Project proposal for:

Development and Validation of a Pasteurization Protocol for an Emulsifying Polysaccharide Solution.

ABSTRACT

Due to the potential pathogenic nature of microorganisms that could contaminate the emulsifying polysaccharide solution being investigated, there is a necessity to thermally inactivate this product in order to ensure that the polysaccharide is safe for human consumption. Accordingly this proposal outlines the procedure to be undertaken in order to develop D-values for both contaminants currently found in the local environment and for the more thermally resistant microorganisms such as Bacillus stearothermophilus. Subsequently, a protocol for operating the pasteurizer in a manner that ensures that the polysaccharide is safe for human consumption will be developed. The literature reviewed also indicates that the methods routinely used for calculating thermal process times and temperatures (both the Arrhenius method and first order reaction kinetics) are subject to discrepancies at high temperatures. Consequently, the only accurate method to determine effective thermal process conditions is to determine these conditions experimentally. It is also important to ensure that this pasteurization protocol does not thermally damage the polysaccharide, which would result in a loss of product efficacy. Accordingly, a product degradation versus process temperature curve will be established in order to investigate the affect of temperature upon product performance.

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INTRODUCTION

Plant biopolymers such as gums, stabilizers, emulsifiers and thickeners are presently imported into Australia and currently used extensively by the food industry in products such as salad dressings, ice creams, soft drinks and syrups. These biopolymers are natural polysaccharides, harvested from plants. However, supplies can sometimes be affected by climatic and political uncertainties. Plant cell culture technology provides an alternative to growing the whole plant. Plant cells are grown in suspension cultures in bioreactors in order to produce biopolymers, which are secreted by the cells into the culture medium.

The Cooperative Research Centre for Industrial Plant Biopolymers was established in 1992 to develop the science and technology to enable these natural plant biopolymers to be manufactured using plant cell culture technology, and subsequently commercialize this technology in order to create a new manufacturing industry based in Australia.

The CRC for Industrial Plant Biopolymers is making the most of this plant cell culture technology to produce these natural biopolymers in a controlled industrial environment. With the right blend of nutrients and growth promoters, it is possible to encourage plant tissue to revert to a friable, amorphous state (somewhat similar to mashed potato in its appearance), which is known as callus. This callus can then be transferred to a liquid medium, it breaks down further to become a fine suspension of individual cells (or small clumps of cells), which can be grown to large volumes in sterile fermenters. As the suspension cultures grow, the biopolymers, which are contained within the cell walls, are secreted into the surrounding liquid.

Once the suspension has been harvested from the bioreactors, the cells are removed by filtration and biopolymer is recovered from the filtrate and processed further. After recovery and down stream processing to remove unwanted salts, the product is then pasteurized for 130°C for about 60 seconds to thermally inactivate any microbial or enzymatic activity. Since this product is currently in the final stages of development and is being released to potential customers for evaluation, it has become necessary to implement steps towards attaining ISO9002 accreditation. One of the first steps towards attaining this accreditation is the development and implementation of a HACCP plan for the entire manufacturing process. Furthermore, one of the critical control points identified within the manufacturing process is the final pasteurization of the finished product prior to aseptic packaging. Hence, there is a real need to correctly validate and document correct procedures for the pasteurization process.

This pasteurizer validation involves, ensuring that the high temperatures currently attained during pasteurization do not adversely affect the emulsifying capability of the biopolymers. It is also necessary to investigate how many decimal orders of magnitude the concentration of contaminating microorganisms will be reduced by during the pasteurization. The microbial contaminants will include those found within the local environment and more thermally resistant microorganisms such *as Bacillus stearothermophilus* which is generally used as the reference strain for any thermal process calculations. This should enable D-values (the time required to reduce the number of contaminating organisms by one decimal cycle) for local contaminants and thermally resistant microorganism to be calculated, and subsequently, allow the pasteurizing process to be adequately validated.

STATEMENT OF AIMS

In order to implement a validated HACCP plan for the manufacturing process used to produce plant biopolymers from plant cell culture, it is imperative to correctly validate and develop a protocol for the pasteurization process, which has been identified as a critical control point (CCP). There are two main strategies involved with validating the pasteurization process. The first strategy is to quantify, and subsequently minimize, any reduction in the efficacy of the product that may be occurring as a consequence of thermal damage from the pasteurization. The second strategy is to ensure that thermal destruction of microbial activity within the product is sufficient enough to minimize the potential risk to consumers by food pathogens that may not otherwise be destroyed. If these studies do not conclusively prove that after pasteurization, the product is still efficacious and safe for human consumption, then various methods of altering the existing pasteurizer configuration will be investigated to allow the required degree of performance to be achieved.

