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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEVELOPMENT OF ANTIMICROBIAL EDIBLE FILM CONTAINING ANTAGONISTIC BACTERIA

M.Sc. THESIS

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Supervisor: **Prof. Dr. Arzu ÇAĞRI MEHMETOĞLU**

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The thesis work titled “Development of Antimicrobial Edible Film Containing Antagonistic Bacteria” prepared by Ali Raza Khan was accepted by the following jury on 18/07/2024 by unanimously/majority of votes as a MSc THESIS in Sakarya University Graduate School of Natural and Applied Sciences, Food Engineering department.

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I declare that the thesis work titled " DEVELOPMENT OF ANTIMICROBIAL EDIBLE FILM CONTAINING ANTAGONISTIC BACTERIA", which I have prepared in accordance with Sakarya University Graduate School of Natural and Applied Sciences regulations and Higher Education Institutions Scientific Research and Publication Ethics Directive, belongs to me, is an original work, I have acted in accordance with the regulations and directives mentioned above at all stages of my study, I did not get the innovations and results contained in the thesis from anywhere else, I duly cited the references for the works I used in my thesis, I did not submit this thesis to another scientific committee for academic purposes and to obtain a title, in accordance with the articles 9/2 and 22/2 of the Sakarya University Graduate Education and Training Regulation published in the Official Gazette dated 20.04.2016, a report was received in accordance with the criteria determined by the graduate school using the plagiarism software program to which Sakarya University is a subscriber, I have received an ethics committee approval document I accept all kinds of legal responsibility that may arise in case of a situation contrary to this statement.

(18/07/2024)

Ali Raza KHAN

To my Respected parents, beloved wife, and sweet Baby

You are the foundation of my life and a source of strength, love, and joy. Each of you has shaped who I am and continues to inspire me every day. Your unwavering support and boundless love mean more to me than words express. I dedicate all my efforts and successes to you.

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Ali Raza KHAN

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ABBREVIATIONS

P. E	: *Penicillium expansum*
B. C	: *Bacillus Clausii*
S. A	: *Staphylococcus Aureus*
E. C	: *Escherichia coli*
WVP	: Water Vapour Permeability
WS%	: Water Solubity Percentage
T. S	: Tensile Strenght
% E	: Percentage Elongation
WPC	: Whey Protein Concentration
WPI	: Whey protein isolate
TSB	: Tryptic Soy Broth
TSA	: Tryptıc Soy Agar
MH	: Mueller Hinton
RH	: Relative Humidity
HCl	: Hydrochloric acid
NaCl	: Sodium Chloride
Wt%	: Weight percentage
PVDF	: Polyvinylidene xiii clüoride
°C	: Degree celsius

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DEVELOPMENT OF ANTIMICROBIAL EDIBLE FILM CONTAINING ANTAGONISTIC BACTERIA

SUMMARY

The microbial spoilage of food products causes significant economic losses. In this study, *Bacillus clausii* (O/C, T, SIN, and N/R) as an antagonistic bacterium was incorporated into an edible film solution based on whey protein concentrate (WPC) and fermented for 24 h to produce antimicrobials and create an antimicrobial edible film.Taguchi test trial (3x3) was used to analyze the optimum condition for the growth of *B. clausii* and its antimicrobial production in the film solution. Nine different growth parameters including temperature (33, 35**,** and 37°C), pH (8.5, 9.0, and 9.5), and protein concentration in the film (6, 8, and 10%) were chosen for that purpose.

The optimum growth of *B. clausii* and the maximum antimicrobial production were observed when protein concentration was 10% (w/v) and pH was 8.5 and 9.0. The film solutions showed antimicrobial effects against *Aspergillus niger, Penicillium expansum, Staphylococcus aureus,* and *Escherichia coli.* The 86% and 87% of *B. clausii* cells survived in the dry film for 14 days at 4 and 25°C, respectively. The highest TS and % E of the film containing *B. clausii* was measured as 3.14 MPa and 27.63%, respectively. Taguchi test trial results showed that the protein content of the film was the most important parameter affecting water solubility (WS) and water vapor permeability (WVP).

In conclusion, the present work showed that *B. clausii* with their spores could be used to prepare WPC films with antimicrobial properties against spoilage of food products.

Keywords: Antimicrobial edible film; biocontrol; antagonistic bacteria; antifungal, *Bacillus clausii*.

ANTAGONİSTİK BAKTERİ İÇEREN ANTİMİKROBIK YENILEBILIR FİLMİN GELİŞTİRİLMESİ

OZET

Gıda güvenliği, üreticiden tüketiciye kadar uzanan gıda zincirinde yer alan herkesin sorumluluğudur ve bu sorumluluğun yerine getirilmesi, insan sağlığının korunması açısından kritik önem taşır. Özellikle risk analizi, gıdalardaki tehlikelere ilişkin verilerin, gıda kaynaklı hastalıklara ait epidemiyolojik verilerle sistematik olarak ilişkilendirilmesini sağlar. Bu sistematik ilişkilendirme, gıdalarda bulunan potansiyel tehlikelerin belirlenmesini ve bu tehlikelerin insan sağlığı üzerindeki risklerinin değerlendirilmesini kolaylaştırır. Gıdalarda meydana gelen mikrobiyal bozulmalar, sadece sağlık açısından tehdit oluşturmakla kalmaz, aynı zamanda önemli ekonomik kayıplara da yol açar. Mikrobiyal bozulmalar nedeniyle gıdaların tüketiciye ulaşmadan önce kaybedilmesi, üreticiden tüketiciye kadar olan zincirde büyük ekonomik zararlara neden olur ve bu durum, gıda sektöründeki tüm paydaşları olumsuz etkiler.

