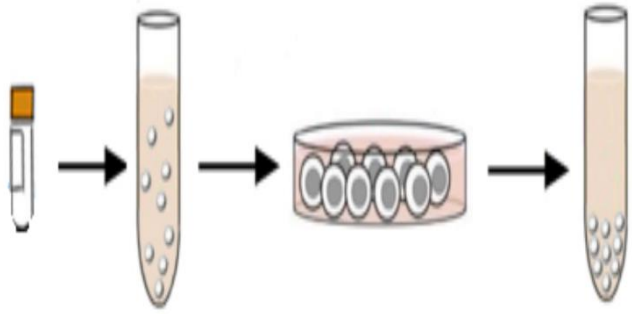
Microbiological work often starts with a transfer of microorganisms from their natural environment to cultures in the laboratory. In nature, different species of microorganisms live together in the same ecosystem while in the laboratory one is almost exclusively dealing with pure cultures i.e. cultures where all cells originates from a single cell. A pure culture must be protected from contamination. Ethanol 70% was used to clean all the working surface. All the media and other equipment that was in contact with the clean cultures was sterile. Both the YPD and MRS media was sterilized by autoclaving. During autoclaving the materials are heated in water steam under high pressure, commonly at 2 atm. The steam temperature reaches 121ºC under this condition. After 20 minutes at 121 ºC, materials will be sterile. Sterile technique was used during all the microbiological analysis steps. Pipettes was used for inoculation of the organisms into the product, and also from product into the dilution serial tubes.

3.4 Inoculum preparation and storage

L. plantarum CCUG 30503 and *Saccharomyces cerevisiae* CCUG 38980 were cultivated from a frozen stage on MRS broth (de Man, Rogosa, Sharp) in 30 degree, sub cultured once more on the MRS plates to be sure that microorganisms survive and once more on the same media before inoculation in the Grill oil Allround product. The same was done for *Saccharomyces cerevisiae* strains but the broth used was YPD (Yeast extract Peptone D-glucose). The overnight cell suspensions were inoculated into the different Grill oil experiments in order to

have an initial population of 10^6-10^7 CFU/ml. All systems were incubated at 20 degree and at selected times, viable cell counts were determined by surface plating on MRS agar at several sample dilutions.

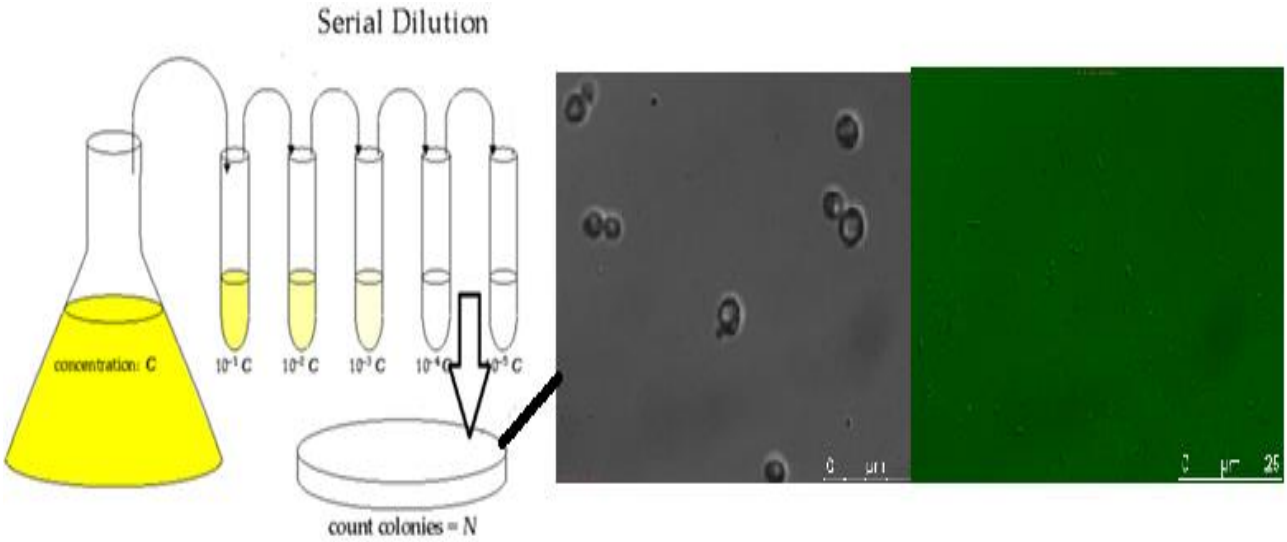
Figure 3. The microorganisms in frozen stage to the ready culture media before inoculation into the GOA product