LITERATURE REVIEW

Human awareness of the presence and the role of microorganisms in foods obviously precedes the establishment of the modern sciences microbiology and bacteriology. Food spoilage and food poisoning must have been prevalent with the commencement of food production some 8000 to 10000 years ago. And as humans sought to overcome these problems in the ensuing ages, techniques such as cereal cooking, brewing, food storage and salt preservation developed. All the large civilizations in history were known to have used some or all of these techniques. Despite these advancements, humans knew very little about the cause of spoilage and food poisoning.

The first person to appreciate and understand the presence, and role, of microorganisms in food was Louis Pasteur. In 1837 he showed that the souring of milk was caused by microorganisms, and in about 1860 he used heat to destroy undesirable organisms in wine and beer for the first time. This process is now known as pasteurization. Pasteurization is used widely in the food industry as a means of inactivating microorganisms and enzyme activity and subsequently extending the shelf life of that product.

From Pasteur's early heat treatments, the modern process of pasteurization evolved. Modern pasteurization is considered a thermal process and calculations can be carried out using first order kinetics to determine thermal death times (TDT) but food science problems generally use the conventional Arrhenius approach and texts such as Atkinson et al. (1983), Miller et al. (1976), Bailey et al. (1977) and Stanbury et al. (1984) use this method extensively. People such as Abraham et al. (1990) and Ramaswamy et al. (1989) have compared the two approaches – both mathematically and experimentally – in terms of temperature dependance and for calculating thermal process time predictions. Mathematical modelling appears to be where the current trend in research is being made, as is evidenced by the more recent literature articles.

It appears that at the high end of the temperature scale (>100°C) the two predictive models diverge with respect to their forecasts. Jonsson et al. (1977) stated that,

> *"theoretically showed the discrepancy in estimating sterilization times by the different models could be quite large at high temperatures"*

Ramaswamy et al. (1989) showed that errors associated between the activation energy (E_a) and z-values were dependant upon the reference temperatures used in the calculations and the range of these reference temperatures used. Atkinson et al. (1983) define the z-value as the number of degrees Fahrenheit required for the thermal destruction curve to move through one $\log_{10}$ cycle. Whereas, Jonsson et al. (1977) showed a good correlation between the activation energy (E_a) and z-values for *Bacillus stearothermophilus* at 121°C. There appears to be a large discrepancy in published values of the activation energy, E_a, of *Bacillus stearothermophilus* spores with values ranging from 67.7 kcal/mole through to 91.2 kcal/mole. This makes it difficult to know which values to use when making predictive calculations and it also adds credence to the notion that experimental values for each particular scenario at high temperatures is imperative.

A linear relationship between log D (or ln k) and temperature T is widely used (in nearly all of the literature) in calculations involving heat sterilization processes. However, some researchers such as Daughtry et al. (1997) found pronounced deviations from this relationship, thus suggesting that the relationship is not linear at all. In comparison, Wang et al. (1964) found that for *Bacillus stearothermophilus* spores, the z-value increased noticeably with an increase in temperature. Whilst Jones (1968) found that there was a difference in the D-value of *Bacillus stearothermophilus* at 140°C of 61% when extrapolating from the D-values used at 100°C.

Haas et al. (1996) confirmed this deviation from the expected linear relationship and also stated,

> "*that D- and z-values can be determined directly from the experimental data; an iterative calculation of an equivalent time is not necessary*"

Jonsson et al. (1977) and Ramaswamy et al. (1989) also state that sterilization D-values for *Bacillus stearothermophilus* vary greatly according to buffer pH, dissolved oxygen concentrations, viscosity and slat concentrations, which could account for some of the variations in the published E_a data.

Daughtry et al. (1997) used a slightly modified Arrhenius model to try and give a somewhat better correlation for calculating thermal process times with a range of microorganisms, but it still appears that there it is no substitute for experimental data.

Therefore, since both models have a reasonably low predictive capacity, it should be considered important that calculations of ultra high temperature (UHT) sterilization process times should ideally be based on experimental results at the actual temperatures rather than simply extrapolating data which was obtained at substantially lower temperatures.

This theory is support by Jonsson et al. (1977), Ramaswamy et al. (1989) and the EDEHG (1993). The EDEHG goes further and states,

> "*process equipment downstream of the holding tube must be aseptic and hence cleanable, sterilizable and bacteria tight*"

If the EDEHG advice is not followed, then microoganisms can re-enter the product as it leaves the holding tube which negates the benefits of pasteurization.