Gıda kayıplarının etkileri geniş bir yelpazeye yayılır. Ekonomik kalkınma süreçlerini sekteye uğratır ve çevresel sürdürülebilirliği tehlikeye atar. Gıda kayıplarının azaltılması, yalnızca ekonomik kalkınmayı desteklemekle kalmaz, aynı zamanda çevresel etkileri de minimize eder. Bu kayıpların azaltılması, tüm paydaşların ortak çabasıyla mümkündür ve bu ortak çaba, gıda güvenliğinin sağlanmasında ve sürdürülmesinde anahtar rol oynar. Gıda kayıplarına engel olabilmek için çeşitli stratejiler ve çözümler geliştirilmelidir. Bu çözümlerden biri, mikrobiyal bozulmalara karşı etkili olan ve aynı zamanda bu çalışmanın da temel amacı olan "antagonistik bakteri içeren antimikrobiyal yenilebilir film geliştirilmesi" olabilir. Bu çalışmada, antagonistik bir bakteri olan *Bacillus clausii* (O/C, T, SIN ve N/R) peynir altı suyu protein konsantresi (PASPK) bazlı yenilebilir bir film çözeltisine eklenmiştir. *B. clausii*, bu tür filmlerde kullanılmak üzere seçilmiş olup, özellikle gıda güvenliği ve koruma amaçlı avantajlar sunmaktadır. Karışım, *B. clausii'nin* gelişimini ve antimikrobiyal madde üretimini teşvik etmek amacıyla 24 saat boyunca kontrollü bir ortamda fermente edilmiştir. Film çözeltisinde *B. clausii'nin* optimum büyüme ve antimikrobiyal madde üretim koşullarının belirlenmesinde Taguchi deney tasarımı (3x3) kullanılmıştır. 6, 8 veya 10 g PASPK, 90.4, 87.2 veya 84 ml saf suda vorteks ile çözündü, ardından pH 0.1 M NaOH ve HCl ile (8.5, 9.0 ve 9.5) olarak ayarlandı. Daha sonra, çözeltinin son beş dakikasında 3.6, 4.8 ve 6 ml gliserol eklenerek, çözeltinin 90 ºC'de 30 dakika boyunca su banyosunda bekletildi. Soğuduktan sonra, her PASPK film çözeltisine pellet halinde 1 ml konsantre *B. clausii* kültürü transfer edildi. Ardından, çözeltiler 33°C, 35°C ve 37°C'de 24 saat inkübe edildi. 24 saat sonra, 100 ml çözelti Teflon kaplı plakalar (çap 22 cm²) üzerine döküldü ve hava kabarcıkları çıkarıldı. Biyolojik güvenlik kabininde %50±5 RH'da 25°C ±1'de 48 saat kurutulduktan sonra plakalarından soyuldular. Çökeltilmiş proteinlerin ve kuru filmlerin antimikrobiyal aktivitesi, disk difüzyon yöntemi

kullanılarak belirlendi. Bu yöntemde, sterilize filtre kağıdı disklerine (çapı 6 mm) 10 µl çökeltilmiş proteinler ve kuru film örnekleri (çapı 6 mm) emdirildi. Bu diskler, *E. coli, S. aureus, P. expansum* ve *A. niger* ile aşılanmış Hilton Agar plakalarının yüzeyine yerleştirildi. Her bir disk özenle yerleştirildikten sonra plakalar mühürlendi ve inkübasyon için hazır hale getirildi. Aşılanmış plakalar, her mikroorganizma türü için belirli inkübasyon koşullarında bekletildi. Bakteri kültürleri için plakalar 35°C'de 24 saat inkübe edildi. Küf kültürleri için ise plakalar 25°C'de 5 gün inkübe edildi. İnkübasyon süresinin sonunda, disklerin etrafındaki inhibisyon zonlarının çapları ölçülerek antimikrobiyal aktivite değerlendirildi. Bu yöntem, çökeltilmiş proteinler ve kuru filmlerin test edilen mikroorganizmaların büyümesini inhibe etmedeki etkinliğini değerlendirmeyi sağladı ve sonuçlar dikkatlice kaydedildi. Filmlerin çekme mukavemeti, yüzde uzama, yüzde su çözünürlüğü ve su buharı geçirgenliği gibi fiziksel ve kimyasal özellikleri, farklı koşullar altında hesaplandı ve gözlemlendi. Bu özellikler, çeşitli çevresel faktörlerin ve formülasyon değişikliklerinin filmlerin performansı ve dayanıklılığı üzerindeki etkisini anlamak için analiz edildi. Sonuçların doğruluğunu ve tekrarlanabilirliğini sağlamak için standart test yöntemleri kullanılarak ölçümler yapıldı.

Deneme sonuçlarına göre *B. clausii' nin* optimum gelişme ve maksimum antimikrobiyal üretimi protein konsantrasyonu %10 (w/v) ve pH 8.5 ve 9.0 değerlerinde gerçekleşmiştir. Elde edilen film çözeltileri, *A. niger, P. expansum, S. aureus* ve *E. coli* gibi mikroorganizmalara karşı antimikrobiyal etki göstermiştir. Ayrıca, *B. clausii* hücrelerinin film içinde sırasıyla 4°C ve 25°C'de 14 gün boyunca hayatta kalma oranları sırasıyla %86 ve %87 olarak ölçülmüştür. Bu yüksek hayatta kalma oranı, filmin biyolojik aktivitesini koruyarak etkin bir koruma sağladığını göstermektedir. *B. clausii* içeren filmin mekanik özellikleri de dikkat çekicidir; filmin en yüksek gerilme mukavemeti (TS) 3,14 MPa ve en yüksek uzama oranı (%E) ise 27,63% olarak belirlenmiştir. Bu sonuçlar, filmin hem dayanıklılığını hem de esnekliğini vurgular ve çeşitli uygulamalar için uygun olduğunu gösterir. Ayrıca, bu değerler filmin çeşitli fiziksel streslere karşı dirençli olduğunu ve uzun süreli kullanım için ideal olduğunu ortaya koymaktadır.