3.5 Microbiological analysis

Grill Oil All-round (100µl) of every experiment were diluted in 900 µl of 0.1% peptone water in sterile plastic tubes, and then homogenized by shaking manually. Serial decimal dilutions were made for respective experiment. The count of *L. plantarum* and *S. cerevisiae* was enumerated by surface plating on MRS and YPD agar plates with a pH in the range of 3.3-3.7. The plates were incubated at 30 degree for 2-3 days before calculation of microorganism's colony and cell forming units per millimeter. Figure 4 illustrate a serial of dilution and picture of microorganisms by digital microscopy.

Figure 4. Serial dilution of inoculated Yeast and Bacterial for calculation of cell forming units per millimeter and used digital microscopy to take picture of Yeast and Bacteria



3.6 Bulk oil-water experiment

In this experiment, the amount of preservative and oil content was changed from the Grill Allround recipe and the weight of these ingredients replaced by water. The oil phase and the aqueous phase were shaken manually in a separatory funnel. This allowed the sorbic acid to reach equilibrium. After shaking for 15 minutes, the separatory funnel was set aside to allow separation of the two phases, with the oil phase on the top, and the aqueous phase at the bottom. After complete separation the aqueous phase was poured into 270 ml glass bottles and carried to Chalmers technical universities system biology laboratory for analysis of the cell forming units.

3.7 Statistical analysis

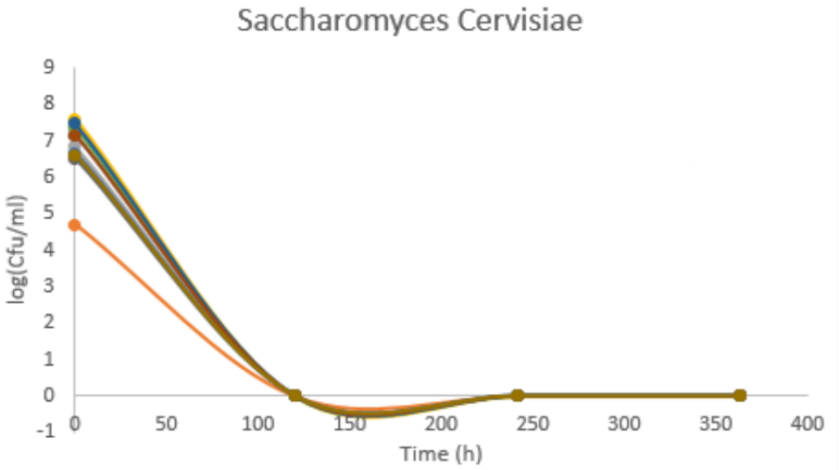
Statistical analysis were conducted using Microsoft Excel. Factorial design equation were calculated to determine the effect of three different parameters (A = pH, B= Oil content and C= Sorbic acid) which affect the growth and reduction rate of the microorganisms. All analyzed data were included in the regression analysis, except data from center point of the experiment. These sampling enabled periods a focus on the growth and reduction of the microorganism after inoculation when log population declines were expected to be linear. All CFU data were transformed ($\log_{10}$) to enable calculation of survival rate over time. Linear regression equations have been regarded as a valuable tool to model the population declines of microorganisms introduced into the oils or water [27].

4 **Result/Discussion**

4.1 Saccharomyces cerevisiae

The yeast growth responses for all of the 10 duplicated experiments are shown in Figure 5. The different experiment showed different initial concentration of yeast, even though they were inoculated with the same level of yeast and had the same formulation. No visual signs of growth were noted in any of the 20 samples inoculated with the yeast Saccharomyces cerevisiae. Figure 5 illustrate the resistance level of the product against yeast (Saccharomyces cerevisiae).According to the Taormina et al. [28] Saccharomyces Cerevisiae can grow at low pH but not in higher than 8% salt content. It is a strong evidence to conclude that yeast is not a problem for shelf life and stability of the GOA containing already 8% salt.

