OPTIMIZED PRODUCTION OF XANTHAN GUM FROM SWEETS AND BAKERY WASTE LEFTOVER USING XANTHOMONAS STRAINS *Pseudo-Xanthomonas* Sp. (NCCP-1183) & *Rhizobium* Sp. (NCCP-1189)

By
Muhammad Hammad Azeez
00000274264

Supervisor
Dr. Waqas Qamar Zaman

Institute of Environmental Science & Engineering
School of Civil & Environmental Engineering
National University of Sciences & Technology
Islamabad, Pakistan
2022

OPTIMIZED PRODUCTION OF XANTHAN GUM FROM SWEETS AND BAKERY WASTE LEFTOVER USING XANTHOMONAS STRAINS *Pseudo-Xanthomonas* Sp. (NCCP-1183) & *Rhizobium* Sp. (NCCP-1189)

By
Muhammad Hammad Azeez
00000274264

Supervisor
Dr. Waqas Qamar Zaman

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Environmental Science

Institute of Environmental Science & Engineering
School of Civil & Environmental Engineering
National University of Sciences & Technology
Islamabad, Pakistan
2022

Approval Certificate

Certified that the contents and form of the thesis entitled

"Optimized production of Xanthan gum from Sweets and Bakery waste leftover using *Pseudo-Xanthomonas* Sp. (NCCP-1183) & *Rhizobium* Sp. (NCCP-1189) strains"

Submitted by

Mr. Muhammad Hammad Azeez

Has been found satisfactory for partial fulfillment of the requirements of the degree of Master of Science in Environmental Engineering.

Supervisor: .....

Assistant Professor

Dr. Waqas Qamar Zaman

IESE, SCEE, NUST

GEC Member: ……

Dr. Muhammad Arshad

Professor

IESE, SCEE, NUST

GEC Member: ……..

Mr. Musharib Khan

Assistant Professor

IESE, SCEE, NUST

Acceptance Certificate

It is certified that final copy of MS/MPhil Thesis written by Mr. M. Hammad Azeez (Registration No: 00000274264) of IESE (SCEE) has been vetted by the undersigned, found complete in all respects as per NUST Statues/Regulations, is free of plagiarism, errors, and mistakes, and is accepted as partial fulfillment for the award of MS/MPhil degree. It is further certified that necessary amendments as pointed out by GEC members of the scholar have also been incorporated in the said thesis.

Supervisor: ……
Asst. Prof. Dr. Waqas Qamar Zaman
Dated: …….

Head of Department: ……

Dated: ……

Principal: …….

Dated: ……..

Declaration Certificate

I declare that this research work titled "**Optimized production of Xanthan gum from Sweets and Bakery waste leftover using *pseudo-Xanthomonas* Sp. (NCCP-1183) & *Rhizobium* Sp. (NCCP-1189) strains**" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources as been properly acknowledged/referred.

Student Signature: ……..

Student Name: …… Mr. M. Hammad Azeez ……

Date: ….…….

Plagiarism Certificate

This thesis has been checked for plagiarism. Turnitin endorsed by the supervisor is attached.

Signature of student: Mr M. Hammad Azeez……..

Signature of Supervisor: ……..

Dedication

This research is dedicated to my loving, caring, and industrious parents and my best friend Junaid Qaisar whose efforts and sacrifice have made my dream of having this degree a reality. words cannot adequately express my deep gratitude to them.

"O My Sustainer, Bestow on my parents your mercy even as they cherished me in my childhood".

Acknowledgment

I am thankful to my Creator *Allah subhanahu* Wa Ta'ala to have guided me throughout this work at every step and for every new thought which You setup in my mind to improve it. Indeed, I could have done nothing without Your priceless help and guidance. Whosoever helped me throughout the course of my thesis, whether my parents or any other individual was Your will, so indeed none be worthy of praise but You.

I am profusely thankful to my beloved parents who raised me when I was not capable of walking and continued to support me throughout in every department of my life.

I would also like to express special thanks to my supervisor Dr. Waqas Qamar Zaman for his help throughout my thesis and to my GEC members Dr. Muhammad Arshad & Dr. Musharib Khan.

I would also like to pay special thanks to Miss Anisa for her tremendous support and cooperation in the Lab. Without her help I wouldn't have been able to complete my thesis. I appreciate her patience and guidance throughout the whole thesis. I am also thankful to Amir Younas and Syed Daniyal Ali for their support and cooperation.

Finally, I would like to express my gratitude to all the individuals who have rendered valuable assistance to my study.

Table of Contents

List of Figures

List of Tables

List of Abbreviation or Keywords

ANOVA	Analysis of Variance
BBD	Box-Behnken Design
BW	Bakery Waste
BW	Bakery Waste Hydrolysate
DNS	Dinitro-salicylic acid
MC	Moisture content
NARC	National Agriculture Research Centre
NCCP	National Center for Culture Preparation
RSM	Response Surface Methodology
Sp.	Species
TS	Total Solids
V.F	Variable Factor
WHO	World Health Organization
XG	Xanthan Gum

Abstract

Xanthan Gum is a bacterial hetero polysaccharide bio-synthesized product that is widely used in food, pharmaceutical and cosmetic industries due to its peculiar functional properties. This study motivates the optimized production of xanthan gum through bakery waste using different strains. Two different types of strains, i.e. *pseudo-Xanthomonas* and *Rhizobium,* were employed for the current research work. At first, the hydrolysis of bakery waste was done using sulfuric acid. Later characterization was done by the DNS method to find the sugary content of the bakery waste and bakery waste hydrolysate. Since the study focuses on optimization of process parameters; therefore, Response Surface Methodology (RSM) in Design Expert Pro was used to analyze the three factorial factors. The selected parameters were bakery waste hydrolysate, Inoculum and agitation, with the ranges 0.5-1.5 %, 5-15% and 150-250 rpm, respectively. The Box-Behnken Design suggested a set of 15 experiments for the fermentation process. The software used the obtained results to calculate the range yield and find the best combination of factors involved in the optimization study. The results showed that the total sugary content of bakery waste hydrolysate was determined as 50mg/ml of the total collected waste; however, the moisture content was 37% for collected bakery waste and 52% for bakery waste hydrolysate. The yield was concluded as a response factor and analyzed using the linear aggression model for *pseudo-Xanthomonas sp.* NCCP-1183 and quadratic aggression model for *Rhizobium sp*. NCCP-1189. The optimized value of *pseudo-Xanthomonas* strain formed at 0.5% Acid Hydrolysate, 5% Inoculum and 200rpm. However, *Rhizobium* strain optimized at 0.5% Acid Hydrolysate, 15% Inoculum and 200 rpm. The *Rhizobium* strain was found relatively better with a yield of 12.6376g/l than the *pseudo-Xanthomonas* strain with a yield of 11.0806g/L, respectively.

Key Words: *Xanthomonas* Strains, *Rhizobium* strain, Response surface methodology, Bakery waste, Xanthan gum

CHAPTER 1

1 INTRODUCTION

This research work has been presented in two parts. The first part is related to the detailed analysis of raw bakery waste material. The objective of this part is to find out the basic raw material constituted by determining the moisture content (MC), total solids (TS), total sugar content and reducing sugar. The second part includes the biosynthesis of this previously determined xanthan gum and to produce xanthan gum in an optimized way with the use of two different types of microbial strains. After that comparison is made between these two strains in terms of optimized yield and yield efficiency.

1.1 Background

Xanthan Gum is a bacterial hetero polysaccharide bio-synthesized by aerobic fermentation of sugars using gram negative bacteria such as *pseudo-Xanthomonas sp.* NCCP-1183 & *Rhizobium sp*. NCCP-1189. It is anionic, highly viscous, and soluble in cold and hot water showing best dissolvability in cold water (Mohsin et al., 2018). Xanthan has both properties of reducing and stabilizing agent. It is the first exo-polysaccharide which is non-toxic, safe to use (biodegradable) and hydrophilic biopolymer (Mohsin et al., 2018). It has been largely used in the food industry for many important reasons, including viscosity enhancer, temperature & emulsion stability, pseudo plastic rheological properties and thawing stability. Because of many functional properties, it is widely used in food, pharmaceutical and cosmetic industries (Palaniraj, 2011). It owns a unique principal characteristic to modify the rheology and flow behavior of solution (Papagianni et al., 2001). Due to these inherited characteristics, the production of xanthan gum reached to about 160,000 tons per year and its market value exceeded to 400 million dollars (Demirci et al., 2019).

Xanthan gum, a very well-known biopolymer, is commercially produced by fermentation of commercial grade sugars such as glucose or starch. However, during recent years, the production of xanthan by using inexpensive substrates and waste material has gained so much interest of industrialists and researchers (Gunasekar et al., 2014). Carbohydrate sources such as sucrose, sugarcane molasses and whey have been

successfully used in the production medium. Other than these, alternative cheap substrates have also been utilized, such as barley, corn flour, acid whey, hydrolyzed rice, coconut juice sugar, sugar cane and sugar cane molasses etc., but glucose is considered as the best with regard to good product quality and best yield product (García-Ochoa et al., 2000; Palaniraj, 2011). Various low-cost waste materials have been used to produce xanthan gum such as kitchen waste, orange peel waste, tapioca pulp, residue of apple juice and cheese whey, etc. and these were very successful for the production of xanthan gum (Mohsin et al., 2018).

In this regard, bakery waste material can be used as a sole substrate to produce valuable biofuels and bio-chemicals. Bakery waste is considered as the most common waste in the Europe and many Asian countries (Melikoglu & Webb, 2013). It is the major food waste and millions of tons of this waste are disposed each year, especially in China around 1.5 million tons of bread waste is disposed to the land on annual basis. Although, the amount of this waste is very high but there are very few attempts to utilize this waste for conversion into valuable compounds. Till now, utilization of this waste into biofuel, succinic acid, lactic acid, enzymes and bio-hydrogen have been successfully produced in previous reports (Ozdal & Kurbanoglu, 2018). Xanthan gum was also produced by utilizing bread waste using enzymatic conversion, but the conversion rate is considered low. Therefore, there is a dire need to utilize this waste for improving the production and total yield of xanthan gum from bakery waste. The production of xanthan gum from different sources is shown in Figure 1.1.

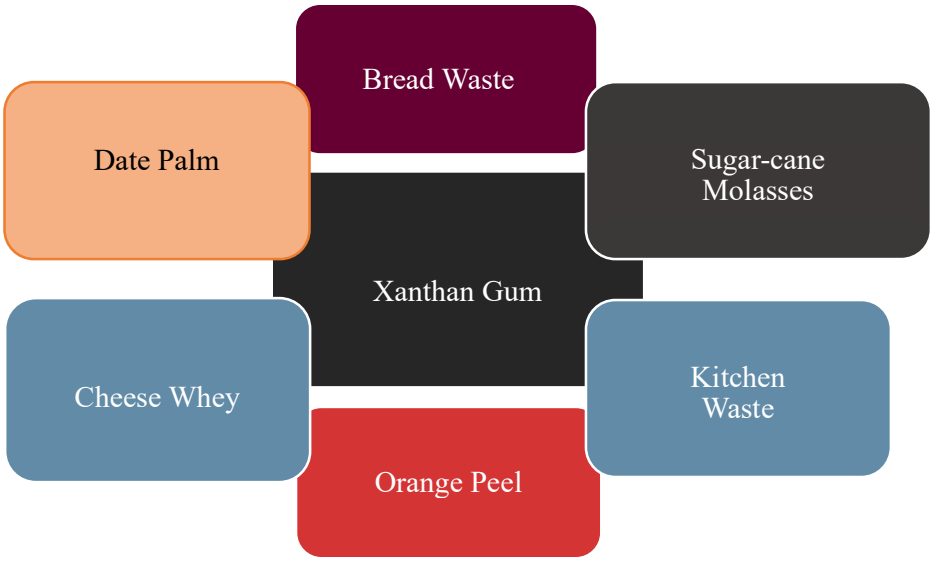


Figure 1.1: Xanthan Gum generated from different waste sources

1.2 Scope

The current study deals with the three-level, three-factorial Box-Behnken experimental design (Design Expert 13) to study the combined influence of Hydrolysate, Inoculum volume and stirring on Xanthan yield and optimized range. The uniqueness of this research work was combined optimization of above mentioned three operating parameters as well as proving the significance of experimental results statistically by analysis of variance (ANOVA) in Response Surface Methodology (RSM). Furthermore, 15 experimental trials performed for both strains separately during the study were considered finished when xanthan yield approaches to its maximum value. In addition, no previous study was investigated related to these three different independent factors i.e. bakery waste hydrolysate WH, Inoculum volume and agitation considering xanthan yield as response parameter through Box-Behnken RSM. The optimal yield obtained after experimental work may serve as a guideline for effective utilization of bakery waste during fermentation process and the desired inoculum range for better quality xanthan production.