Taguchi deneme modeli, çeşitli parametrelerin (pH, sıcaklık, protein90 konsantrasyonu gibi) film çözeltisinde *B. clausii'nin* performansı üzerindeki etkisini sistematik olarak değerlendirmeyi mümkün kılmıştır. Deney tasarımı sayesinde, en uygun koşullar belirlenerek, film çözeltisinin antimikrobiyal etkinliği maksimum düzeye çıkarılmıştır. Bu kapsamlı analizler, *B. clausii* içeren antimikrobiyal yenilebilir filmlerin geliştirilmesi ve gıda güvenliğine katkı sağlama potansiyelini ortaya koymuştur. Aynı zamanda,Taguchi deneme modelinin sonuçları, filmin protein içeriğinin su içindeki çözünürlük (SÇ) ve su buharı geçirgenliği (SBG) üzerindeki etkisinin en önemli parametre olduğunu ortaya koymuştur. Bu bulgular, filmin performansını optimize etmek için protein içeriğinin ayarlanmasının kritik olduğunu vurgular. Ayrıca, protein içeriğinin artırılması, filmin genel mekanik özelliklerini de iyileştirerek daha güçlü ve dayanıklı hale gelmesini sağlar. Protein içeriğinin artırılması, su buharı geçirgenliğini azaltarak filmin nem karşısındaki direncini artırır ve bu da filmin yapısal bütünlüğünü uzun süre korumasına yardımcı olur. Sonuç olarak, bu ayarlamalar, film uygulamalarında uzun ömürlü ve etkili sonuçlar elde edilmesine katkıda bulunur ve çeşitli çevresel koşullarda daha güvenilir bir çözüm sunar.

PASPK bazlı yenilebilir filmlerde, *B. clausii* tüm test koşullarında kolayca çoğaltılabilmiş olup, antimikrobiyal maddeler üretebilmektedir. Film fermantasyon

çözeltisinde üretilen antimikrobiyal madde, *A. niger*, *P. expansum*, *E. coli* ve *S. aureus'un* büyümesini inhibe etme yeteneğine sahiptir. Kuru filmde Bacillus cluasii hücrelerinin canlılık oranı, 14 günlük 4°C ve 25°C'de depolama sırasında %87 civarında olduğu tespit edilmiştir. Filmin çekme dayanımı (TS) ve kopma uzaması (%E) gibi mekaniksel özellikleri, %8 protein içeren filmde en iyi ölçümlerde elde edilmiştir. Filmin pH'ının artırılması hem TS'yi hem de su buharı geçirgenliğini (WVP) iyileştirmiştir. Protein içeriğinin azalması ve artmasıyla birlikte filmin WVP ve ağırlıkça su şişme (%WS) özellikleri de sırasıyla iyileşmiştir. Sonuç olarak, bu çalışma *B. clausii* sporlarının gıdaların bozulmasına karşı antimikrobiyal özelliklere sahip PASPK filmler hazırlamak için kullanılabileceğini göstermiştir. Fermentasyon süreci boyunca, bakteri hücreleri aktif olarak çoğalmış ve antimikrobiyal bileşikler üretmiştir. Bu bileşikler arasında özellikle bakteriyosinler gibi antimikrobiyal maddeler bulunmaktadır. Bu bileşikler, elde edilen yenilebilir filmin mikrobiyal bozulmalara karşı koruyucu bir bariyer oluşturabilme potansiyeline sahip olduğunu göstermiştir. Filmin bu özellikleri, hem raf ömrünü uzatmada hem de gıda güvenliğini artırmadaki etkisi sonraki çalışmalarda ortaya konulması gerekmektedir. Ayrıca, bu antimikrobiyal etkinin, filmin gıda uygulamalarında kullanım süresini ve etkinliğini artırabileceği öngörülmektedir.

Anahtar Kelimeler: Antimikrobiyal yenilebilir film; biyokontrol, antagonistik bakteri; antifungal, *Bacillus clausii*

1. INTRODUCTION

Food product safety and quality are determined by the number and type of spoilage and pathogenic bacteria existing on the food surface [1]. One of the preservation techniques that can increase the shelf life of products while assuring customer safety is antimicrobial edible packaging [2]. Edible films also can offer sterile surfaces and stop the loss of other crucial components [3]. These films can also create a modified atmosphere by preferentially permitting the exchange of respiration gases like oxygen (O_2), ethylene, and carbon dioxide (CO_2) with food and their environment [4].

Awareness of the environment has increased the demand for edible films and coatings made of whey protein concentrate (WPC). Because of its multiple beneficial qualities, including as high elasticity, good look, and moderate oxygen barrier, the manufacturing of films based on WPC has been extensively investigated [5]. In addition, these films act as carriers for nutraceuticals, antibiotics, and antioxidants in addition to being edible packaging materials. Research studies in this area have started to gain interest in the field of antimicrobial film research in the past few years as consumers' knowledge of natural antimicrobials has grown [6]. The study of antagonistic microorganisms, commonly referred to as "bio-control," is considered the main research area for natural antimicrobials. Antagonism microorganisms regularly alter their environment with their metabolic byproducts, making it challenging for competing other species to exist[7]. Environmentally friendly and resistant to extreme conditions microbial antagonists can be used to protect food against pre-harvest illnesses and microbial deterioration in storage.