METHODOLOGY

The experimental methodology that will be primarily employed throughout this research project, is to be a one group pretest – posttest design. This will entail testing each sample prior to exposure to the experimental procedure being trialled, and then posttested to glean the nett effect of each experimental trial. Once the data is compiled, it is anticipated that linear and/or polynomial regression (and correlation) will be employed in order to develop the appropriate relationships.

The primary piece of equipment being validated is the purpose built pasteurizer which is located at the CRC for Industrial Plant Biopolymers Scale-Up Facility, situated in Yarraville. A copy of the system layout for the pasteurizer and a copy of the Standard Operating Procedures (SOP) for this piece of equipment is enclosed in Appendix 4 of this research proposal.

<u>Subproblem one.</u>

The first part of the problem to be investigated is the issue of how effective, with regard to microbial destruction, the pasteurization process is. This entails determining by how many decimal cycles the concentration of contaminating microorganisms will be reduced at a variety of temperatures.

The current practice is to pasteurize at 130°C at a flow rate of 10 litres per minute. It should be noted that a flow rate of 10 litres per minute equates to a resonance time, t, of 66.9 seconds (holding tube length = 5.5 metres and has an internal diameter of 2” → 0.0508m, this means that the hold-up volume of the holding tube is 11.15 litres and at 10 litres/minute this means that the hold-up time is 66.9 seconds).

It is planned to test the emulsifying polysaccharide produced (after downstream processing) at a range of temperatures from about 60°C to 135°C whilst keeping the flow rate, and hence resonance time t, constant. Since only one variable is being altered, temperature, it will enable the affect imparted by only temperature to be established. The polysaccharide to be tested will be raised in temperature to about 30°C, aerated with unfiltered air and sucrose (10g/L) is to be added and allowed to sparge with this dirty air overnight. This will create favourable conditions for microorganism growth and provide an ideal baseline, by simulating the worst case scenario likely to be encountered with contaminants found in the local environment.

A pretest sample will be collected just prior to each trial in a gamma irradiated sterilize sample jar and ‘Standard Plate Counts’ (SPC) will be undertaken to establish the level of contamination. Aseptic posttest samples will be taken in similar sample jars for SPC analysis. Then posttest samples will be taken aseptically, this is achieved by steam sterilizing a Teflon lined stainless steel braided hose of about 3m in length to the outlet of the pasteurizer’s holding tube. Steam is switched off from this hose immediately prior to the test, which enables the sample to be taken aseptically and is steam sterilized between each trial. The ‘Standard Plate Counts’ (SPC) analysis is to contracted out to the Australian Government Analytical Laboratories (AGAL) since it is anticipated that a large number of samples will be generated.

Once the SPC data is analyzed it will reveal how many decimal orders of microorganism concentration reduction is being achieved. From this data it is hoped that an Arrhenius plot can be established which will allow some estimation of D-value for local environment contaminants. It is hoped that this trial will prove that the practice currently employed (130°C at 10L/min) at a bare minimum adeequate and at best it is extreme overkill, and as a result, the temperature can be lowered to achieve the same level of consumer safety.

It is also planned that a similar trial will take place using flow rate, and subsequently resonance time, as the variable to be altered. This will enable the investigation of the affect of resonance time upon the effectiveness of pasteurization. This trial will probably be undertaken after an effective temperature upper limit has been established and undertaken at a variety of flow rates (eg. 5, 10, 15 and 20L/min) at two or three temperatures around this upper limit temperature.

This pasteurization regime will form the basis of the HACCP plan recommendations for the normal manufacturing heat treatment process. It should be noted that no material will be released to customers until that particular batch has undergone a ‘Standard Plate Count’ to determine its level of safety for human consumption. It is envisage that an upper limit from the Standard Plate Count will be in the order of 10^2 cells/mL, although this is yet to be confirmed.

Subproblem two.

The outcomes of subproblem one will establish an effective pasteurization regime for typical contaminants (i.e. those presently found locally in the manufacturing environment). However, this does not mean that more thermally resistant strains could not enter the local environment at any time, and therefore, contingency conditions must be developed in order to overcome the worst case scenario – which would be a particularly heavy contamination of *Bacillus stearothermophilus* (> 10^6 cells/mL). It was initially planned to obtain a sample culture of *Bacillus stearothermophilus* from the University of Melbourne, School of Microbiology. The plan would then be to grow this culture up, and use it to contaminate a sample of polysaccharide and then use the method outlined in subproblem one to determine the effectiveness of pasteurization. However, the CRC for Industrial Plant Biopolymers management have strongly advised against this since they do not want to risk introducing *Bacillus stearothermophilus* colonies into the local environment in the Scale-Up Facility and subsequently create a long term problem. In hindsight, this is probably not an unreasonable request.