1.3 Aims of Study

The main objectives of the current study are as follows

- To effectively utilize the bakery waste by using green path recycling process.
- To enhance the mass production of xanthan gum using Response Surface Methodology (RSM).
- To simulate the model and get the desired results.

1.4 Limitations of the Study

Following are some of the limitations of the current study

- This study only considered bakery waste as a source of xanthan production and other types of waste were not used for xanthan production.
- Glucose concentration factor was not directly involved in optimization technique.
- The pyruvate & acetyl content of xanthan gum was not determined in the current study.
- This study also did not find the viscosity of the xanthan gum.

- This study is limited only to the acid hydrolysis of the Bakery waste and did not consider the enzymatic hydrolysis.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Introduction

Xanthan Gum, a polysaccharide used for industrial purposes as food additive in creams, fruit juices, chicken, fish and meat as well as thickener in syrups and ice cream. Non-food items are paints and lotions. Xanthan has a major stability in prevention of ingredients from separating. To initiate the green path processing of products, many industries are finding new ingredients which are environment friendly. Majorly, production of xanthan gum is taking out in conventional stirred tank fermenters but on laboratory scale orbital shaking flasks are used under controlled conditions. Xanthan gum quality can be maintained by selecting the most suitable Bacterial strains and fermentation conditions such as Xanthan Gum produced by Xanthomonas sp. Such as *Xanthomonas malvacearum and Xanthomonas phaseoli* during aerobic fermentation , *Xanthomonas pelargonii* and *Xanthomonas (X.) campestris* (Habibi & Khosravi-Darani, 2017). The suitable Stains are majorly *pseudo-Xanthomonas* strains, most likely to be accepted. Factors affecting the fermentation are temperature, aeration, agitation, pH, substrate, nitrogen and carbon sources which determine the quality, production, molecular mass and composition of the xanthan gum (Palaniraj, 2011). There is no continuous process in the production of xanthan gum through microbial activity at any industrial scale. However, industrial xanthan production often uses substrates (sucrose and glucose) as a carbon source, and these substrates form high productive quality products. The xanthan gum market value exceeds from 400 million dollars whereas the usage has increased from 6 to 12 % per year in various sectors and reached up to yearly production of 150 to 160 tons/year.

2.2 Food Safety

In US the major producers of xanthan gum are Pfizer and Merck. So, the production of xanthan gum was legalized for commercial use as food additives (emulsifier & stabilizer) by the U.S Food and Drug Authority without any restrictions (Ghashghaei et al., 2016). Xanthan gum, the first naturally occurring biopolymer to be mass-produced

on a large scale commercially. For this purpose, the gum's safety has been thoroughly investigated. For acute toxicity test, the effect of xanthan gum was studied on animals. In those investigations, xanthan gum doses up to 19 g/kg body to weight had no discernible harm. The digestibility tests revealed the non-digestibility in humans whereas the caloric test shows facilitation of food through the upper intestinal tract. However, in some circumstances, it caused increased bowel frequency. When taken in significant amounts, increased stool output, softer stools, increased gas, and altered gut microbes occur. Long-term feeding studies on albino rats and beagle dogs found no significant differences in developmental parameters between test and controls (xanthan gum in the diet at dosage levels of 0, 0.25, 0.50, and 1 g/kg body to weight per day for 2-year studies on albino rats and at dosage levels of 0, 0.25, 0.37, and 1 g/kg body to weight per day for 2-year studies on beagle dogs.

2.3 Xanthan Structure

The Xanthan molecule's fundamental chain is made up of a 1, 4-linked D-glucose backbone with a charged tri-saccharide side chain comprising a residue (glucuronic acid) between two mannose entities at every alternate glucose residue's (C-3) position. The terminal β—D-mannose is coupled to the glucuronic acid through β1,4 and the glucuronic acid is attached to the (α-D)-mannose through α-(1,2) (Wang et al., 2017). A pyruvic acid moiety is connected to the (O4) and (O6) locations on around half of the terminal mannose residues via a ketal linkage. At the (O6) position of the non-terminal mannose, acetate groups are present as substituents. Acetate groups typically originates on 60-70% of internal mannose residues, while pyruvate residues are found on 30–40% of terminal mannose residues (Preichardt & Klaic, 2016). Since its introduction in the 1960s, the most desired high molecular weight anionic biotechnological polysaccharide, xanthan gum (XG), has been widely used in a range of industrial applications. The XG molecule has a backbone of (l→4)-linked β-d-glucopyranose (like cellulose) and a three-sugar side chain with two mannose and one glucuronic acid connected at (C-3) to alternate glucose residues Side chains and characteristics of the gum's most desirable properties, such as its outstanding hydration property, make up about 60% of the molecule. The molecular weight of xanthan is estimated to be between 2×10^6 and 50×10^6 Da, although the actual figure is unknown (Berninger et al., 2021).

The chain structure of xanthan linked through oxygen is shown in Figure 2.1.

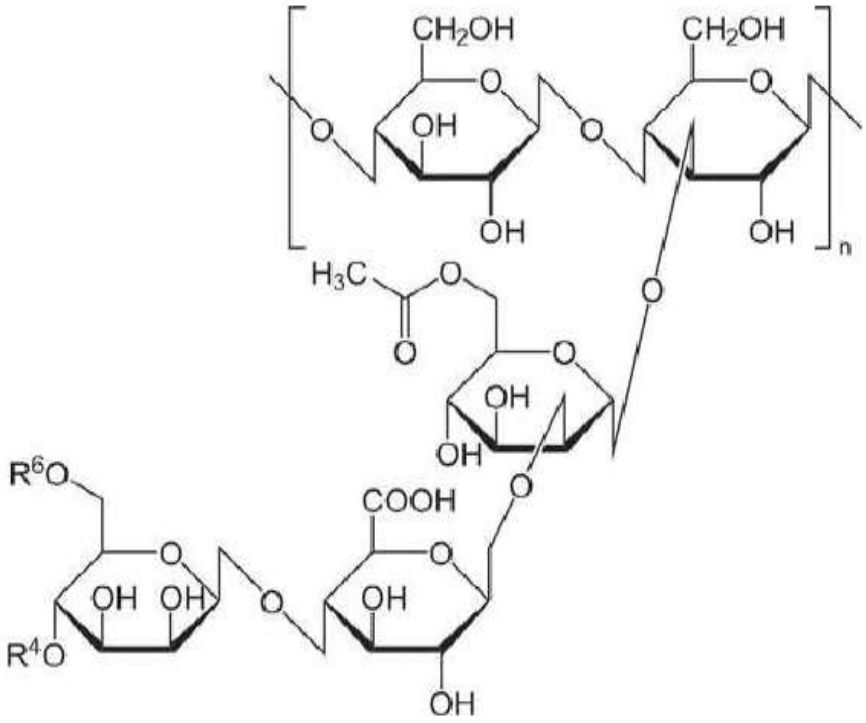


Figure 2.1: Xanthan structure in a chain of carbohydrates linked through oxygen group to form a chemical formula of $C_{35}H_{49}O_{29}$

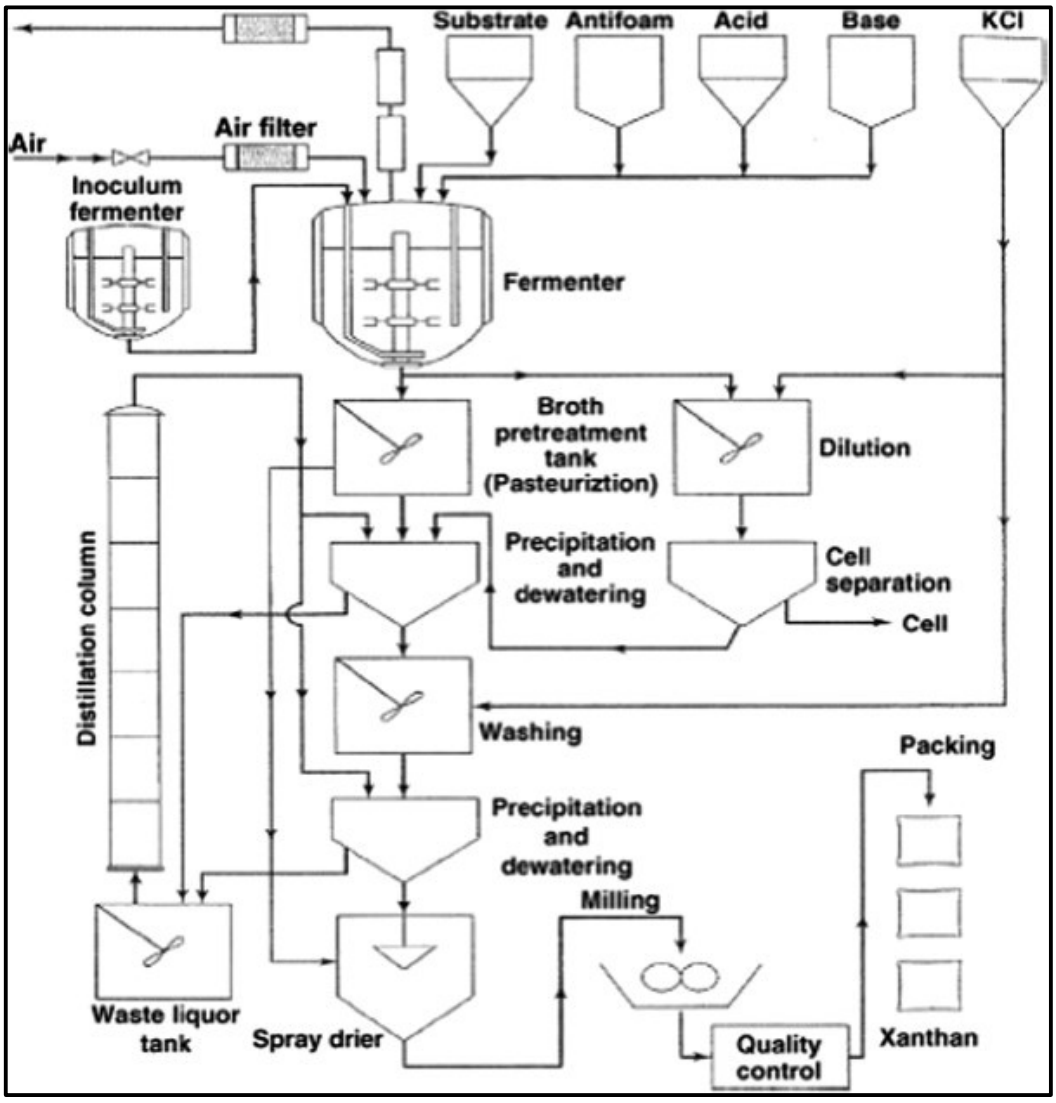
2.4 Xanthan Production

Xanthan gum is made from glucose and mostly invert sugars, with companies favoring batch over continuous production (Leela & Sharma, 2000; Letisse et al., 2001). X. campestris inoculums are created in appropriate fermentation mediums processing standard batch through involuntarily agitated vessels in a normal xanthan production method. The aerated culture that passes through the aerobic process normally kept at the following pH 7, Temperatures: 28–30 °C, an aeration rate of at least 0.3% (v/v), and a particular power input of 1 kW-m^3. To disseminate the injected air equally throughout the medium, proper agitation is required. The rate at which the movement of nutrients alongside the cell membrane is improved, by agitating the medium results in support of the microorganism's growth rate.

Xanthan is manufactured on an industrial large scale using low-cost substrates and fertilizers. Sucrose, whey (Silva et al., 2009) and sugarcane molasses, have all been

successfully utilized as carbohydrate sources in the manufacturing medium. Also, Whey contains enough nitrogen and growth nutrients. For cost-effective conversion of carbon "C" sources to desirable polyose synthesis, a high carbon to nitrogen ratio (C/N) is required. Ammonium or nitrate salts are suitable inorganic nitrogen sources, as are several complex nitrogen sources such as soy-meal peptone, yeast extract and soybean whey. Cereal grains are used to create beneficial output. Batch cultivation with different conditions is preferred in most cases (Psomas et al.). Simple synthetic media, on the other hand, can be employed. It takes two days to cultivate a batch of plants. Because Xanthomonas species are known for their variability, maintaining a Xanthomonas campestris stock culture is essential for xanthan synthesis uniformity. Polysaccharide accumulation begins during the early stages of growth and continues afterward. During fermentation, the pH lowers due to the creation of organic acids. The generation of xanthan is dramatically reduced when the pH dips below 5.0 (Gumus et al., 2010). As a result, a buffer (acid or base) must be added during the process to keep the fermentation mixture at the ideal pH of 7.0 (Kerdsup et al., 2011).