Numerous antimicrobial substances are produced by the *Bacillus* genus of bacteria, including *B. clausii* strains that produce protease and bacteriocins that work against Gram-positive bacteria [8]. As a spore-producing bacteria with quick growth in liquid culture, extreme heat tolerance, and the capacity to develop resistance, *B. clausii* can be used as an antagonistic agent [9].

To enhance the antimicrobial properties of the film by increasing the production of bacteriocins from that kind of antagonistic species within the film, it is possible to

subject the film solution to fermentation with these bacteria before allowing it to dry. This method may enable more effective integration of bacteria into the film matrix, strengthening their antimicrobial features.

This study aims to determine the optimal conditions for the growth and antimicrobial production of *B. clausii* in the WPC film solution using the Taguchi test method. Additionally, the study aims to investigate the optimal conditions for the mechanical properties, water vapor permeability, and antimicrobial film formation of films produced from this solution. Fermentation variables have been selected as pH, temperature, and protein content.

1.1. Motivation

The main goal of this research is to create an ecologically acceptable and biodegradable packaging material that prolongs the shelf life of food items but also prevents the growth of harmful microorganisms by utilizing the natural antimicrobial properties of whey protein. This innovation reduces plastic waste and promotes the principles of the circular economy, which not only addresses concerns about food safety but also contributes to the global sustainability agenda.

1.2. Problem Statement

The increasing demand for sustainable and eco-friendly packaging solutions to improve food safety and lessen environmental impact by creating antimicrobial edible films with whey protein concentrate (WPC). Our goal is to address the problems of microbiological contamination and plastic waste in the food industry by investigating the potential of WPC as a sustainable packaging material. This research aims to evaluate how well WPC-based films prolong shelf life and support creative, environmentally responsible packaging solution.

2. LITERATURE REVIEW

2.1. Background of the Food Packaging

The theory of food packaging has arisen with the human desire to consume food for ages and has been regularly adapted to the commercialization and industrialization processes. In ancient times, people ate the food freshly on the exact days as raw ingredients were harvested or hunted from the orchard with no preservation difficulties [10]. Through the advancement of time and the tendency of the people alive in communities, food preservation requirements emerged as solutions for food packaging were found. However, glass was the first material ever used for food packaging and served as a model for paper, which the food industry currently uses widely. As one of the most discussed packaging containers nowadays, plastic items were found in specific studies starting in the 1870s [11]. Today's food industry has been facing a challenge in meeting customer demands for fresh, healthy, and minimally processed items.

2.1.1. Demand of the consumers

Consumers are also conscious of the consequences of packaging on the environment due to the requirement to have suitable possibilities that improve their lifestyle, particularly the shorter time spent grocery shopping and cooking food. Customers are accustomed to acquiring those goods at any time and normally for a reasonable price thanks to globalization's enhanced accessibility of several goods worldwide [12]. Because of such factors, as well as others, including cost-effectiveness safety, and marketing, manufacturers carefully consider the packaging they select for their commodities. In support of the argument, consumers are aware that the cost of the packaging is included in the finishing cost of the food. This is one of the reasons it is challenging to introduce a new packaging idea into the market, even if it is affordable or the user is aware of its advantages [13].

2.1.2. Global Aspects

One of the biggest industries in the world is food packaging, accounting for almost 50% of all packaging sectors globally in 2009. Its estimated value is US$380 billion. $9.5 billion for plastic, $4 billion for paper, $1.6 billion for glass, and $0.3 billion for wood are the estimated costs associated with importing packaging materials. The Food and Agricultural Organization of the United Nations (FAO) conducted a survey that found that the food packaging sector, estimated to be worth US$15.4 billion, has a substantial economic impact on both industrialized and developing countries [14]. In general, a product's package has an important role in all stages of its journey, including shipping, distribution, retailing, and most importantly, preservation and protection. Nevertheless, despite all of these efforts, a World Packaging Organization (WPO) analysis reveals that food goods more than 25% are wasted due to faulty packaging, including limited storage protection, wrong dose, and a lack of a reclosing mechanism, or reduced package sizes [15].

2.1.3. Future in the food packaging

The improvements in food packaging are constantly improving to address these specific issues that are directly linked to the worldwide challenge of food waste. Moreover, manufacturers in the packaging industry are always forced to create innovative packaging designs in response to changing customer demands [16]. Consumer demands are changing constantly, thus manufacturers in the packaging sector are continuously under pressure to design new package models. Foods that are ready to eat yet have a longer shelf life due to innovative methods, such as smart, intelligent, active packaging ideas, which are the latest trends on supermarket shelves [17]. To this aspect and sustain product quality, it is necessary to utilize fresh approaches in manufacturing and new ways of strategic packing. There are several methods for extending the shelf life of items, e.g., nanomaterials time-temperature devices, absorbers or CO2 emitters, the addition of chemicals, O2 indicators, and biosensors with barcode labels.

2.2. Processing of Edible Packaging Materials

Two processing techniques, known as dry and wet procedures, can be used to create an edible film [18]. Compression molding, extrusion, heat pressing, or injection are

popular methods for carrying out dry processing [19]. Extrusion is more frequently used in dry processing for thermoplastic materials like starches linked with a plasticizer [20]. High temperatures are utilized during extrusion to identify the package into the appropriate shape. Processing of high temperatures might change the final product's quality in several biopolymers [21].