As an alternative, Food Science Australia (formerly CSIRO Division of Food Science and Technology) in North Ryde, Sydney, have a pasteurizer computer simulation model. It appears that this is a very sophisticated tool and when programmed with the right conditions (such as pH, protein content, polysaccharide content, physical data about he pasteurizer, etc.) it provides an accurate series of D-values for particular microorganism. In this instance it is envisaged that a grid of ranging temperatures and resonance times will be calculated for thermally resistant strains such *as Bacillus stearothermophilus, Bacillus cereus* and *Escheria coli*. Particular detail will be paid to the method of calculation employed and where their reference data for each strain was originated. This obviously assumes that Food Science Australia will be willing to provide such information. As a consequence of this, the final HACCP document will have contingency pasteurization regimes for worst case scenarios and these will have been legitimately validated.

Subproblem three.

The third aspect of the project is to quantify the affect of temperature upon the emulsifying polysaccharide being produced. The pasteurization process is the only time that the product is exposed to temperatures greater than about 40°C and consequently there is a necessity to determine if there is a detrimental affect upon the emulsification efficacy of the polysaccharide.

The emulsification efficacy can be measured by a reasonably simple method which is outlined in Appendix 5. The emulsifying polysaccharide is being touted as a replacement for Gum Arabic which is currently used extensively in the food industry, but is susceptible to radical price and quality fluctuations. Therefore this product is to be marketed as a standardized substitute without any of the fluctuations that Gum Arabic is susceptible to. Therefore, the CRC for Industrial Plant Biopolymers has devised a method for comparing our product to Gum Arabic. This method uses a correlation previously determined to yield an inferred droplet size – the target is 1.4μm which is achieved by a 20% w/v solution of Gum Arabic.

This test, abbreviated as the OD_{500} test, is the means by which the efficacy of the polysaccharide is determined. It is planned to test the efficacy of the emulsifying polysaccharide produced (after downstream processing) at a range of temperatures from about 60°C to 135°C whilst keeping the flow rate, and hence resonance time, t, constant. Since only one variable is being altered, temperature, it will enable the affect imparted by only temperature to be established. This will enable a product degradation (%) versus temperature curve to be established, since the OD_{500} will be measured before and after pasteurization for each of the samples generated at the various temperatures.

Due to the fact that this polysaccharide is still expensive to produce, it is extremely difficult to obtain the large quantities required for this experiment, and since customer evaluation and toxicology trials are currently being undertaken, there will not be enough polysaccharide available for the evaluation of the effect of varying the resonance time (as flow rate) whilst maintaining a constant temperature.

Confirmation of Results.

Once the ideal conditions for pasteurization have been established, a confirmation trial will be undertaken to ensure that adequate microbial destruction is occurring to protect human consumers from food pathogens whilst ensuring that the efficacy of the emulsifying polysaccharide is maintained.

This will involve a pasteurization trial at the ideal conditions and pretesting and posttesting the polysaccharide for both microbial activity (via standard plate counts at AGAL) and emulsification efficacy (using the OD_{500} method in-house). Upon satisfactory completion of this confirmation trial, the pasteurization process will be effectively validated and correctly documented and subsequently, satisfy the requirements needed to gain HACCP accreditation.

✳ *Except where stated otherwise, all experimental work and analysis will be performed by myself.*

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APPENDIX 1

ACTIVITY LIST

A. Microbial Destruction Trial #1 (various temperature & constant flow rate)
B. Analysis of Trial #1
C. Microbial Destruction Trial #2 (various temperature & constant flow rate)
D. Analysis of Trial #2
E. Microbial Destruction Trial #3 (various flow rate & constant temperature)
F. Analysis of Trial #3

G. Temperature vs Product Degradation Trial #1
H. Analysis of Product Degradation Trial #1
I. Temperature vs Product Degradation Trial #2
J. Analysis of Product Degradation Trial #2