The fermentation process takes around 100 hours and transforms about half of the glucose/sucrose and fructose into the finished product. Inoculum making is divided in various stages, each of which necessitates the use of a reactor with a capacity ranging from 10 L for the initial germ to 100 m^3 for the building stage, during which the capacity is typically increased by tenfold. Cells would expand exponentially as the fermentation progressed, resulting in rapid nitrogen consumption. After the fermentation stage, there would be a series of multi-step downstream operations. Figure 2.2 shows the xanthan gum manufacturing process that involves multiple downstream phases (Rosalam et al.,). The xanthan gum is usually precipitated using a lot of alcohol, then sprayed dry or re-suspended in water and re-precipitated. When cell-free xanthan gum is required, cell centrifugation is facilitated by diluting the fermentation broth to improve cell separation. Cell separation from a highly viscous xanthan solution requires a lot of time and is expensive procedure. (Balows et al., 1992). A common method is to combine alcohol and salt to increase precipitation by creating reverse effect charges. The xanthan gum acquired in wet solid form would be dewatered and cleaned to reach the requisite purity.



.

Figure 2.2: Process Flow Diagram of Xanthan Gum Production (Adapted from Palaniraj and Jayaraman, 2011)

2.5 Xanthan Applications

Xanthan gum can thicken and stabilize emulsions, foams, and suspensions. Xanthan has the applications in food (e.g., as stabilizer, emulsifier and thickening agent) (Reinoso et al., 2020), pharmaceutical (Singhvi et al., 2019), cosmetic (e.g., as binding agent) (Balkrishna et al., 2018), and biomedical (Nagaraja et al., 2021). By consistently suspending the solid component in fungicides, herbicides, and insecticide formulations, xanthan has been employed in agriculture as well to increase flowability. Drift is reduced, and pesticide cling and persistence are increased, thanks to the unique rheological features of xanthan gum solution. USEPA has recently authorized several

"tolerance exemptions" for the use of xanthan gum as a surfactant in pesticide formulations. Because of its capacity to spread and hydrate quickly, as well as the fact that it is non-polluting and produces good colour output, xanthan is also used in jet injection printing. Xanthan gum has recently been incorporated to the composition of new generations of thermal coatings to meet the problems of generating environmentally friendly goods. In the petroleum industry, xanthan gum is utilized in oil drilling, fracturing, pipeline cleaning, and work-over completion. Xanthan gum is also useful as a drilling fluid additive because of its high compatibility with salt and resistance to thermal breakdown. Because of the pseudo plasticity of its solutions, low viscosity near the drill bit, where shear is high, and high viscosity in the annulus, where shear is low, would be the consequence. As a result, xanthan performs two functions: it speeds up bit penetration while also suspending cuttings in the anchor rings. Approximately two barrels of oil are left in the ground for every barrel produced. As a result, xanthan gum will be used extensively in enhanced-oil recovery (EOR) in the coming years. The primary approach used is to improve oil and water separation to increase oil retrieval. However, the quality of the xanthan gum is important, as significant contaminants will make refining the oil more difficult. Micellar polymer flooding is a xanthan gum-based ultimate oil recovery technique. The surfactant slug is pushed into permeable reservoir rock with the polymer thickened brine to mobilize residual oil; the polymer stops the drive water from bypassing the surfactant band and offers good area sweeping. Other specialized applications for xanthan gel include removing rust, welding rods, wet slag, and other debris from gas pipes. Many more xanthan gum applications are likely to emerge in the future. Non-Newtonian qualities, especially high yield viscosity at lower concentrations (500–2000 ppm), minimal compassion of viscosity to salinity fluctuations, mechanical degradation resistance, temperature stability (up to 91 °C) and a biodegradability substance make xanthan gum an environmentally acceptable product (Palaniraj, 2011). Xanthan gum has much industrial importance, as it has applications on small and medium scale industries. In food items it is used as a thickening agent like in fruit juices, ice cream and for baking purpose because of adhesive properties. Xanthan gum is a good stabilizing, thickening, and emulsifying agent. As it has numerous uses, xanthan can be used in food and non-food items. Also, it has several pharmaceutical uses. Xanthan is very useful in food business just because of its thickening, stabilizing and jelling properties. Xanthan can

be used in ice creams and other milk-based items. Confectionery products and noodles can also be the food making items using xanthan. Salad dressing is a far a good way step for routine usage of xanthan on commercial scale. Non-alcoholic beverages now are days are also using xanthan for its stable property capability. Sweets and noodles, salad dressings, and nonalcoholic beverages, all contain xanthan gum. Soups, sauces, gravies, drinks, syrups, relishes include it as a suspending agent somehow dressings and cake mixes contain it as a suspending agent. In Pakistan most of the xanthan gum is imported subject pharmaceutical industries are also using xanthan gum as chemical with name Vanzon NF. Xanthan is also used in paints, toothpastes, and lotions.

2.6 Economic Aspects

In order to save money, subsidiary sources of needed substrate, such as hydrolyzed rice, maize flour and sugar cane have recognized to be effective (Palaniraj, 2011). These days, commercialized xanthan production has shifted to using low-cost substrates such as (Silva et al., 2009), crude glycerol (Rončević et al., 2020), kitchen waste (Li et al., 2016), olive-mill wastewaters (López et al., 2001) and tapioca pulp. Additionally, experiments centered in response surface methodology (RSM) on an optimizing tool for further process economization have been done for maximizing production. The cost of the substrate showed a big impact on the fermented xanthan gum production. In terms of cost-effective xanthan production, using a less expensive fermentation medium rather than a more expensive one will almost certainly yield superior results. Bakery waste is high in soluble and insoluble carbohydrates, particularly fermentable sugars. During the creation of sweets, millions of tons of bakery waste are generated each year (10–25 million tons), with 20% of the sweets and biscuits ending up as waste in the form of useless material. 65 percent of the total volume of xanthan produced worldwide is used in the food business, 15% in the oil sector, and roughly 20% in other applications. Such demand is growing, with an annual growth rate of 5–10 percent predicted. China (Fufeng Group, Shandong Province, and Deosen Biochemical, Zibo City) and Austria (Fufeng Group, Shandong Province, and Deosen Biochemical, Zibo City) are the two largest manufacturers of this raw material. The local market of Pakistan consumes approximately 175 tonnes of xanthan gum per year, according to Pak trade data. Because there are no xanthan gum producing companies in Pakistan, the

need is met entirely by multinational enterprises. As can be seen, manufacturing these goods locally would help some countries develop and empower themselves.

CHAPTER 3

3 METHOD AND MATERIALS

An optimization technique needs validation. This validation can be done through re-running the experiments in the optimized condition. Research Surface Methodology is used for this purpose. In RSM the best suitable results are selected and validated through re-running the experiment in the desired conditions.

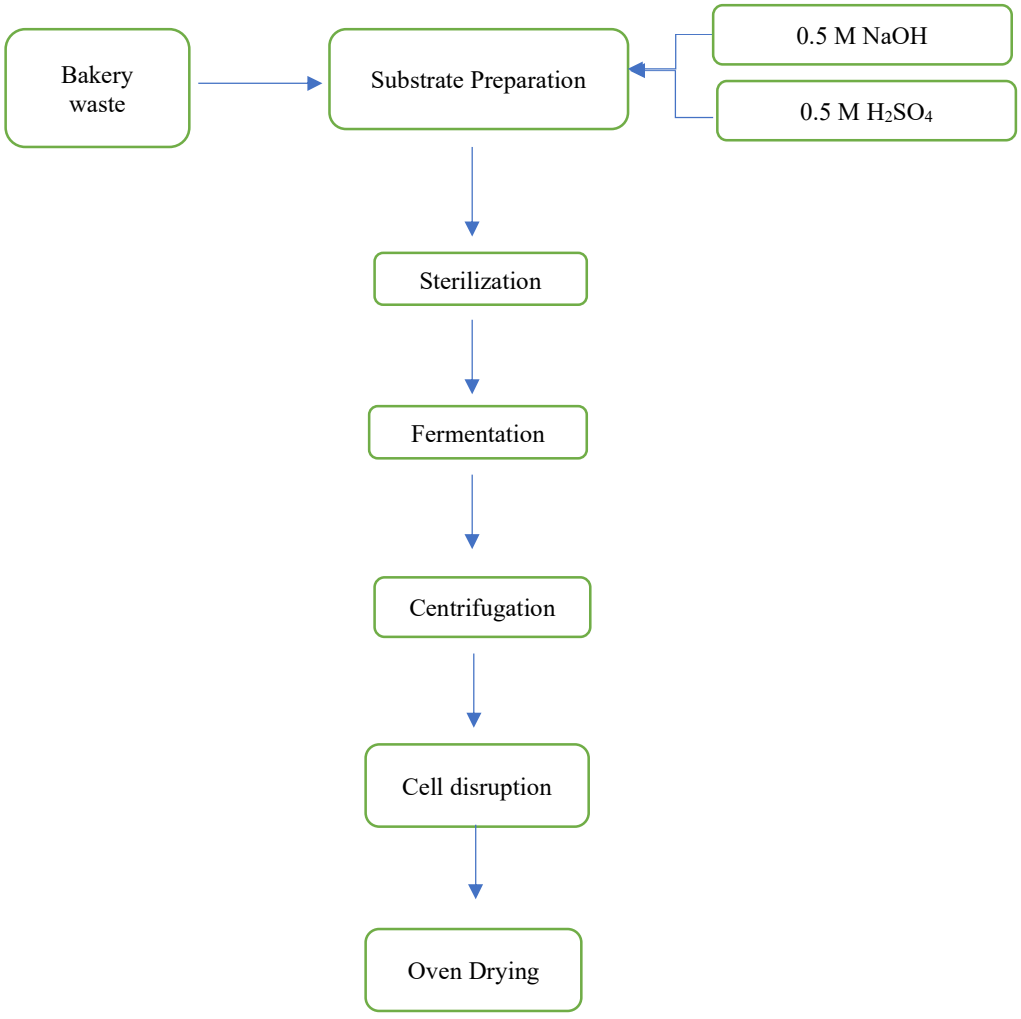


Figure 3.1: Methodology Step for Xanthan production using Xanthomonas strains

3.1 Study area for collection

The bakery waste was collected from the Rawalpindi city. The various locations of the collection points are shown in the figure. Bakeries were targeted for this study instead of their warehouses. The location map of study area and selected waste collection sites are shown in figure 3.2

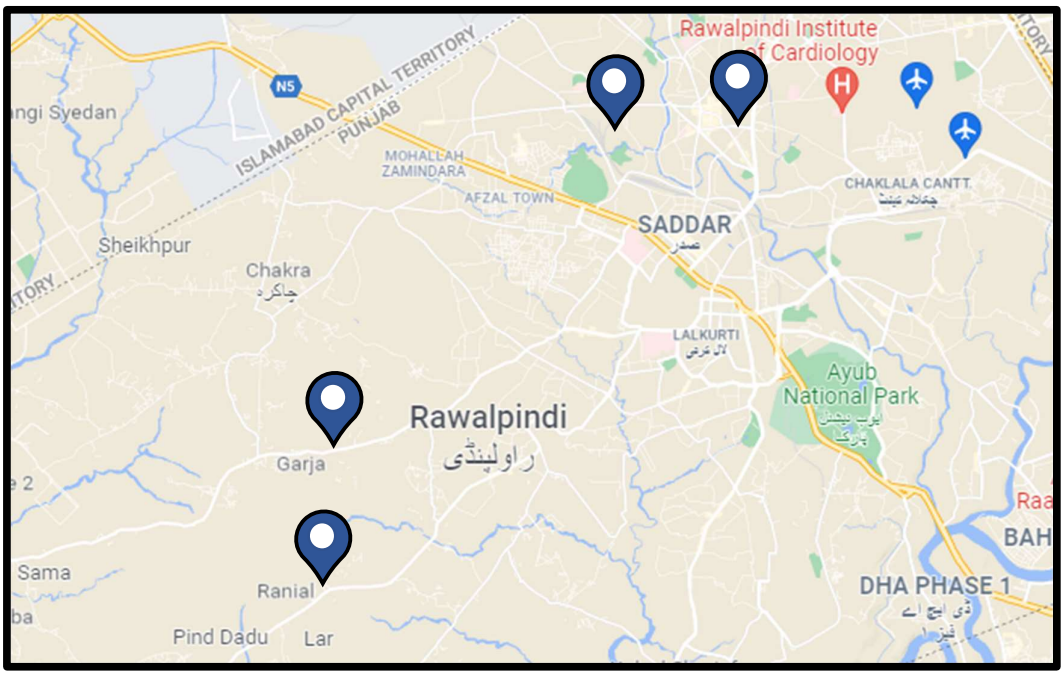


Figure 3.2: Map of the waste collection points (Rawalpindi City)

The purpose of selecting bakery waste from different areas of Rawalpindi is to collect the maximum waste for maximum sugary content. The bakery waste does not contain any of dried fungal breads, rotten eggs, & expired material. The Segregation of bakery waste was done at the spot.