2.2.1. History of edible films

Wax was first utilized in China around the 12th century to keep fruit from drying out, which led to the advancement of food preservation films. However, Japan's earliest soymilk-based edible films were employed for fruit preservation and to create a glittering surface sixteen centuries ago [22]. There was little interest in this kind of package at the time because of the limited selection of available materials to cover fruits and vegetables. Edible packaging has never gotten more attention than freezing, modified/controlled environments, heat or radiation sterilization, or smoking [23].

Naturally, food preservation techniques have improved significantly, providing limitless chances to prepare, preserve, and any type of food to consume throughout any season. However, EP may presently be used to preserve a wide range of food items, and it does so in a way that is more specific, personalized, and creative than traditional food preservation methods [24].

2.2.2. Edible Films and Coatings

Any kind of substance that is used to enrobe (i.e., wrap or coat) different foods, to increase the product shelf life is regarded as an edible film or coating. These contain substances that can be removed and eaten together with the food, with or without further processing [25]. Edible films replace and/or fortify natural layers to reduce moisture loss. They also allow for the controlled interchange of certain gases, like oxygen (O_2), ethylene, and carbon dioxide, which are essential for the transport of oxygen. Surface sterility can also be provided by a film/coating, which can help stop the loss of other essential components. In most cases, it is thinner than 0.3 mm [26].

The advancement of edible films is accelerating today in an effort food quality and increase food safety. An effective edible film should maintain the structure's integrity and protect it against impurities and food-borne diseases [19]. In edible films Generally Recognized as Safe (GRAS) materials must be utilized so that they may be

used alongside food. These films may transfer antioxidants, vitamins, food additives, and antibacterial substances. Because edible films and coatings are biodegradable, provide protection, and may have active substances like antioxidants and antimicrobials, the scientific community is very interested in them. This is particularly accurate to allowing them in food and also in food packaging. Depending on how they are utilized, the phrases edible films and coatings are defined as follows: Although coatings are created immediately onto the product surface, films are added after being formed [27].

2.2.3. Protein-based edible film

The good barrier and mechanical qualities of protein-based edible films have led to a surge in their popularity in the past few years. By regulating the pace at which antibacterial and antioxidant compounds diffuse from the surface to the food, they may be made to delay food decomposition [28]. Depending on the food's qualities they can be used for both multilayer and individual packing. In nature, proteins can either be globular or filamentous. They should be organized in a long structure to produce a film. To make an edible protein film, heat, acid, base, or solvents must denature proteins. Protein chains in the film matrix can then create covalent, ionic, and hydrogen bonds [29].

The arrangement of amino acid residues and the degree of chain extension affect the film's characteristics. The barrier qualities of films become stronger, less flexible, and weaker as the chain-to-chain contact increases [30]. Both proteins from animal origin and proteins from plants are put into use in these degradable films and covering layers. They combine it with plasticizers and solvents to create an edible film. The kind of protein, plasticizers, and used solvent, as well as external elements that affect the final quality of films, including temperature, pH, ionic strength, relative humidity, drying conditions, and others [31]. Gelatin, casein, whey protein, egg albumen, meat protein, and feather keratin are examples of animal protein sources. Wheat protein, soy protein, pea protein, and maize zein are examples of plant protein sources that may be utilized to create edible films [32].

2.2.4. Whey protein-based edible film

Approximately 20% and 80% of the proteins in milk are whey protein and casein respectively. Whey proteins are proteins that are still soluble at pH 4.6 and 20°C after

caseins have been extracted from milk [33]. Whey possesses the greatest biological value (BV) of any protein source and is highly bioavailable. Therefore, if you're seeking a protein supplement that your body can easily utilize to gain muscle, a whey product can be a great option [34]. Whey protein concentration (WPC) in an industrial setting typically includes between 25 percent and 80 percent protein. After converting into whey protein isolate (WPI) which contains roughly 90% protein [35].

The popularity of consumable coating and films based on whey proteins has grown together with environmental awareness. Furthermore, these films perform several tasks besides being edible packaging material, such as acting as a carrier for nutraceuticals, antimicrobials, or antioxidants [36]. Degradable films based on whey protein demonstrate sufficient mechanical, sensory, and optical qualities as well as resistance to O_2 permeability, but they are moisture sensitive. In this regard, investigations examining the outcomes and various traits of these edible films have been outlined in the literature[37].

Barrier characteristics, mechanical strength, and thermal properties of the whey protein film are all enhanced by the addition of plasticizers [38] [39]. The structure of the plasticizer must be suitable for the polymer when selecting one. A plasticizer having properties comparable to the polymer's solubility, polarity, and hydrogen bonding would typically have good compatibility [40]. Some plasticizers could be harmful because of the phthalate migration. Thus, edible films must employ natural and biodegradable plasticizers [41]. Several plasticizers, such as oleic acid, glycerol, xylitol, sorbitol, and PEG 200 & 400, have been investigated in the past in combination with whey protein-based film [42], [43].

The researchers concluded that among the most efficient are glycerol plasticizers and glycerol is a protein miscible, water-soluble, polar, non-volatile, and has a high boiling point [44], [45]. Due to these capabilities, glycerol can be utilized as a plasticizer in conjunction with whey protein or another comparable water-soluble polymer[46].