Figure 5. Reduction of spoilage yeast Saccharomyces cerevisiae under 0- 363 h in model cold-filled GOA.



4.2 Lactobacillus plantarum

According to figure 6 the population of Lactobacillus plantarum in every experiment declines in different level after fifteen days. Figure 6 also demonstrate that when the pH is in high level 3.7 and oil content in low level (20%) amount of sorbic acid 0-2000 is of minor importance and the inclination of the plot unaffected. When the pH is 3.7 the population of microbial spores will decrease in a similar manner, but after initial lag period. The curves of different experiments are referred to as microbial survivor curves. Although the shape of these curve is

often described by a first order model, there is an increasing evidence that alternate models are more appropriate when the application is design of a preservation process [28].

When the pH is 3.3, oil content is 20% and no preservative, the reduction is from 7 log CFU/ml to 5 log CFU/ml. This show that the pH variable is most importance with optimization of preservative. Alternative 1&2 indicate that the GOA manufactured forward and reverse and there are not much differences of the plots. Figure 7a shows how the growth behavior of *L. plantarum* was affected by different levels of the variables in GOA systems and followed the reference. The growth of *L. plantarum* could also be followed in systems with 35% oil, pH (3.5) and 1000 ppm (Figure 7b). These two plots indicate once again that addition of preservative into the GOA system is not necessary when the pH is low and oil content in the high level. If the oil content less than 50 %and pH 3.5 it requires addition of preservative in order to could follow the reference product. To take into consideration combination of these three variables from this thesis give possibility to eliminate or optimize the preservative from GOA product. These condition were confirmed by the evaluate data see (Table 3).

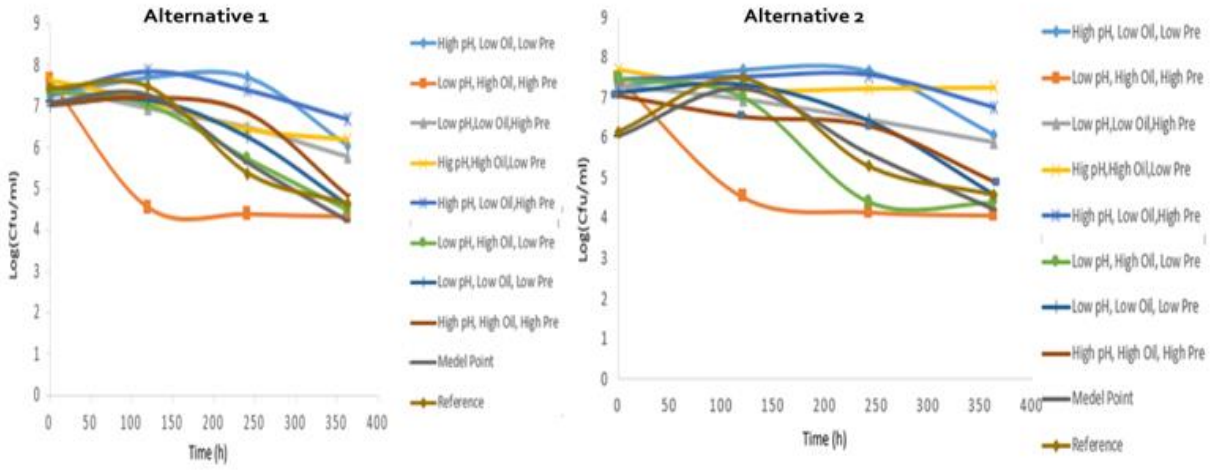
To calculate the reduction rate of the microbial population equation (1) was used. The data of initial concentration and final concentration were used to determine the rate constant (K_d) of the model. Equation (1) describe the reduction in the microbial population as function of time. The values of Bacteria concentration and calculated rate constant (K_d) illustrate in (Table 2)

$$N = N^0 e^{-Kd*t} \qquad (1)$$

Table 2. The calculated rate constant (K_d) from initial and finale concentration of L. plantarum.