3.2 Waste Collection Method

Three steps were involved in this process of waste collection

- At first, the permission letter was obtained from NUST for acquiring Bakery waste.
- After that, the bakery waste was collected from various bakeries at different locations from the Rawalpindi city.

- Finally, the collected waste was transported to Environmental Biotechnology Laboratory, Institute of Environmental Sciences and Engineering (IESE), National University of Sciences and Technology (NUST), Islamabad.

3.3 Characterization of Bakery Waste

3.3.1 Determination of reducing sugar & total Sugar

Many of the reagents are reduced when sugars are reduced. A reducing sugar is one that generates an aldehyde or ketone in a basic solution. 3, 5-dinitrosalicylic acid (DNS) is converted to 3-amino-5-nitrosalicylic acid, which is the reduced form of DNS, by the aldehyde group of glucose. During the reaction, water is used up as a reactant, and oxygen gas is released. At 540 nm, the production of 3-amino-5-nitrosalicylic acid causes a shift in the amount of light absorbed.

Total sugar and reducing sugar are determined using the 3, 5 Dinitro-salicylic Acid (DNS Method). A spectrometer and DNS reagent were used in the procedure. The DNS reagent is made by combining 10 grams of 3, 4-dinitrosalicyclic acid with 200 milliliters of distilled water. NaOH was dissolved in 150 mL of distilled water after continuous stirring. To get a clear solution, the mixture was incubated at 50 degrees Celsius. Potassium sodium tartrate hydrate was slowly added until the entire 403g of $KNaC_4H_4O_6.4H_2O$ was dissolved. The entire solution was filtered out in the end. And kept at 4 °C in an amber bottle for further use.

The procedure involves as

- At first glucose standard solutions were prepared ranging from 0.2-2.00 µmol/ml in 0.05 M acetate buffer (pH 4.8).
- 1 mL standard solution was added in each tube. Each tube was filled with 0 0.05 M acetate buffer (pH 4.8).
- Preparation of unknown samples was carried out in an appropriate dilution.
- 1 ml of 0.05 M acetate buffer (pH 4.8) was added to each tube and stirred.
- 3 mL of DNS reagent was poured in each test tube and thoroughly mixed.
- All the tubes were boiled for 5 minutes using water bath.
- The tubes were allowed to cool to room temperature and the absorbance at 540nm was found. The absorbance of unknown dilution helped in determining the conc. of unknown sample.

3.3.2 Determination of Moisture Content

Moisture content was determined by drying the bakery waste solution in oven at 105 degrees Celsius for 24 hours duration Percentage was fined by the formula as mentioned.

$$\mathrm{MC} = \frac{\text{weight before drying } - \text{weight after drying}}{\text{weight after drying}} \times 100$$

3.4 Response Surface Methodology

Using design expert pro version 13, RSM to be taken out through Box-Behnken Design (BBD). BBD uses three center nodes and 12 middle edge nodes to fit a second order equation. Both BBD and CCD become full factorial with 3 extra samples taken at the center. Points were placed on the midpoints of the edges of the cubical design zone, as well as in the center, in Box-Behnken designs. Three inputs given as Bakery waste Hydrolysate (0.5-2.5%), inoculum volume (5-15%), Agitation rate around 150 to 250rpm.

3.5 Hydrolysate Preparation

Hydrolysis of bakery waste was being done using Sulfuric acid. 50 gram of bakery waste was taken in 250ml of culture bottles. According to RSM the number of experimental runs for each strain were 15. Each experimental run contained fixed amount of sulfuric acid in 50 gram of bakery waste distilled to 100ml of solution using deionized water. Four bottles were prepared with sulfuric acid 0.5% each. Seven bottles were prepared while taking 1.5% sulfuric acid. Other four bottles contain 2.5% of sulfuric as recommended by the Box-Behnken Design in Design Expert Pro. All the 15 bottles were incubated at 121°C for 1 hour. 3 g of powdered activated carbon was added in each bottle after inoculation to remove any dissolved inhibitor by keeping culture bottles in orbital shaker for 2 hours. Filtration of this solution was carried out through 0.45µm filter paper to remove any undissolved substrates. Later, this Bakery waste Hydrolysate was stored in Refrigerator at 4°C, to be used as fermentation media.

3.6 Fermentation Process

In recent years, starch and molasses have been employed as feedstock for fermenting simple sugars into Xanthan Gum. The presence and removal of various by-products of

hydrolysis processes and yeast metabolism, such as hydroxymethyl furfural (HMF), were the key operational variables impacting xanthan output and fermentation efficiency (Hu et al., 2012). However, choosing the right fermentation medium and yeast, detoxifying the hydrolysate, and separating/removing the fermentation by-products helps to boost fermentation productivity. The carbon source, in instance, accounts for up to 60% of the operating costs. As a result, high substrate conversion yields are necessary, high productivity procedures must be established, and the use of a low-cost, convenient carbon source must be provided. This explains the widespread use of molasses, whey, or industrial leftovers as well as initiatives to employ fossil fuels as substrates, depending on local availability. Four strains were gifted to NUST from NARC (National Agriculture Research Center). Two of them shows proper growth. *Pseudo-Xanthomonas* NCCP-1183 and *Rhizobium* sp. NCCP-1189 were selected for fermentation. Both strains were grown on agar slant media for duration of 2 weeks. After 2 weeks re-growth of these strains is done & the process continues. Best culture was selected from the agar slant media and this media was inserted in the inoculum and incubated for 24 hours to get the total growth of 10^9mg/ml cells. Firstly 40 gram of Bakery Waste Hydrolysate ranging from 0.5 to 1.5% acid was taken separately in 1000ml of conical bottles. These bottles were inserted with inoculum ranging from 5 to 15 percent of the total solution. Agitation of these bottles was done at different RPM's, which is 150, 200 & 250rpm. The fermentation time was kept 72 hours as after 72 hours the cells shows constant growth. The fermentation process was carried out at 32 degrees Celsius while maintaining the pH 7. 10 ml of sample was collected after 72 hours and centrifuged at 10,000 rpm, 4 °C for a total duration of 30 minutes. The biomass was collected from the bottom whereas the remaining substrate was washed with ice cold iso-propanol in 1:3 (volume substrate/volume iso-propanol) proportion. The resulting solution was kept at 4°C for a duration of 24 hours and centrifuged again at 6000 rpm for 30 minutes to precipitate out xanthan gum. The resultant gum was then dried in dry oven at 55°C for 24 hours, to remove all the moisture present in the resulted Xanthan Gum.

3.7 Optimization

The optimization technique has dependency on the number of factors involved. In fact, the more variables that influence a desired result, the more complex the optimization process gets. In such instances, Response Surface Methodology (RSM) is frequently cited as providing useful tools for response optimization (Myers H Raymond, 2016). Accordingly, RSM is selected for the present study to maximize xanthan production. The individual and interactive effects of the inoculum size (5–15%), Acid Hydrolysis of Bakery waste (0.5-2.5%) and shaking (150-250 rpm) on xanthan production from 2 different strains are studied. To determine the significant experimental variables and develop a response surface for medium optimization, the major factors mentioned above are further investigated by Box-Behnken Design (BBD). The experimental range for each factor was selected based on results obtained from 15 preliminary experiments carried out by BBD. Actual variables were selected for the experimentation. The standard deviation graph at 200rpm is shown in fig 3.3. The obtained response values

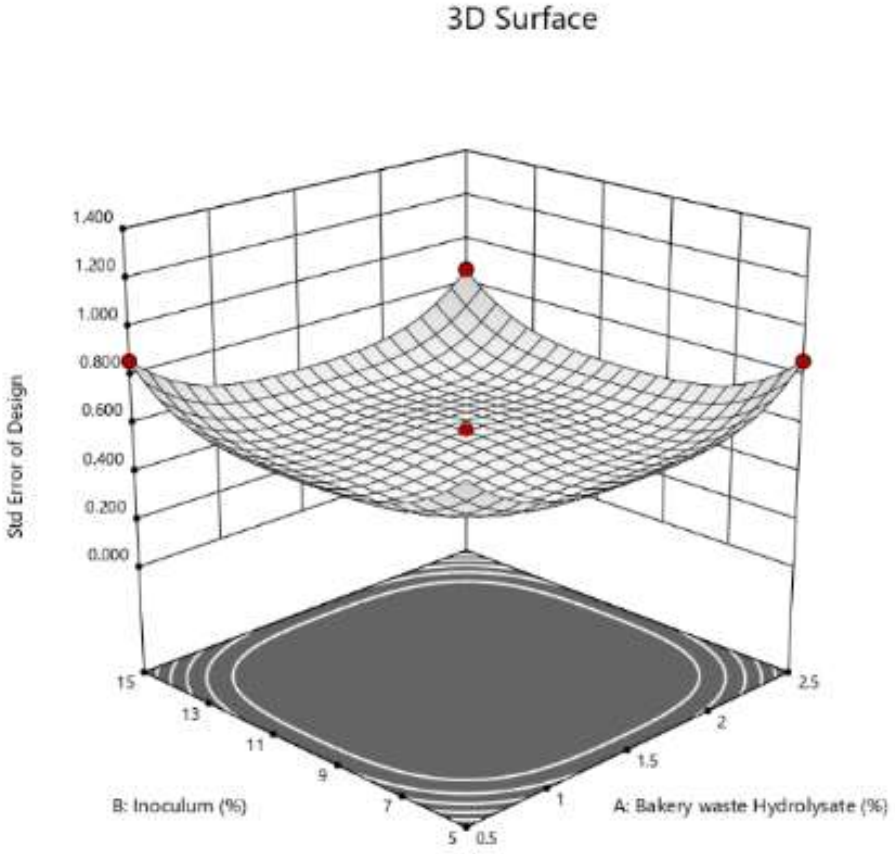


Figure 3.3: Standard error deviation for the selected ranges of the three independent variables.

are used to estimate the model coefficients using the least square approach, utilizing the experimental design tool Design Expert Pro version 13. Before they can be used, mathematical models must be tested to ensure that the results are correct and reliable. In the case of the composite design used in this study, the model is validated by using analysis of variance to examine the data (ANOVA). The graphs of actual and predicted values for both strains were depicted and are used for finding experimental errors.

CHAPTER 4

4 RESULTS AND DISCUSSION

4.1 Determination of reducing sugar, total sugar, moisture content & total solids

4.1.1 What is reducing sugar?

"A sugar that serves as a reducing agent due to its free aldehyde or ketone functional groups in its molecular structure. Examples are glucose, fructose, glyceraldehyde, lactose, arabinose and maltose, except for sucrose."

4.1.2 Determination of reducing sugar, total sugar, MC, and total solids

At initial stage reducing sugar was determined by drawing a standard curve using glucose. An equation was drawn through the graph and then we find the concentration level at the required absorption. Total sugar determined by the following formula. Which is 0.56 mg of reducing sugar after hydrolysis obtained by checking the curve multiplying with dilution factor 1 and constant value 0.9 into 100.

$$y = 4.4687x + 0.0565$$

Reducing sugar, $x = 0.058138$

The reducing sugar comes out to be 0.058138. However, the total sugar is determined after hydrolysis of bakery waste.