2.3. Antimicrobial Edible Films

Edible films containing antimicrobial properties are a potential development in food preservation. They are recognized as a safe, ecologically friendly, and biodegradable substitute for traditional plastic films made of petroleum. The main biological

materials used in these films include lipids, proteins, and polysaccharides. Proteins and polysaccharides serve as the primary fundamental components of antimicrobial edible films, whereas lipids generally work as plasticizers and transporters of active ingredients in composite films [47]. Extracts or oils from plant-based components including rosemary, cinnamon, bergamot, and lemon, are added to whey protein films. Essential oils give health advantages in addition to protection, which makes it more important for their use [19]. Whey-based films have also included additional antibacterial agents from sources such as titanium oxide and silver nanoparticles [48].

Antimicrobial packaging, one of the active packaging innovations, is a novel packaging solution that improves food safety by lowering the amount of living bacteria in food [49]. Antimicrobial hygiene also stops the growth of microbes on surfaces that come into touch with food, even while packaging programs work to release antimicrobials into foods in an effective way. Antimicrobials can be added to edible films and coatings to increase their protective barrier effect and prevent or delay microbial development [50].

Edible films can also contain natural preservatives like bacteriocin and chitosan, in addition to chemical substances or phenolic compounds like sodium benzoate, sorbic acid, propionic acid, and potassium sorbate, which are frequently used in food[50] [51], [52]. The antimicrobial effect of cellulose-based films containing natamycin on *Penicillium roqueforti* was demonstrated by Oliveria et al. (2007) in their study on the effect of whey protein-based edible films containing a mixture of sorbic acid and p-aminobenzoic acid against the growth of *Escherichia coli O157:H7, Salmonella Typhimurium, and Listeria monocytogenes*. This means that edible films offer regulated diffusion of natural or artificial antimicrobial components into the food and provide an excellent carrier environment for them.

2.3.1. Antagonistic microorganisms

Over the last ten years, consumer worries about the effects of artificial compounds on food and the environment have grown significantly. There is a chance that these chemicals may come into direct contact with people and animals when they consume certain foods [53]. Researchers and food organizations have been pushed by consumer demand for products that are healthful, safe, high-quality, and contain few or no chemical preservatives [54]. However, biological control has been utilized for

centuries, but it wasn't until recently as a chemical alternative. In biocontrol applications, Numerous agents are available for use, such as yeast, bacteria, and mold [55].

2.3.2. Benefits and drawbacks of using biocontrol applications

Comparing the usage of biocontrol agents to chemical preservatives, there are several benefits and drawbacks.

Advantages

Bio-controlling organisms are picky, while selected pests and closely linked organisms are generally the only ones they affect.

1. They essentially leave no hazardous residue behind. It is an environmentally beneficial strategy in this sense.
2. Biocontrol agents' development costs are less than those of artificial chemical preservatives.
3. It is economical.
4. It is a functional, effective, and unique application. [56].

- **Disadvantages**

1. Compared to conventional chemical preservatives, the rate of pathogen killing is low.
2. Due to its sensitivity to unfavorable environmental factors, it has a short shelf life.
3. It can't sufficiently replicate the effect it has in the lab in meals.
4. The impact on the product’s internal quality is yet not completely understood [57].

2.3.3. Edible films with antagonistic yeast

Some of the yeast can be used as an antagonistic agent. The existence of a secretion known as fatal toxin, lethal factor, or zymosin, which while inhibiting sensitive strains, does not affect some yeast species, is well recognized. These fungi are referred to as killer yeast [58]. The deadly impact of *Saccharomyces cerevisiae* on other strains of the same species was originally documented. Before being used further in biocontrol

field experiments, the microorganism needs to be evaluated for several selection criteria [59].

The inability of the chosen antagonistic microorganism strains to produce substances toxic to biological systems and their propensity to multiply, colonize, and survive are crucial selection criteria that guarantee the chosen strains will be safe and beneficial when introduced into the environment [60]. For that purpose, we highlight some reviews which are following.

When it comes to food preservation, innovative methods are constantly being developed to compete for spoilage and extend shelf life. One promising approach involves the use of yeast-infused edible films, which not only protect food from contamination but also enhance its longevity.

In the case of cereal grains, mycotoxins, which are byproducts of fungal metabolism, pose significant health risks. Researchers have discovered that antagonistic yeast, such as *P. anomala*, can be a formidable ally in this battle. This yeast effectively prevents the notorious *P. roqueforti* from spoiling feed grains [61], [62]. Furthermore, *P. anomala* has been utilized to reduce contamination of wheat stored with *P. verrucosum* and *P. ochratoxin* A (OTA), highlighting its versatility and effectiveness in various storage conditions [63].

In another study, *C. guilliermondii* and a species of Debaryomyces were tested using cellulose-based films to preserve oranges. These films, particularly those made with methylcellulose, significantly slowed down the decay of "Pineapple" and "Valencia" oranges during storage. The results were impressive, with some treatments preventing deterioration for up to four weeks. This demonstrates the potential of yeast-infused coatings to maintain the quality of fresh produce [64].

Candida utilis was combined with chitosan films to prevent pathogens like *A. alternata* and *G. candidum* from causing deterioration in tomatoes. When compared to controls, all tested treatments (mixtures of chitosan at 0.25% and 0.5% with or without *C. utilis*) significantly reduced infection (by *G. candidum* and *A. alternata)* and lesion breadth. The best outcomes were obtained with *C. utilis* and chitosan 0.5%. After 168 hours, the amount of yeast per wound had increased from 105 to 106 CFU[65].

For strawberries, *C. laurenti* was incorporated into alginate-based coatings. These coatings not only reduced degradation by 25% compared to control groups but also

improved the firmness and appearance of the fruit. The addition of ingredients like glycerol and palmitic acid further enhanced the coating's performance (low impact on strawberry's external color and weight loss) showcasing the multifaceted benefits of yeast-infused films in fruit preservation [66].