	Replicate 1		kd 1	Replicate 2		kd 2
a	1,80E+07	1,10E+06	1,86E-01	1,88E+07	1,20E+06	1,83E-01
bc	4,11E+07	2,10E+04	5,05E-01	3,00E+07	1,20E+04	5,22E-01
c	3,11E+07	6,00E+06	1,10E-01	1,96E+07	8,00E+05	2,13E-01
ab	4,20E+07	1,60E+06	2,18E-01	5,00E+07	1,80E+07	2,28E-01
ac	2,00E+07	5,00E+06	9,24E-02	2,91E+07	6,00E+06	1,05E-01
b	2,24E+07	3,00E+04	4,41E-01	3,00E+07	2,50E+04	4,73E-01
1	1,11E+07	4,00E+04	3,75E-01	1,37E+07	4,00E+04	3,89E-01
abc	1,13E+07	7,00E+04	3,39E-01	1,13E+07	8,00E+04	3,30E-01

Figure 6. Inhibition of Lactobacillus Plantarum in Alternative 1& 2 in different degree



According to the experimental data lower pH by adding acetic acid is an important food preservative technique to prevent the growth of spoilage organisms. Increase oil content and reducing water activity is another widely used preservation technique used this thesis. Combination of lowering pH and increasing oil content could the sorbic acid be optimized this thesis. Combination of reduced water activity, reduced pH, increase oil content showed to be a key element in the stability of shelf-stable GOA. According to SNFA 2000 ppm is highest acceptable level in food product when the oil content is below 60%. According to this study there is not necessary adding of preservative when the pH is 3.3 and oil content 50%. The company should take both fat content and pH into account when designing guidelines for the use of preservative in food products. Figure 6 indicate that the addition of preservatives less importance when the pH is in the high level and oil content in the low level. This is most probably due to the relatively low pH and ingredients antimicrobial activity which result in a lower concentration of sorbic acid in the aqueous phase.

Figure 7. a Growth of *L. plantarum* in Grill Oil Allround with no preservative, low pH (3.3) and 50% oil content b) Growth of *L. plantarum* in 35% Grill Oil Allround with pH 3.5 and 1000ppm preservative

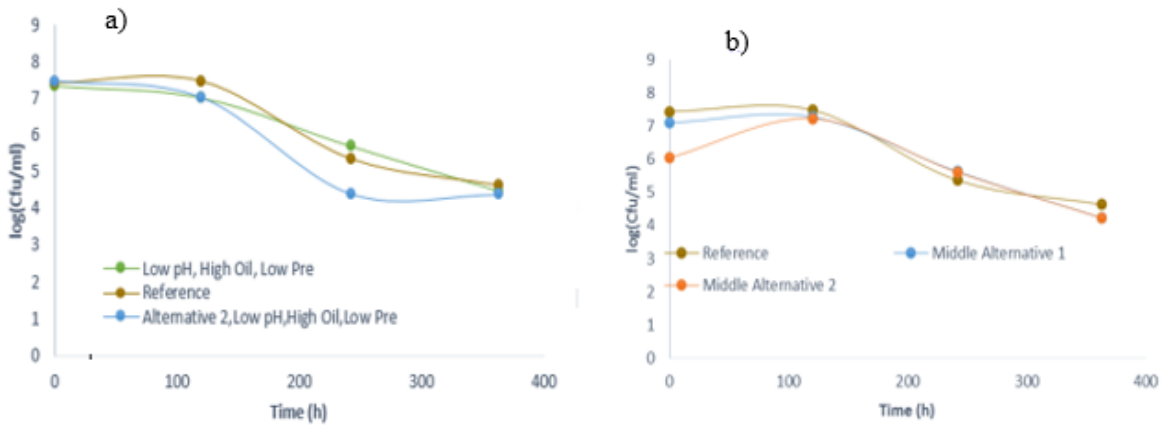


Figure 7a indicate that low pH and high oil content was enough to inhibit growth of *L. plantarum* and follow the reference level in the emulsion which was inconsistent with effect of pH and Oil content in the Castro et al. [29].

In GOA with 35% oil and pH 3.5 there is need of 1000ppm sorbic acid to cause reduction of *L. plantarum* and follow the reference level (figure 7b).

4.3 Evaluation

Evaluation show significance effect of the experimental data by experimental design. Table 3 shows the response and contrast of the experimental data. Anova table illustrate significance effect and interaction of interested variables A, B and C. pH and oil content is statically significance variables but preservative is not statically significance according to the experiment design calculation.