The absorbance of unknown concentration sample was determined through spectrophotometry. This comes out to be $2.5853\mu m$. After putting it in equation the unknown concentration comes out to be 0.56. While putting this value in the formula, the total sugar comes out to be 50.4mg/ml.

$$y = 4.4687x + 0.0565$$

If, $y = 2.5853$

$x = 0.56$

Total sugar= milligrams of reducing sugar after hydrolysis obtained by checking the curve × dilution factor × 0.9 ×100

$$\text{Total sugar} = 0.56*1*0.9*100$$

$$= 50.4 \text{ mg/ml}$$

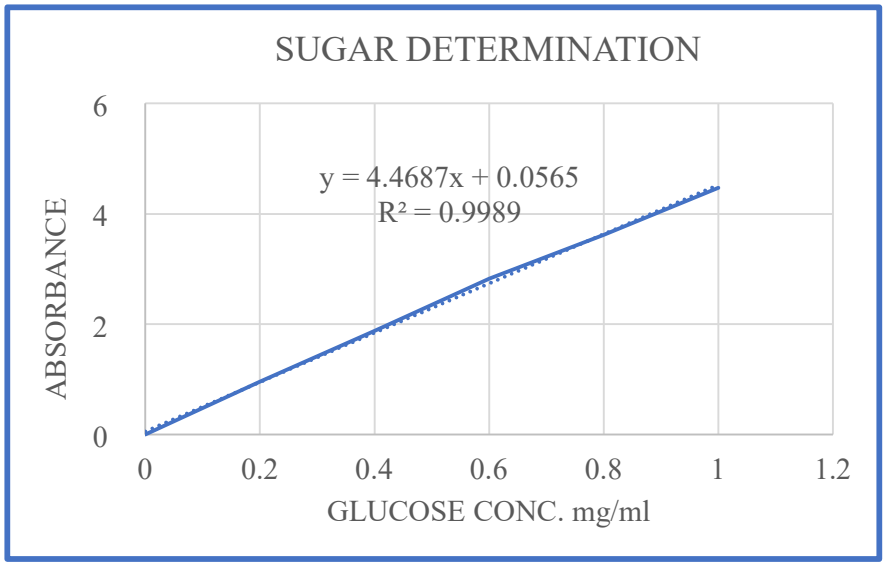


Figure 4.1: Reducing Sugar determination

Table 4.1: Characterization of BW & BWH

Sample	Reducing Sugar	Total Sugar	Moisture Content	Total Solids
Bakery waste	0.058	-	37%	80g
Bakery Waste Hydrolysate	-	50.4mg/ml	121%	44.45g

The moisture content is determined by the formula

$$MC = (weight\ before\ drying - weight\ after\ drying)/weight\ after\ drying \times 100$$

Calculations for bakery waste moisture content

Weight before drying = 44.44

Weight after drying = 36.25

Calculations for bakery waste hydrolysate moisture content

Weight before drying = 50.442

Weight after drying = 22.24

The moisture content of Bakery waste hydrolysate came out to be 121%.

Total solids equal the mass left after drying at 105 degrees Celsius as mentioned in the table 4.1.

4.2 Optimization of Fermentation Process

The experimental design and analysis were carried out using Design Expert pro software and response surface methodology (RSM) using Box Behnken Design (BBD) (version 13The experimental design included three independent variables: acid hydrolysis of Bakery waste (A, percent), inoculum (B, percent), and agitation (C, rpm). Bakery waste hydrolysate (0.5, 1 & 1.5) percent, inoculum volume (5, 10 & 15) percent, and agitation (150, 200 & 250) rpm are all chosen at three levels, with each factor having three coded values of 1, 0 and 1, as indicated in Table 4.3. There are 15 total runs with three mid-points. These three variables, along with their aspect, were discovered to be crucial parameters for maximizing the R variable's response.

Table 4.2: Build Information for the Box-Behnken Design

Software Version	13.0.5.0		
Sort of Study	Response Surface	Subtype of Study	Randomized
Kind of Design	Box-Behnken	No. of runs	15.00
Model	Quadratic	Total no. of blocks	No Blocks

The initial status of the Design Expert pro software is mentioned in the Table 4.2. Box-Behnken Design was selected for the Experimental variables to arrange them in a Quadratic pattern with 3 center points in common. In table 4.2, three factors in given ranges are initialized for experiments. The Factor ranges were kept as recommended by the (Mohsin et. al). The values were coded high (- 1) and coded low (+1). The mean for

bakery waste is 1.5, for inoculum its 10.00 and for agitation, it is about 200. The Standard deviation is shown in Table 4.3.

Table 4.3: Coded values and St. Deviation for the selected ranges of all the three variables.

Factor	Name	Units	Min	Max	Coded Low	Coded High	Mean	Std. Dev.
A	Bakery waste Hydrolysate	%	0.50	2.50	-1 ↔ 0.50	+1 ↔ 2.50	1.50	0.7559
B	Inoculum	%	5.00	15.0	-1 ↔ 5.00	+1 ↔ 15.00	10.00	3.78
C	Mixing Rate	rpm	150.	250.	-1 ↔ 150.00	+1 ↔ 250.00	200.00	37.80

Table 4.4 defines that Response surface methodology, applied on the given ranges of the defined variables and provides the basic information to initiate the optimization steps. These variables were randomized to total number of 15 runs to initiate the experiments.

Table 4.4: Experimental design runs for both strains (*pseudo-Xanthomonas* sp. and *Rhizobium* sp.)

Run	V.Factor 1 A:Acid Hydrolystae %	V.Factor 2 B: Inoculum %	V.Factor 3 C: Agitation rpm
1	1.50	10.00	200.00
2	2.50	10.00	250.00
3	2.50	15.00	200.00
4	0.50	5.00	200.00
5	2.50	10.00	150.00
6	1.50	15.00	150.00
7	1.50	5.00	250.00
8	0.50	15.00	200.00
9	1.50	10.00	200.00
10	1.50	15.00	250.00
11	1.50	10.00	200.00
12	2.50	5.00	200.00
13	0.50	10.00	150.00
14	1.50	5.00	150.00
15	0.50	10.00	250.00

4.3 Analysis of Variance for *pseudo-Xanthomonas* Strain NCCP-1183

The P values for xanthan gum is shown in table. ANOVA was applied for the linear model. In linear model only 3 variables were involved, and the variables involved independently in graph formation.

Table 4.5: p-value and F-value for *pseudo-Xanthomonas* sp. (NCCP-1183)

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	50.33	16.78	2.91	0.0823	not significant
A-Bakery Waste Hydrolysate	37.68	37.68	6.54	0.0267	
B-Inoculum	5.51	5.51	0.9557	0.3493	
C-Agitation Rate	7.14	7.14	1.24	0.2895	
Residual	63.41	5.76			
Lack of Fit	55.91	6.21	1.65	0.4328	not significant
Pure Error	7.51	3.75			

The Model F-value of 2.91 implies that there is an 8.23 percent chance that noise is to cause for such a high F-value. If the P-values of model terms are less than 0.0500, they are considered significant. In this case, A is an important model term. If the model terms have values greater than 0.1000, they are not significant. If there are a lot of insignificant model terms, model reduction may help (not including those needed to sustain hierarchy). The Lack of Fit has an F-value of 1.65, indicating that it is not statistically significant when compared to the pure error. Noise has a 43.28 percent risk of causing a significant Lack of Fit F-value. Because we want the model to fit, a slight fit issue is acceptable.

Table 4.6: Production yield as response factor R1 (g/L) for *pseudo-Xanthomonas* sp. (NCCP-1183)

Std.	Run	A:Acid Hydrolystae %	B: Inoculum %	C: Agitation rpm	Response 1 Xanthan Gum g/L
14	1	1.50	10.00	200.00	8.8577
8	2	2.50	10.00	250.00	3.865
4	3	2.50	15.00	200.00	4.8454
1	4	0.50	5.00	200.00	11.0806
6	5	2.50	10.00	150.00	4.0356
10	6	1.50	15.00	150.00	3.5091
11	7	1.50	5.00	250.00	2.72
3	8	0.50	15.00	200.00	8.7881
15	9	1.50	10.00	200.00	8.8577
12	10	1.50	15.00	250.00	9.2
13	11	1.50	10.00	200.00	5.502
2	12	2.50	5.00	200.00	1.8549
5	13	0.50	10.00	150.00	4.3643
9	14	1.50	5.00	150.00	4.0483
7	15	0.50	10.00	250.00	7.73

The response factor comes out to be minimum at 2.72 g/L at 250rpm, 5% inoculum and 1.5 % BWH as reported in table 4.6. So, the runs were arranged in order and the difference between actual and predicted values is shown in table 4.7.

Table 4.7: *Pseudo-Xanthomonas* sp. (NCCP-1183) predicted vs. actual

Run Order	Actual Value	Predicted Value
1	8.86	5.95
2	3.87	4.73
3	4.85	4.61
4	11.08	7.29
5	4.04	2.84
6	3.51	5.84
7	2.72	6.07
8	8.79	8.95
9	8.86	5.95
10	9.20	7.73
11	5.50	5.95
12	1.85	2.95
13	4.36	7.18
14	4.05	4.18
15	7.73	9.07

The table predicts that this total number of 15 runs were analyzed for the selected variables. The actual values came after running the experiment whereas the predicted ones were driven by the software based on prediction from the experimental results as in table 4.7. The predicted vs. actual value is shown in figure 4.2 related to *pseudo-Xanthomonas* species strain NCCP-1183.

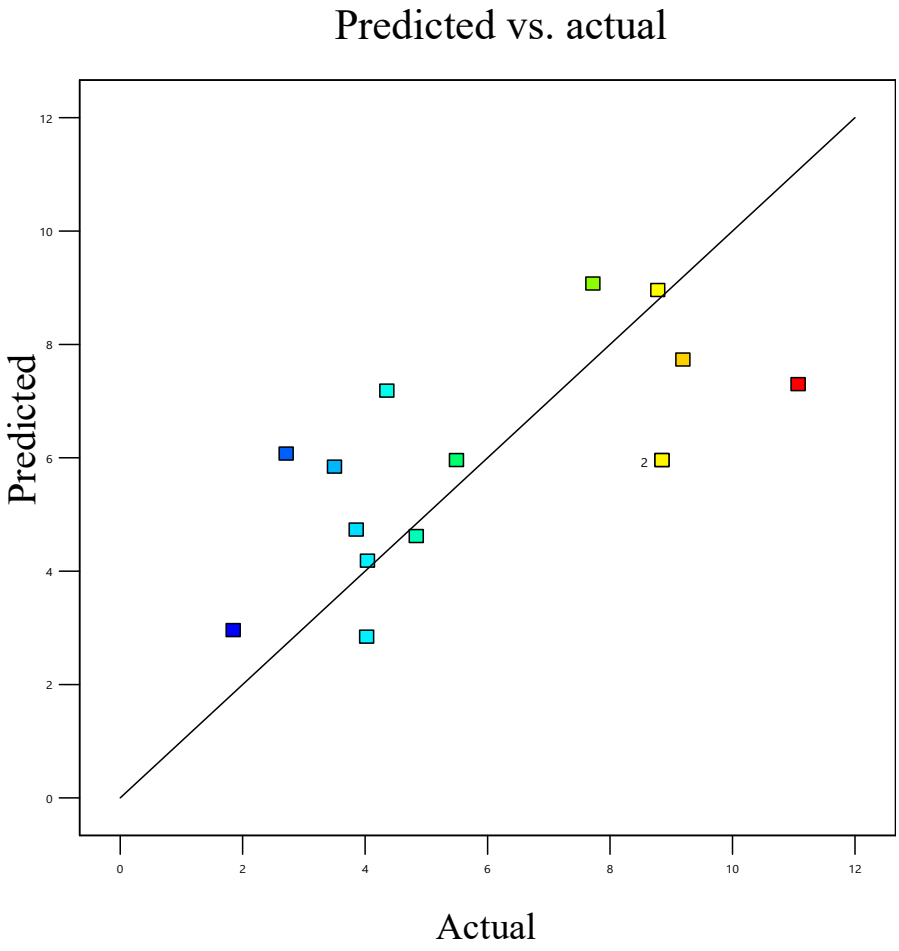


Figure 4.2: Actual vs. predicted yield of *pseudo-Xanthomonas* sp. (NCCP- 1183)

Run 1, 4, 7 and 14 shows much difference in the predicted values and the actual values. Actual yields at runs 7 & 13 are less whereas run 1 and 4 shows less predicted values. The graph between actual values and the predicted one is depicted in the figure 4.2. Predicted values are taken along y-axis whereas x- axis gives the actual values.

The following 3d graph shows the maximum yield of the xanthan Gum at 200 rpm. Increasing or decreasing the Agitation rate, results in decrease of the Xanthan yield. So, 200 rpm is considered as the optimized point for xanthan yield when 0.5% of BWH is taken along with 5% of inoculum during fermentation. So, the values of these three factors provides the optimum amount of response "R".