McGuire and Baldwin (1994) reported that the population of yeast remained steady when *C. oleophila* was added to cellulose. For hydroxypropyl and methylcellulose, the storage time was extended by nine and eleven days, respectively. Only after 157 and 151 days did half of the fruits covered with these compounds and free of yeast begin to rot [67]. The population of yeast remained steady when *C. oleophila* was added to cellulose formulations (methylcellulose or hydroxypropyl cellulose), with hydroxypropyl cellulose displaying superior performance versus methylcellulose. It also examined the impact of including preservatives in the coatings, and the scientists found that *C. oleophila* did not suffer from the inclusion of potassium sorbate at the maximum permitted concentration of 0.15 % [68].

Lastly, brewer's yeast was used in coatings made of alginate and carboxymethyl cellulose to preserve grapes. These coatings effectively decreased spoilage and weight loss while maintaining the sensory qualities of the grapes and minimizing the loss of vitamin C during storage. These findings underscore the potential of baker's yeast-infused coatings to extend the shelf life of perishable fruits [69].

These all studies illustrate that the integration of yeast into edible films presents a compelling solution for food preservation. By harnessing the natural antagonistic properties of yeast, we can create coatings that protect against spoilage and pathogens while extending the freshness and quality of a wide range of food products.

2.3.4. Edible films with antagonistic bacteria

In recent years, the application of edible films and coatings has emerged as a promising strategy for managing infections within food systems. These films incorporate various elements such as *Lactic acid bacteria (LAB)* and yeast, which have been demonstrated to exert biocontrol effects. Live *LAB*, for instance, can effectively inhibit pathogen introduction through competitive exclusion mechanisms and the production of antimicrobial compounds. Similarly, the integration of yeast into these films addresses degradation issues caused by post-harvest pathogens like *Penicillium* species, particularly in fruits and vegetables [68].

An example illustrating this concept involves the use of antibacterial peptide Nisin alongside *LAB* strains in alginate-based films and coatings. Research has shown that the combination of Nisin with strains of *Carnobacterium maltaromaticum* generates bacteriocin-like compounds, enhancing biocontrol efficacy against *L. monocytogenes*. Experimental findings on salmon chunks wrapped with these treated films demonstrated a significant bacteriostatic effect compared to untreated controls over a 28-day storage period at 4 °C [70].

Further studies have explored the incorporation of probiotic strains into films made from whey protein isolate (WPI). Pereira et al. investigated films containing *B. animalis* or *L. casei,* noting substantial reductions in cell viability after storage at 23 °C, albeit with some viability retention even after extended periods. The films maintained probiotic viability under varied storage conditions, indicating potential applications in food preservation [71].

In the context of bakery products, edible coatings have been studied for their impact on probiotic viability and product quality. Studies evaluating films made from sodium caseinate and *L. sakei* showed enhanced bacterial survival under certain storage conditions, contributing to reduced levels of pathogens like *L. monocytogenes* in beef products[72] (Gialamas et al., 2010).

Moreover, comparative studies between different film materials, such as sodium alginate and whey protein concentrate (WPC), highlighted variations in their ability to protect probiotics like *L. rhamnosus* GG during simulated digestion. Films made from WPC demonstrated effective probiotic delivery, suggesting their potential utility in producing probiotic-enriched bakery items [74].

Overall, the integration of probiotic bacteria into edible films and coatings presents a viable strategy to enhance food safety and extend shelf-life by leveraging the antimicrobial properties and protective capabilities of these films. However, the effectiveness of such applications depends on various factors including film composition, storage conditions, and specific microbial strains, underscoring the importance of tailored approaches in optimizing their efficacy in food systems [75].

2.4. *Bacillus Clausii (Alkalihalobacillus Clausii)*

One of the members of the spore-forming probiotic bacteria that is being researched extensively after *Bacillus coagulans* is *Alkalihalobacillus clausii* (formerly known as *Bacillus clausii*). It is described as an *alkaliphilic*, rod-shaped, aerobic, motile Gram-positive bacteria. Additionally, its *alkaliphilic* nature adds to the survival of alkaline environments ranging from pH 7 to 10.5 at 40°C [76]. *B. clausii* 088AE is a commercial spore probiotic (GRN 971) that is generally recognized as safe (GRAS) and has been shown to help treat human diarrhea caused by antibiotics [77], [78]. Probiotics (live microorganisms) have been shown to enhance human health when ingested in sufficient quantities [79] [80]. Spore-forming probiotic bacteria have gained a lot of attention because of proven health claims and remarkable survivability at room temperature [81] Based on clinical reports, *A. clausii* relieves rotavirus, Helicobacter pylori, upper respiratory tract infections, allergic rhinitis, late-onset sepsis, severe diarrhea, endotoxemia, and recurrent aphthous stomatitis[76]. In addition, *A. clausii (B. clausii)* possesses antimicrobial activities and immunomodulatory as well as improving gut immune function [82]. Even so, there was little information available on the substances exhibiting antimicrobial activity [83]. The strains of *B. clausii* are an ideal choice for taking probiotics together with antibiotic therapy since they possess a non-transferrable antibiotic resistance characteristic [84]. The non-spore formers comparison such as *Lactobacillus* spp., spores offer a variety of benefits, including the capacity to be dried and kept at room temperature without negatively affecting viability. Another advantage is that, unlike other *Lactobacillus* species, the spore can survive the low pH of the gastric barrier [85].