Interpretation is to go from low level to high level of A give decreased rate constant (K_d) in equation (1). It determines the reduction of microbial. Negative signs of the significance effect shows that reduction is high when the pH is in low level. Positive significance effect of oil content demonstrate opposite, the reduction is faster in high level of oil content see (Table 3). By these combination the amount of sorbic acid could be optimized. Logistic regression was

used to create the predictive model. The pH and oil content and interaction of oil content and potassium sorbate concentration were found to be significant factors controlling the probability of microorganism's reduction. At lower oil contents a low pH is important together with high concentrations of preservative. At higher oil contents there is a need for more preservative when the pH increases, and in 50% GOA preservatives is of minor importance probably due to partitioning into the oil phase. According to [8], there is a need for higher concentrations of sorbic acid when the oil content is low and the pH is high.

Table 3. The significance effect and interaction of variables, A= pH, B = Oil and C = preservative

Anova	SS	df	MS	F	Effekt*100		
A	0,1344	1	0,1344	1015,4	-18,33		
B	0,1024	1	0,1024	773,5	16,00		
C	0,0015	1	0,0015	11,5	-1,95		
AB	0,0021	1	0,0021	16,2	-2,32		
AC	0,0042	1	0,0042	31,5	3,23		
BC	0,0429	1	0,0429	324,4	10,36		
ABC	0,0001	1	0,0001	0,7	-0,48	F(1,8,.9)=	3
error	0,0011	8	0,0001			F(1,8,95)=	5
tot	0,2887	15				F(1,8,.99)=	11

Figure 8: Interaction between oil content and preservative. It actually describes if one factor effects the performance in other factor.

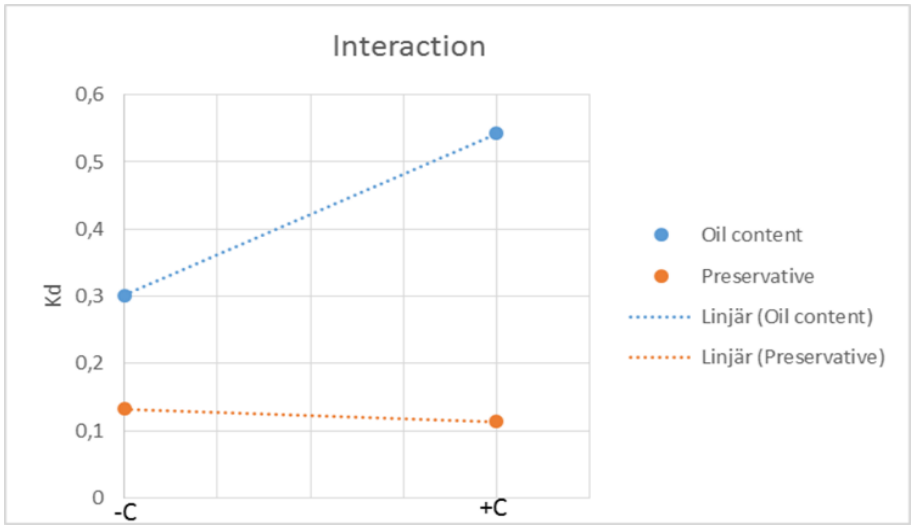


Figure 8 shows that plot of both variables are parallel and it means that there are not interaction between preservative and oil content. The interaction curve show the differences of the two difference level. Difference of oil content is larger than difference of the sorbic acid indicating that the effect of sorbic acid could be ignored.

4.4 Development of G/NG Model

Separate logistic regression models were developed for all different experiments, taking all the variables and interaction into account. The model were established for all data collected after 363 hour *for L. plantarum*. The variables were estimated with their standard deviation shown in the table 3. [A] is -1 alt +1, represent low and high level of pH respectively. [AB] interaction of A=pH and B= Oil content and C= Preservative (Table 4). The developed model show the reduced velocity of microorganisms. The main effect of the interested variables are divided by two because there are differences in low levels and high levels of the variables. (b1, b2, b12, b13, b23) are the effect of variables which shows in table 3. Figure 9 shows the residual between model and experiment data. Correlation and regression only describes linear relationship. Residuals and residual plots determine how well a line describe the data. Residuals are the vertical distance between the regression line and recorded data. The residual plot of this thesis show even distribution of observed and predicted data.