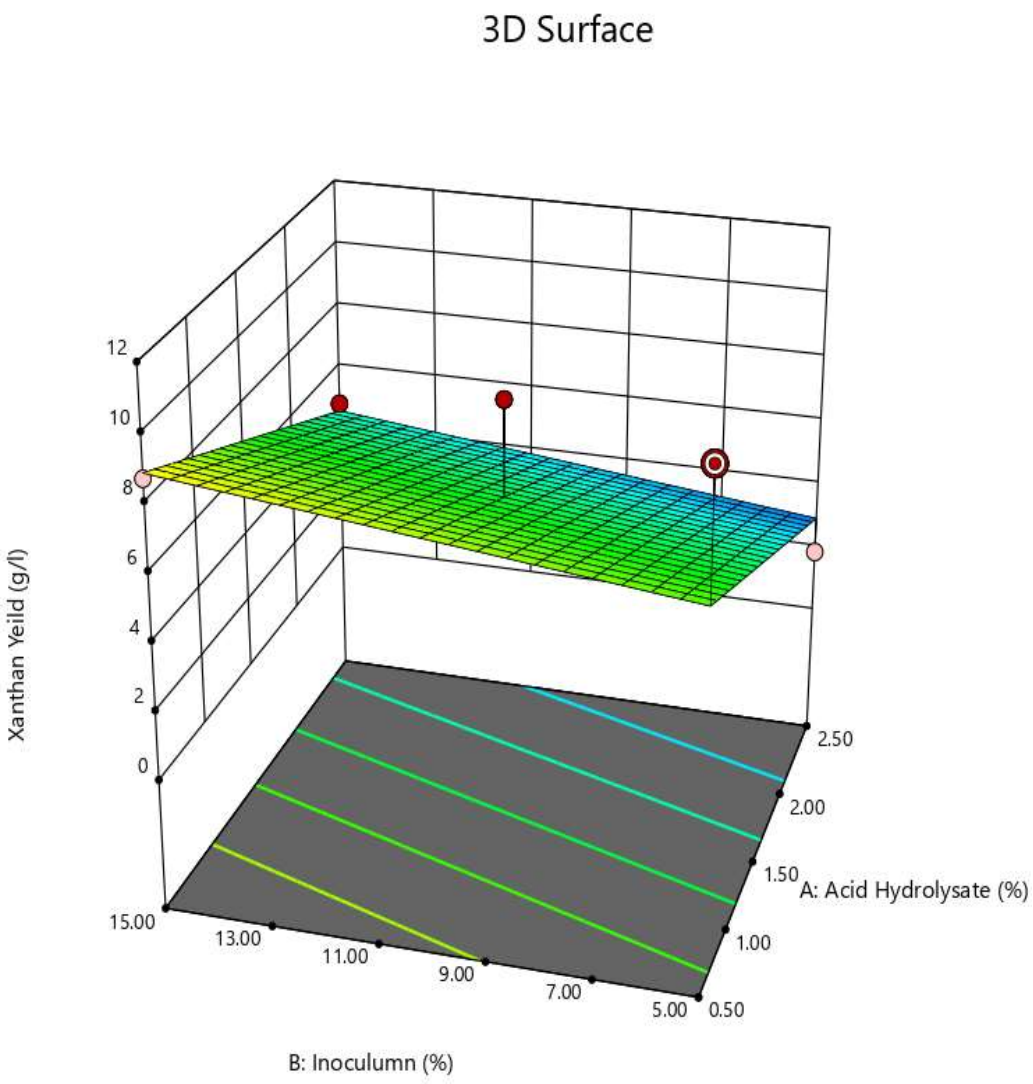


Figure 4.3: Response graph for *pseudo-Xanthomonas* sp. NCCP- 1183 Strain

Table 4.8: p-value with resulted constants considering *strain pseudo-Xanthomonas* sp. (NCCP- 1183)

Source	p-value	Xanthan Yield
Model	0.0823	+3.76742
A-Bakery waste Hydrolysate	0.0267	-2.17026
B-Inoculum	0.3493	+0.165970
C-Mixing Rate	0.2895	+0.018894
Lack of Fit	0.0307	Not significant

A, B, C are the factor values involved in table 4.8. The equation 1 shows the optimized design formula. By putting the values of constants, A, B and C we can reach the desire yield we need.

The coded equation came out to be

Xanthan yield = 5.95058 -2.17026(A) + 0.82985(B) + 0.94471(C) ..... (1)

4.4 Analysis of Variance for *Rhizobium* Strain (NCCP-1189)

The p and f values for xanthan gum is shown in table 4.9. ANOVA was applied for the Quadratic aggression model. In Quadratic model three variables were involved, and the variables involved independently in graph formation. Table 4.9 shows the p and F-values for the selected model of the *Rhizobium Sp*. strain. The F-value for Quadratic

model comes out to be 0.5884. However, p-value 0.6790 this much lower shows the validity of the selected model. Quadratic model is suggested for the selected *Rhizobium* strain and applied whereas cubic model is aliased in this case.

Table 4.9: F and p-value for Model selection related to *Rhizobium* sp. (NCCP-1189)

Source	Sum of Squares	Mean Square	F-value	p-value	
Linear	99.82	11.09	0.9226	0.6214	
2FI	96.56	16.09	1.34	0.4868	
Quadratic	21.22	7.07	0.5884	0.6790	Suggested
Cubic	0.0000				Aliased
Pure Error	24.04	12.02			

The p values are shown in table 4.9. Lack of fit is non-significant which is good. So, the model applies well for the selected model. The sum of square comes out to be 21.22 with deferential of 3, mean of the square 7.07, the f-values 0.5884 less than 1. With p-value of 0.6790 quadratic model is suggested for the runs and applied. The selected model should have insignificant lack of fit which is good for the model.

Table 4.10: Response (xanthan yield g/L) for *Rhizobium* sp. (NCCP-1189)

Std	Run	A: Acid Hydrolysate %	B: Inoculum %	C: Agitation rpm	Response 1 Xanthan Gum g/L
10	1	1.5	15	150	4.3527
7	2	0.5	10	250	5.015
15	3	1.5	10	200	3.088
9	4	1.5	5	150	3.5677
3	5	0.5	15	200	12.6376
11	6	1.5	5	250	1.715
1	7	0.5	5	200	9.388
2	8	2.5	5	200	4.1104
12	9	1.5	15	250	1.375
14	10	1.5	10	200	5.9173
8	11	2.5	10	250	2.3
13	12	1.5	10	200	9.9856
6	13	2.5	10	150	2.4746
5	14	0.5	10	150	2.2716
4	15	2.5	15	200	5.5557

Three factors were involved in the Box-Behnken Design. The maximum yield in table 4.10 was obtained at run 5 presenting a total yield of 12.6376 for the *Rhizobium* strain.

After applying the ANOVA Quadratic Model Aggression was obtained for the optimized condition of the Xanthan Yield. The model F value of 1.35 indicates that the model is insignificant due to noise. Model terms are significant if the P-VALUE is less than 0.05. C square is a significant model term in this case. Values greater than 0.1 indicate that the model terms are unimportant. Which is advantageous for the model's fit.

Table 4.11: p and f value for the resulted xanthan when quadratic model fitted for *Rhizobium Sp.*

Source	f-value	p-value	
Model	1.35	0.3872	Not significant
A bakery waste Hydrolysate	3.05	0.1410	
B-Inoculum	0.3648	0.5722	
C-Mixing Rate	0.0706	0.8010	
AB	0.0899	0.7764	
AC	0.2351	0.6482	
BC	0.0349	0.8591	
A^2	0.3510	0.5793	
B^2	0.1803	0.6887	
C^2	7.34	0.0423	P-value $\leq$ 0.05
Lack of Fit	0.5884	0.6790	Not Significant

The coded equation (2) for xanthan yield came out to be the one from assembled table 4.11

Xanthan yield = 6.3303 - 1.85894(A) + 0.64248 (B) - 0.2827(C) - 0.45107(AB) - 0.7295(BC) - 0.28125(CA) + 0.92766(A^2) + 0.66496(B^2) - 4.24266(C^2) …… (2)

Table 4.12: Predicted vs. Actual values of Response "R" related to *Rhizobium* Strain (NCCP-1189)

Run Order	Actual Value	Predicted Value
1	4.35	3.96
2	5.01	5.32
3	3.09	6.33
4	3.57	2.11
5	12.64	10.88
6	1.72	2.11
7	9.39	8.69
8	4.11	5.87
9	1.38	2.83
10	5.92	6.33
11	2.30	0.1442
12	9.99	6.33
13	2.47	2.17
14	2.27	4.43
15	5.56	6.26

Run orders 3, 11, 12 and 14 shows much variation in the actual yield than that predicted by Design Expert Pro version 13. The graph between predicted values and the actual one is shown in figure 4.4.

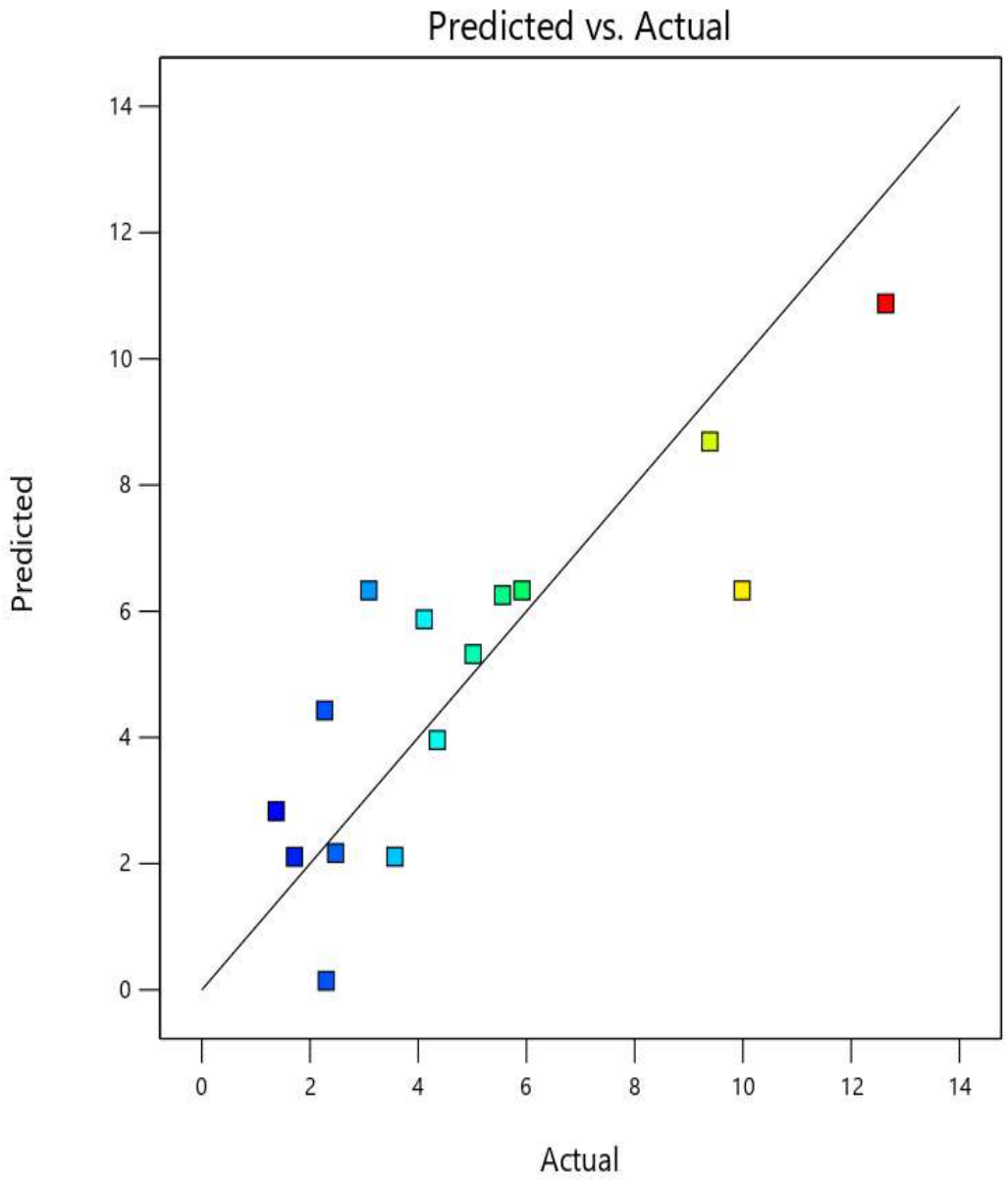


Figure 4.4: Predicted vs. Actual value graph for *Rhizobium* sp. Strain NCCP-1189

To develop a 3D graph, Bakery waste was taken along x axis, Inoculum along y-axis whereas the xanthan gum yield was driven along third axis which is z-axis. The graph in figure 4.5 depicted the optimized value of the xanthan gum when the actual factor

was kept 200. Meaning the maximum yield at 200 rpm was obtained according to run 5. The following dotted circled red points shows the optimized values of the 2 variables AB of the topmost xanthan yield achieved at 200rpm. By increasing or decreasing the rpm the xanthan yield decreased. The range for optimized production was between 9.2 g/l to max yield at relevant factors.