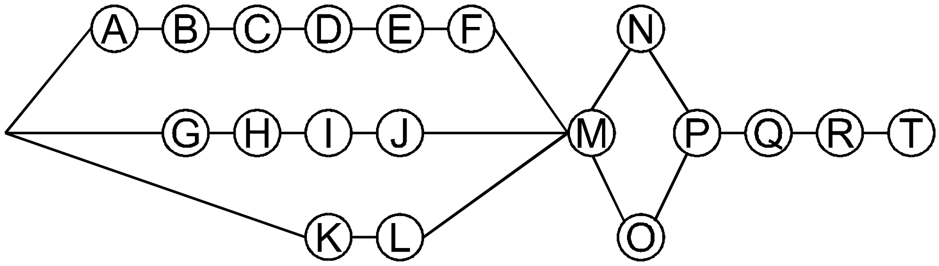
K. Computer simulation of thermally resistant pathogens
L. Interpretation of the Computer Simulation

M. Confirmation Trial
N. Microbial Analysis of Confirmation Trial
O. Analysis of Product Degradation

P. Write Final Report
Q. Prepare Report Copies and Presentation Material
R. Prepare Oral Presentation
S. Give Oral Presentation

Critical Path Analysis

Using the activity list above the network diagram is as follows:



The critical path is :

A-B-C-D-E-F-M-N-P-Q-R-S

This would, according to the timeline in the Gantt chart (see attached charts), take 102 days to complete the project at best.

Note to the Reader:
The Gantt charts that accompanied the original document could not be included with this version for technical reasons. We hope to provide a copy of them on the web at a later date.

APPENDIX 2

Resources and Costing

All equipment required for this research project is currently owned by the CRC for Industrial Plant Biopolymers and accordingly the availability of all equipment when required will not be a problem and any associated maintenance and depreciation costs will be in-kind contributions by the CRC for Industrial Plant Biopolymers.

- All consumables (such as disposable lab products and chemicals) required will be supplied by the CRC for Industrial Plant Biopolymers.

Estimated cost : $1000

- Outsourcing of Standard Plate Counts will be paid for by the CRC for Industrial Plant Biopolymers and performed by the

Australian Government Analytical Laboratories (AGAL)
51 – 65 Clarke Street
South Melbourne
Victoria 3205.
Contact: Julia Maczuga or Karen Baines

Estimated number of samples – 50 at $22 per sample.

Estimated cost : $1100

- Contract computer simulation of microbial destruction of thermally resistant microorganisms will be paid for by the CRC for Industrial Plant Biopolymers and performed by

Food Science Australia
Delhi Road
North Ryde
NSW
Contact : Edward Jansson

Estimated cost : $800

Human Resouces

Name	**Est Time**	**Hourly Rate**	**Total Cost**
Andrew Redman	200 hours	$50 per hour	$10 000
Dr. David McManus[#]	10 hours	$110 per hour	$1100
Dr. Tissa Habrakada[#]	10 hours	$110 per hour	$1100

[#] These are estimations of the time each of these two CRC for Industrial Plant Biopolymers staff will be involved in the project – mainly in discussions and receiving advice about this project.

The salary component of the resources is an in-kind contribution by the CRC for Industrial Plant Biopolymers.

SUMMARY

Total costs charged to the CRC for Industrial Plant Biopolymers (including in-kind contributions)	**$15 100**
Total costs charged to Deakin University	**NIL**
TOTAL COST OF PROJECT	**$15 100**

APPENDIX 3

Literature search methodology.

The first step taken in searching for relevant literature was to examine the reasonable selection of texts accumulated at work that may have covered this topic. Several were identified that had chapters within them that were particularly useful. The next step undertaken was to examine the references within these texts to investigate whether any of the references were likely to provide more in depth information. Several were identified and added to the list of journal articles to be obtained.

The next step was to do a search of electronic databases for relevant articles using some of the following keywords:

Pasteuri* (truncated to pick up spelling variations such as pateurizer, pasteuriser and pasteurization)
Sterili*
Arrhenius
Thermal Inactivation Kinetics
Thermal Death Time
D-value
Emulsi*
Microbial Inactivation
Death Kinetics

The following databases were searched using the above listed keywords:

- Applied Science and Technology Index
- Medline
- Academic Press Journals Online
- Cambridge Scientific Abstracts
- EI Compendex
- General search of the internet.

Each time a relevant abstract or title was found, it was added to the growing list. I soon developed a reasonable collection of abstracts. Occasionally, a full text article which was relevant was found and was subsequently printed. After the list of desirable articles was compiled, I obtained several of the articles from the University of Melbourne medical and undergraduate libraries (I generally obtained articles from common journals like Journal of Food Science). The remainder of the articles were obtained through the CRC for Industrial Plant Biopolymers librarian who was able to obtain the most obscure references.

APPENDIX 4

Pasteurizer Details and Standard Operating Procedures

APPENDIX 5

Method for Performing the OD_{500} Assay.