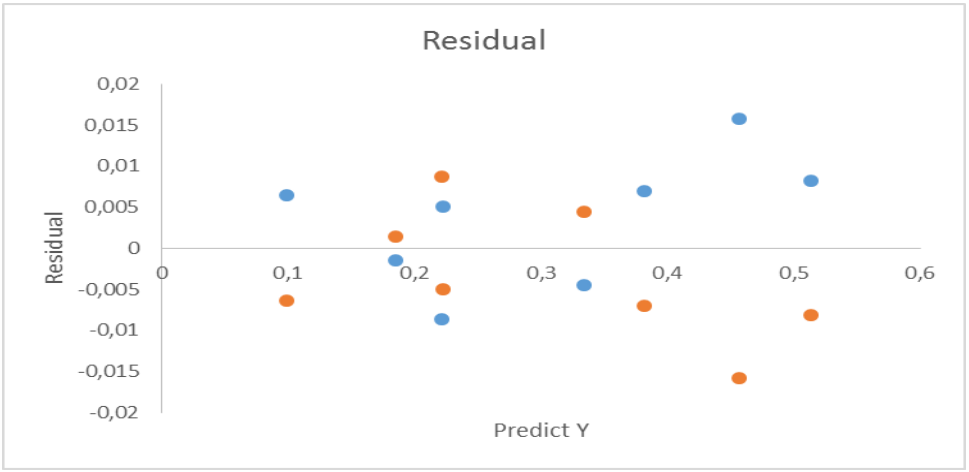
Residual = Y^{obs}- Y^{pred}

$$Y = \text{bo} + \frac{\text{b1[A]+b2[B]+b12[AB]+b13[AC]+b23[BC]}}{2} \qquad (2)$$

Table 4: Differences between the experimental and developed model data

Variables effect	[A]	[B]	[AB]	[AC]	[BC]	Observed Y 1	Observed Y 2	Predicted Y
b0=0,030	-1	-1	1	1	1	0,375	0,389	0,382
b1=-0,18	1	-1	-1	-1	1	0,186	0,183	0,185
b2=0,16	-1	1	-1	1	-1	0,441	0,473	0,457
b12=-0,023	1	1	1	-1	-1	0,218	0,228	0,223
b13=0,032	-1	-1	1	-1	-1	0,231	0,213	0,222
b23=0,10	1	-1	-1	1	-1	0,092	0,105	0,099
	-1	1	-1	-1	1	0,505	0,522	0,513
	1	1	1	1	1	0,339	0,330	0,334

Figure 9: The residual plot between experiment data and model data



The result of factorial design experiments for studying the effect of three dependent variables, pH, Oil content and sorbic acid concentration were presented in the Table 4 along with the mean observed and predicted response (log cycle reduction for cells of L. plantarum). By applying multiple regression analysis on the experimental data (Table 4), the response variable and the test variables were related by the polynomial equation (2). All linear regression analyses of L. plantarum survival in GOA and field plots were significant ($P< 0.05$). In some instances, regression models accounted for relatively low amount of variation in the data, although analysis of regression residual indicated linear regressions generally provided good model for L. plantarum survival. Population level of bacteria declined over all observation periods.

According to Santa Maria AB quality standards, all product should be below the following microbial values (Table c). Optimized sorbic acid product and reference product was send to the Eurofins and the result shows in (Table 4 d). Theses result indicate that optimized product for tested microorganism's concentration is below 1 and it is acceptable.

Table 5 (a-b) Santa Maria Guarantees, microbial data for product should not exceed the following data. b) Experiments result from Eurofins for all microorganisms in Grill Oil All-round.

5a)

Total Plate count	< 6 log cfu/ml
Bacillus cereus	< 3 log ctu/ml
Yeast	< 4 log ctu/ml
Mould	< 4 log cfu/ml
Salmonella	< negative ctu/25ml
E.coli	< 1 log ctu/ml

5 b)

Total Plate Count	< 3.3-4.1 log cfu /ml
Bacillus cereus	< 1 log cfu/ml
Yeast	< 1 log cfu/ml
Moulds	< 1 log cfu/ml
Salmonella	Not detect / 25ml
E coli	< 1 log ctu/ ml
Lactic Acid Bacteria	< 1 log ctu/ml

5. Sensory Test

The experiment without preservatives gave the desirable result and was tested by sensory test. It is very important that the product should keep the original quality characteristic .Sensory test needs to be addressed when applying antimicrobials is their potential impact on the sensory characteristics of a food. Obviously, compounds that negatively affect flavor and odor or contribute inappropriate flavors and odors would be unacceptable.