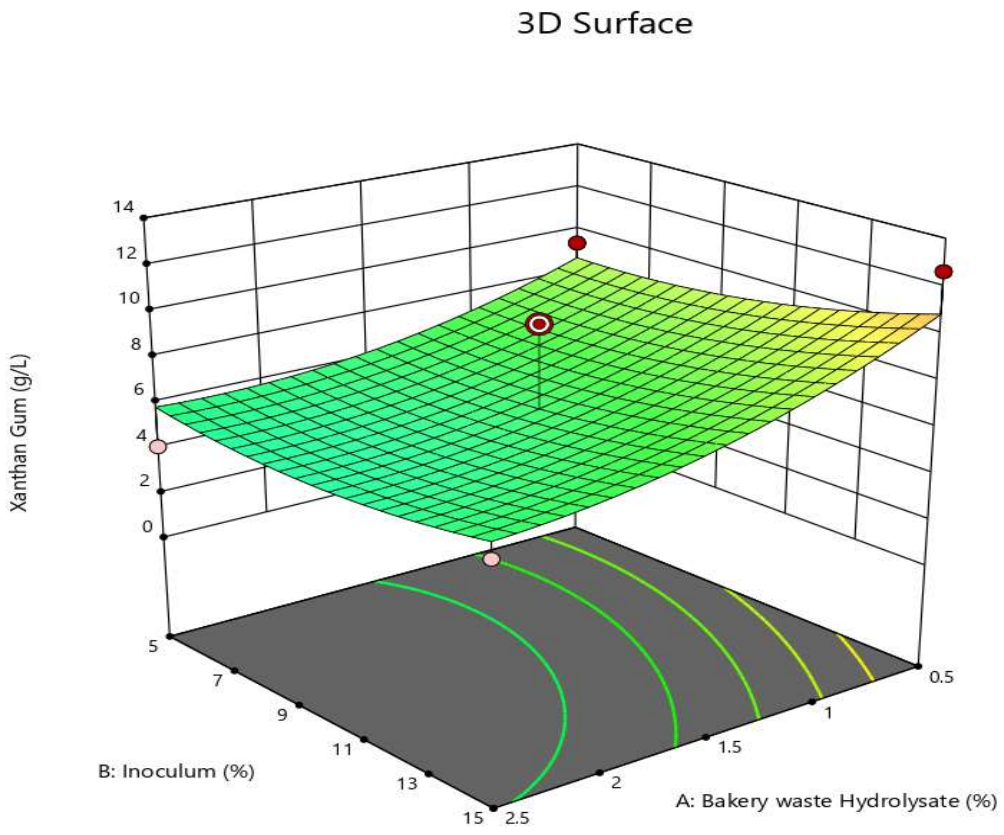


Figure 4.5: *Rhizobium* sp. NCCP-1189 strain yield graph (A) based on Quadratic model

Following 3D graph is presented in figure 4.6, when A and C factors were collectively combined. Graph for maximum yield of xanthan Gum obtained is shown when inoculum was kept constant at 15. On Increasing or decreasing the inoculum percentage will result in decrease of xanthan yield.

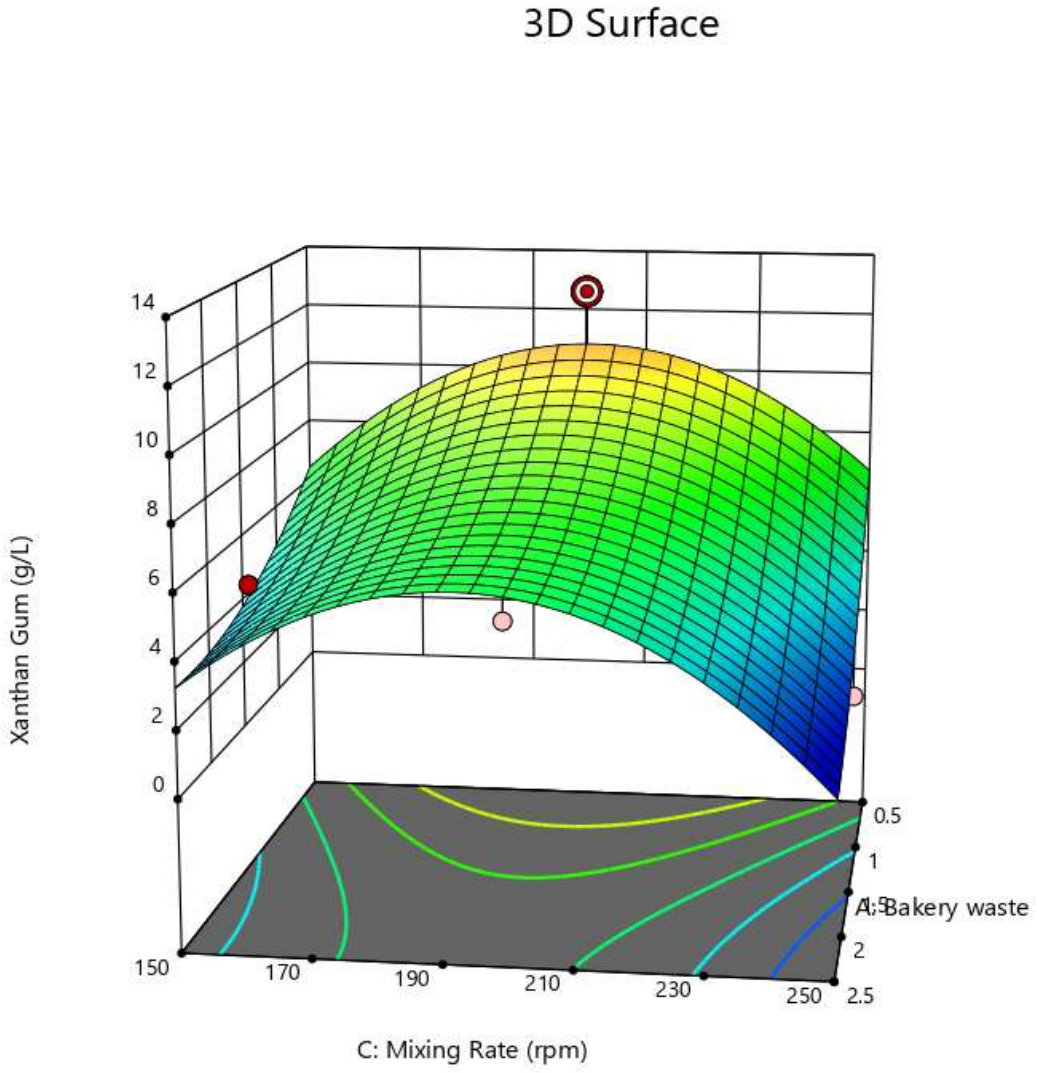


Figure 4.6: *Rhizobium* sp. NCCP-1189 strain yield graph (B) based on Quadratic model

The graph obtained between the relation BC by fixing the 0.5 percent of BWH. We got the maximum yield of xanthan yield. By varying the factor “A” the xanthan yield decreased.

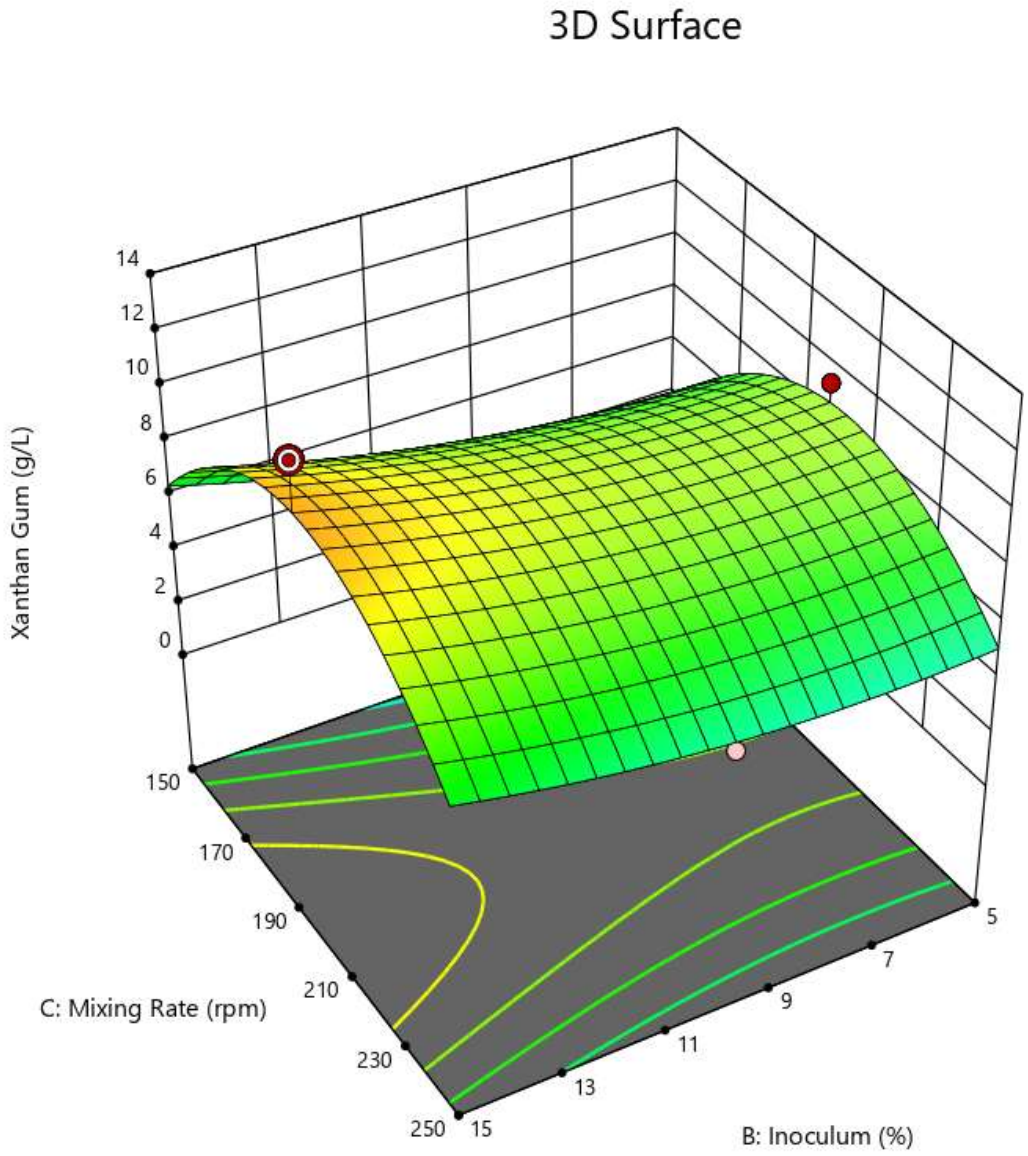


Figure 4.7: *Rhizobium* sp. NCCP-1189 strain yield graph (C) based on Quadratic model

So, in all these 3 graph you have seen that at run 5 we got the optimized production of xanthan gum. Combining the 2 variables and varying the 3rd variable showed the result on the xanthan yield.

4.5 Mass Production:

For assurance xanthan yield was taken out along with 24 hours. In Graph time was taken along x axis whereas the xanthan yield was taken along y axis in g/l. the max yield found is shown in the figure 4.8. At 24 hours interval the xanthan yield of both strains was almost similar. At 48 hours, xanthan yield showed little variation. And after 72 hours, the xanthan yield of both strains shows deviation same as in previous conducted research on optimized production of xanthan gum from bread waste when using 4 different types of strains.