During a tetrad test, a panellist is presented with four samples of which two are the same and the other two are also the same. The panellists must state which sample belong to each other. The results should indicate whether or not a detectable difference exists between two samples. The method is statistically more efficient than the triangle test. Only 28 % could determine that difference exist between two samples but 72% could not make a difference between reference and sample in interest. It means that there is not any significance difference in taste and odour between both of the Grill Oil product (Table 6).

Table 6. Summarize result of sensory test

(Alpha) = 0.719(when Alpha<0.05: samples are significant different.

	1	2
codes	418, 593	213, 845
Description	Grillolja REF	Grillolja prov 6
Description3	Grillolja REF	Grillolja prov 6
image	noimage.jpg	noimage.jpg
sessionid	*	*
wizard	product1	product2

	Correct	InCor.	Total	Density	Min 5%	P<5	P>=5	p<0.1%	p<1%	p<5%	p<10%
1	5	12	17	0.522	10	0.281	0.719	FALSE	FALSE	FALSE	FALSE

	Total	P<0.001	P<0.01	P<0.05	P<0.10
1	17	13	11	10	9

6. Recommendations

Combination of food ingredients structure is the main intrinsic factor when formulating Grill oil Allround recipe stable against microbial spoilage. The use of a high concentration of preservative in 50% Grill oil Allround may not have desired effect when the pH is low.

In Grill oil with 20% oil there is need for both low pH and high concentration of preservative to keep the products shelf stable. Lowering pH and increased oil content in all the system make opportunity to decrease the intracellular pH and prevent the spoilage of microorganisms. When the pH of the system is high the addition of preservative is necessary. Saccharomyces cerevisiae is not recommended in further studies when the salt content is above 7.5%. Low pH is importance for product safety.

By applying developed models, stability of acidified foods with characteristics within investigated pH and oil content can be evaluated. In addition, defined no growth region can be used as guidelines for food developers formulate new shelf stable products without using preservatives. The models also provide to manufacturers useful indications regarding the effect of inhibitory factors, so that the producers can implement an efficient preservation method ensuring both stability and sensorial quality of food products

With respect to the significances between oil content and preservative use of center point experiment give more the expected result. By use this experiment the company can optimize their preservative, oil content and also provides enterprise profits.

Acknowledgements

I would like to thank my supervisors Torbjörn Lilliebjelke and Martin Lindgren (Santa Maria) heading me through this work.

Peter Blomgren (Santa Maria) for his credible ideas during the project.

My examiner Professor Claes Niklasson at Chalmers University for his input and assistance during the project.

Christer Larsson Professor in microbiology at Chalmers University for letting me analyze the products in the System biology laboratory

I would also like to express my appreciations to all the members of the R&D (Santa Maria) department for making me feel welcomed and helping me with practical staffs.

I would like to thank my wife and family for their love and encouragements.

References

[1] Blackburn CdW, Knovel (e-book collection). Food spoilage microorganisms. Cambridge, England; Boca Raton: Woodhead Publishing;

[2]SHAW, D. J. 1992. Introduction to colloid and surface chemistry, Butterworth-Heinemann.

[3]Martini, M.C. and Seiller, M. Actifs et additifs a cosmetology 3e edition (Tec et Doc ed), Lavoisier, France (2006) pp. 763–793.

[4] Wilson PD, Wilson DR, Waspe CR. Weak acids: dissociation in complex buffering systems and partitioning into oils. Journal of the Science of Food and Agriculture. 2000; 80(4):471-6.

[5] Sofos, J. N., Busta, F. F., Bhothipaksa, K., Allen, C. E., Robach, M. C., and Paquette, M. W. 1980b. Effects of various concentrations of sodium nitrite and potassium sorbate on Clostridium botulinum toxin production in commercially prepared bacon. J. Food Sci. 45:1285–1292.

[6] Sofos, J. N., Pierson, M. D., Blocher, J. C., and Busta, F. F. 1986. Mode of action of sorbic acid on bacterial cells and spores. Int. J. Food Microbial. 3:1–17.

[7] Wedzicha, B. L. and Brook, M. A. 1989. Reaction of sorbic acid with nucleophiles: preliminary studies. Food Chem. 31:29–40.