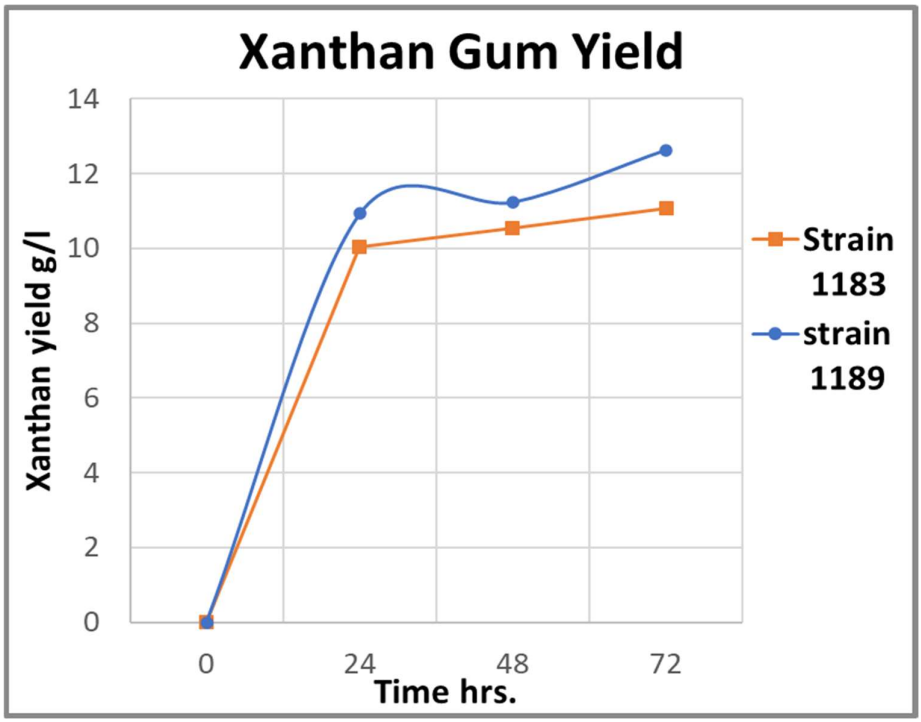


Figure 4.8: Xanthan Yield with respect to time.

CHAPTER 5

5 Conclusion and Recommendation

5.1 Conclusions

- RSM used by DESIGN Expert Pro recommends linear model for *pseudo-Xanthomonas* NCCP-1183 & Quadratic model for *Rhizobium* sp. NCCP-1189.
- The xanthan yield decreases at higher RPM. Lower RPM below 200 is found to be best for xanthan production.
- The production of xanthan gum decreases with increase in percentage of acid in acid hydrolysis.
- *Rhizobium* sp. NCCP-1189 is more effective in terms of amount of xanthan production i.e. (0.3 g of xanthan gum per ml of bakery waste hydrolysate) relatively Compared to *pseudo-Xanthomonas* NCCP-1183 (0.275 g of xanthan gum per ml of bakery waste hydrolysate).

5.2 Recommendations:

- Xanthan production had only been produced from limited organic peel waste. Considering the seasonal generation of Mango peel xanthan gum needs to be produced from mango peel in future.
- Studies can be enhanced in Response Surface Methodology (RSM) by directing involving the glucose concentration as a variable factor. This gives much absolute values.
- Acid hydrolysis has been done in this research. In addition, enzymatic hydrolysis can be taken out in future research to check the production feasibility on xanthan yield.

References

Balkrishna, A., Agarwal, V., Kumar, G., & Gupta, A. K. (2018). Applications of Bacterial Polysaccharides with Special Reference to the Cosmetic Industry. *Microbial Bioprospecting for Sustainable Development*, 189–202. https://doi.org/10.1007/978-981-13-0053-0_9

Balows, A., Trüper, H., Dworkin, M., & Harder, W. (1992). *The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*. https://link.springer.com/book/10.1007/978-1-4757-2191-1

Berninger, T., Dietz, N., & González López, Ó. (2021). Water-soluble polymers in agriculture: xanthan gum as eco-friendly alternative to synthetics. *Microbial Biotechnology*. https://doi.org/10.1111/1751-7915.13867

Demirci, A. S., Palabiyik, I., Apaydın, D., Mirik, M., & Gumus, T. (2019). Xanthan gum biosynthesis using Xanthomonas isolates from waste bread: Process optimization and fermentation kinetics. *LWT*, *101*, 40–47. https://doi.org/10.1016/J.LWT.2018.11.018

García-Ochoa, F., Santos, V. E., Casas, J. A., & Gómez, E. (2000). Xanthan gum: production, recovery, and properties. *Biotechnology Advances*, *18*(7), 549–579. https://doi.org/10.1016/S0734-9750(00)00050-1

Ghashghaei, T., Soudi, M. R., & Hoseinkhani, S. (2016). Optimization of Xanthan Gum Production from Grape Juice Concentrate Using Plackett-Burman Design and Response Surface Methodology. *Applied Food Biotechnology*, *3*(1), 15–23. https://doi.org/10.22037/AFB.V3I1.9984

Gumus, T., Demirci, A. S., Mirik, M., Arici, M., & Aysan, Y. (2010). Xanthan gum production of Xanthomonas spp. isolated from different plants. *Food Science and Biotechnology*, *19*(1), 201–206. https://doi.org/10.1007/S10068-010-0027-9

Gunasekar, V., Reshma, K. R., Treesa, G., Gowdhaman, D., & Ponnusami, V. (2014). Xanthan from sulphuric acid treated tapioca pulp: Influence of acid concentration on xanthan fermentation. *Carbohydrate Polymers*, *102*(1), 669–673. https://doi.org/10.1016/J.CARBPOL.2013.11.006

Habibi, H., & Khosravi-Darani, K. (2017). Effective variables on production and structure of xanthan gum and its food applications: A review. *Biocatalysis and Agricultural Biotechnology*, *10*, 130–140. https://doi.org/10.1016/J.BCAB.2017.02.013

Hu, L., Sun, Y., & Lin, L. (2012). Efficient conversion of glucose into 5-hydroxymethylfurfural by chromium(III) chloride in inexpensive ionic liquid. *Industrial and Engineering Chemistry Research*, *51*(3), 1099–1104. https://doi.org/10.1021/IE202174F

Kerdsup, P., Tantratian, S., Sanguandeekul, R., & Imjongjirak, C. (2011). Xanthan Production by Mutant Strain of Xanthomonas campestris TISTR 840 in Raw Cassava Starch Medium. *Food and Bioprocess Technology*, *4*(8), 1459–1462. https://doi.org/10.1007/S11947-009-0250-7

Leela, J. K., & Sharma, G. (2000). Studies on xanthan production from Xanthomonas campestris. *Bioprocess Engineering 2000 23:6*, *23*(6), 687–689. https://doi.org/10.1007/S004499900054

Letisse, F., Chevallereau, P., Simon, J. L., & Lindley, N. D. (2001). Kinetic analysis of growth and xanthan gum production with Xanthomonas campestris on sucrose, using sequentially consumed nitrogen sources. *Applied Microbiology and Biotechnology*, *55*(4), 417–422. https://doi.org/10.1007/S002530000580

Li, P., Li, T., Zeng, Y., Li, X., Jiang, X., Wang, Y., Xie, T., & Zhang, Y. (2016). Biosynthesis of xanthan gum by Xanthomonas campestris LRELP-1 using kitchen waste as the sole substrate. *Carbohydrate Polymers*, *151*, 684–691. https://doi.org/10.1016/J.CARBPOL.2016.06.017

López, M. J., Moreno, J., & Ramos-Cormenzana, A. (2001). Xanthomonas campestris strain selection for xanthan production from olive mill wastewaters. *Water Research*, *35*(7), 1828–1830. https://doi.org/10.1016/S0043-1354(00)00430-9

Melikoglu, M., & Webb, C. (2013). Use of waste bread to produce fermentation products. *Food Industry Wastes*, 63–76. https://doi.org/10.1016/B978-0-12-391921-2.00004-4

Mohsin, A., Zhang, K., Hu, J., Tariq, M., … W. Z.-C., & 2018, U. (2018). Optimized biosynthesis of xanthan via effective valorization of orange peels using response surface methodology: A kinetic model approach. *Elsevier*. https://www.sciencedirect.com/science/article/pii/S0144861717313620

Myers H Raymond, M. C. D. & A.-C. C. C. (2016). *Response Surface Methodology: Process and Product Optimization Using ...* -. 894. https://www.wiley.com/en-ca/Response+Surface+Methodology%3A+Process+and+Product+Optimization+Using+Designed+Experiments%2C+4th+Edition-p-9781118916018

Nagaraja, K., Rao, K. M., Krishna Rao, K. S. V., Riazunnisa, K., & Suresh Reddy, K. V. N. (2021). Polysaccharides of Natural Gums-Based Biomedical Devices for Drug Delivery Application. *Nanotechnology in the Life Sciences*, 507–554. https://doi.org/10.1007/978-3-030-84262-8_18

Ozdal, M., & Kurbanoglu, E. B. (2018). Valorisation of chicken feathers for xanthan gum production using Xanthomonas campestris MO-03. *Journal, Genetic Engineering & Biotechnology*, *16*(2), 259–263. https://doi.org/10.1016/j.jgeb.2018.07.005

Palaniraj, A. (2011). *Production, Recovery and Applications of Xanthan gum by Xanthomonas campestris*. https://www.researchgate.net/publication/229404612_Production_Recovery_and_Applications_of_Xanthan_gum_by_Xanthomonas_campestris

Papagianni, M., Psomas, S. K., Batsilas, L., Paras, S. V., Kyriakidis, D. A., & Liakopoulou-Kyriakides, M. (2001). Xanthan production by Xanthomonas campestris in batch cultures. *Process Biochemistry*, *37*(1), 73–80. https://doi.org/10.1016/S0032-9592(01)00174-1

Preichardt, L., & Klaic, P. (2016). Xanthan gum application in food. In *Xanthan Gum: Applications and Research Studies* (pp. 2–31).

Psomas, S., … M. L.-K.-B. E., & 2007, undefined. (n.d.). Optimization study of xanthan gum production using response surface methodology. *Elsevier*. Retrieved April 4, 2022, from https://www.sciencedirect.com/science/article/pii/S1369703X07000435?casa_token=OyVa0LWLS9wAAAAA:bygcUUe5-u90s_-fKCSJ7NBrB0xBi5ErNpqUXA4IrdajhDbupJ_qpVwCBgAjzfBucs1NruAHCQ

Reinoso, D., Martín-Alfonso, M. J., Luckham, P. F., & Martínez-Boza, F. J. (2020). Flow behavior and thermal resistance of xanthan gum in formate brine. *Journal of Petroleum Science and Engineering*, *188*, 106881. https://doi.org/10.1016/J.PETROL.2019.106881

Rončević, Z., Bajić, B., Vlajkov, V., Dodić, S., Grahovac, J., Jokić, A., & Dodić, J. (2020). Optimisation of xanthan production on glycerol-based medium using response surface methodology. *Brazilian Journal of Chemical Engineering 2020 37:4*, *37*(4), 617–627. https://doi.org/10.1007/S43153-020-00062-6

Rosalam, S., Technology, R. E.-E. and M., & 2006, undefined. (n.d.). Review of

xanthan gum production from unmodified starches by Xanthomonas comprestris sp. *Elsevier*. Retrieved April 4, 2022, from https://www.sciencedirect.com/science/article/pii/S0141022905004631?casa_token=sVpe7hkfS5AAAAAA:_PY4BoBw_XEx9XkSOajE9bpvuiKTDDrT9ULHMk-sIHAybOhO935kgmKyQfU5rmgRH-cqHLEKXQ

Silva, M. F., Fornari, R. C. G., Mazutti, M. A., de Oliveira, D., Padilha, F. F., Cichoski, A. J., Cansian, R. L., Di Luccio, M., & Treichel, H. (2009). Production and characterization of xantham gum by Xanthomonas campestris using cheese whey as sole carbon source. *Journal of Food Engineering*, *90*(1), 119–123. https://doi.org/10.1016/J.JFOODENG.2008.06.010

Singhvi, G., Hans, N., Shiva, N., & Kumar Dubey, S. (2019). Xanthan gum in drug delivery applications. *Natural Polysaccharides in Drug Delivery and Biomedical Applications*, 121–144. https://doi.org/10.1016/B978-0-12-817055-7.00005-4

Wang, Z., Wu, J., Zhu, L., & Zhan, X. (2017). Characterization of xanthan gum produced from glycerol by a mutant strain Xanthomonas campestris CCTCC M2015714. *Carbohydrate Polymers*, *157*, 521–526. https://doi.org/10.1016/J.CARBPOL.2016.10.03