[8] Davidson PM, Sofos JN, Branen AL, CRCnetBASE (e-book collection), Not MarcUpdate (EBS). Antimicrobials in food. 3rd ed. Boca Raton: Taylor & Francis; 2005.

[9] Martoadiprawito, W. and Whitaker, J. R. 1963. Potassium sorbate inhibition of yeast alcohol dehydrogenase. Biochim. Biophys. Acta 77:536.

[10] Wilson PDG, Brocklehurst TF. The role of lipids in controlling microbial growth. Grasas y Aceites. 2000; 51(1):66-73.

[11] Sofos, J. N. 2000. Sorbic acid. In Natural Food Antimicrobial Systems, Naidu, A. S., Ed., CRC Press, Boca Raton, FL, pp. 637–659.

[12]Wind, C. E. and Restaino, L. 1995. Antimicrobial effectiveness of potassium sorbate and sodium benzoate against Zygosaccharomyces bailii in a salsa mayonnaise. J. Food Prot. 58:1257–1259

[13] Songsermsakul P. Mycotoxins contamination of food in Thailand (2000-2010): Food safety concerns for the world food exporter. International Food Research Journal. 2015; 22 (2):426.

[14] Freese, E. and Levin, B. C. 1978. Action mechanisms of preservatives and antiseptics. In Developments in Industrial Microbiology, Underkofler, L. A., Ed., Society for Industrial Microbiology, Washington, D.C., pp. 207.

[15]Freese, E., Sheu, C. W., and Galliers, E. 1973. Function of lipophilic acids as antimicrobial food additives. Nature (London) 241:321–325

[16] Robinson RK, Batt CA. Encyclopedia of Food Microbiology. England; United Kingdom; Academic Press; 2014; 1999; 2000;

[17] Fialová J, Chumchalová J, Miková K, Hrůšová I. Effect of food preservatives on the growth of spoilage lactobacilli isolated from mayonnaise-based sauces. Food Control. 2008; 19(7):706-13.

[18]BRAVO 2015-10-08. http://www.bravo.nu/bravofriscus/.

[19]PROVIVA 2015-10-08. http://www.proviva.com/sv/Produkter/Fruktdryck/

[20] Baird-Parker, A. C., R. L. Buchanan, F. F. Busta, M. B. Cole, M. P. Doyle, M. Eyles, J. Farkas, R. S. Flowers, J. L. Jouve, S. Mendoza, et al. 1998. Soft drinks, fruit juices, concentrates and fruit preserves, p. 440-460.

[21] Deak, T., and L. R. Beuchat. 1994. Use of indirect conductimetry to predict the growth of spoilage yeasts, with special consideration of Zygosaccharomyces bailii. Int. J. Food Micro. 23:405-417.

[22] Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16, 675–729.

[23]Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods -a review. International Journal of Food Microbiology, 94, 223–253.

[24] De Azeredo, G.A., Stamford, T.L.M., Nunes, P.C., Gomes Neto, N.J., De Oliveira, M.E.G. & De Souza, E.L. (2011). Combined application of essential oils from Origanum vulgare L. and Rosmarinus officinalis L. to inhibit bacteria and autochthonous microflora associated with minimally efflux

[25] NIKO-PAAVOLA, M. L., Laitila, A., Mattila-Sandholm, T., and Haikaara, A. 1999. New types of antimicrobial compounds produced by Lactobacillus plantarum. J. Appl. Microbial. 86:29.

[26] Jenkins P, Poulos PG, Cole MB, Vandeven MH, Legan JD. The Boundary for Growth of Zygosaccharomyces bailii in Acidified Products Described by Models for Time to Growth and Probability of Growth. Journal of Food Protection. 2000; 63(2):222-30.

[27] Hurst CJ. Modeling the Fate of Microorganisms in Water, Wastewater, and Soil. In: ASM Press; 2007. p. 355-64.

[28] Taormina PJ. Implications of Salt and Sodium Reduction on Microbial Food Safety. Critical Reviews in Food Science and Nutrition. 2010; 50(3):209-27.

[29] Castro M, Garro O, Gerschenson LN, Campos CA. Interaction Between Potassium Sorbate, Oil And Tween 20: It's Effect On The Growth And Inhibition Of Z Bailii In Model Salad Dressings. Journal of Food Safety. 2003; 23(1):47-59.