For several decades, spore-forming *Bacillus* species have been utilized to produce fermentation products and spore-based probiotic supplements. For the last fifty years, the Italian product *Enterogermina* was first approved as an over-the-counter (OTC) dietary supplement in 1958. But only in the past fifteen years, scientists have been interested in using *Bacillus* species as probiotics, and just a few major reviews have addressed the topic [85]. A limited number of Bacillus strains have been identified to be safe and available for commercial purposes [86]. *B. clausii* is one of the most widely used *Bacillus* species in terms of clinical application [87]. The only commercial strains known to generate the antimicrobial substance *clausin* are *Bacillus clausii* UBBC-07

and *Bacillus clausii* O/C [88]. *Clausin* is a ribosomally synthesized class I lanthipeptide (lantibiotic) with a low molecular weight that resembles epidermin [89]. Additionally, the existence of thioether linkages, peculiar amino acids, and stereochemistry makes *clausin* stable in a range of environmental circumstances [90] Numerous Gram-positive bacteria are inhibited by *Clausin*, including various kinds that are resistant to antibiotics[91].

It is presently being researched concerning several gastrointestinal conditions as well as respiratory infections. Researchers have discovered that the gram-positive bacteria *Enterococcus faecium*, *Clostridium difficile*are, and *Staphylococcus aureus* are all susceptible to the antibiotic compounds produced by *B. clausii* [92]. However, *Bacillus* species were once thought to be soil microorganisms, *Bacillus* spp. bacteria should be reevaluated as gut commensals due to their growing number in animal feces[86].

Compared to non-spore formers like Lactobacillus spp., spores offer benefits such as being dried and stored at room temperature without losing viability. Unlike Lactobacillus species, spores can survive the low pH of the gastric barrier, ensuring that the full dose of ingested bacteria reaches the small intestine intact without refrigeration [93].

3. EXPERIMENT AND METHODOLOGY

Research methodology and experimental procedures are discussed in this section. Materials were prepared, morphologically, and electrochemically characterized in the laboratory facility. Details of all types of equipment and materials are also presented in this chapter.

3.1. Experimental Setup and Materials Used:

The following materials and microorganisms are used for the preparation of edible films as well as for different stages:

NaOH, HCl, glycerin, ammonium sulfate, silica gel balls, and Whatman no:1 filter paper were supplied from a Sigma-Aldrich distributor (Turkey). The whey protein concentrate (80%) was provided by Malkara Birlik Süt ve Süt ürünleri A.Ş. Turkey. The agar and broth of Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB), Casein Pepton Agar, and Mueller Hilton Agar were supplied by Merck distributor (Turkey). The microbial strains *Bacillus clausii* (Enterogermina, O/C, T, SIN, and N/R), *Staphylococcus aureus* ATCC 25923, *Penicillium expansum* MRC 200806, *Aspergillus niger* MRC 200806, and *Escherichia coli* O157:H7 NCTC 12900 were supplied from culture collection of the Department of Food Engineering at Sakarya University (Turkey).

3.2. Applications of the Taguchi Trial Model

In this study, the Taguchi trial model (L9, 3^3) was designed to investigate the optimum production of antimicrobials by *B. clausii* and the optimum properties of the films at 3 different factors (pH, temperature and the amount of protein in the film solution) with 3 effective levels (Table 1).

3.2.1. Culture preparation

In this research, *Bacillus clausii* strains were cultivated in TSB at 30 °C for 24 hrs. The bacterial cell was collected through centrifugation at 9000 rpm for 15 min at 4 °C.

After removing the supernatant, the bacterial cells underwent two rounds of washing in sterile distilled water.

Table 3.1. Design for Taguchi trial model (L9, 3^3)

Sample code	pH of the film solution BI (AI)*	Incubation temperature (°C)	Whey protein in the film solution (%) (w/v)
1	8,5 (7.52±0.5)	33	6
2	8,5 (7.63±0.9)	35	8
3	8,5 (6.94±0.7)	37	10
4	9,0 (8.09±0.9)	33	8
5	9,0 (7.96±1.1)	35	10
6	9,0 (8.00±0.6)	37	6
7	9,5 (8.16±1.2)	33	10
8	9,5 (8.43±0.8)	35	6
9	9,5 (8.65±0.5)	37	8

*BI: before incubation, AI: after 24 h of incubation

3.2.2. Preparation of whey protein concentration films

6, 8, or 10 g of (WPC) whey protein concentrate was dissolved in 90.4, 87.2-, or 84-ml distilled water through a vortex then the pH was adjusted to (8.5, 9.0, and 9.5) with 0.1 M NaOH and HCl. [6]. Further, the solution was put in the water bath for 30 min at 90 ºC, adding 3.6, 4.8, and 6ml glycerol to the solution with 6, 8, or 10 g WPC in the last 5 min, respectively. After cooling 1 ml of concentrated *B. clausii* culture (7.2±0.2 $\log_{10}$ CFU/ml vegetative cells, 6.9 log±0.09 $\log_{10}$ CFU/ml spore cells) in the pellet was transferred to each WPC film solution. Then, the solutions were incubated at 33°C, 35°C, and 37°C for 24 h. After 24 h 100 ml of solutions were cast on the Teflon-coated plates (diameter of 22 cm^2) and air bubbles were removed through the hot loop. Following drying under a biological safety cabinet at 50±5 % RH at 25°C ±1 for 48 h they were peeled from the plates (Figure 1).

Figure 3.1. WPC film after drying at 25C, 50%±5 RH for 72 hours

3.2.3. Time affects the bacterial cells in film (4°C and 25°*C)*

For checking bacterial cells in edible film, two different stages (0, and 14 days) were determined through the dilution process the antifungal activity and spore count. Make ready and cut the films (0.1g) put in 9.9ml sterile distilled water mix by vortex then using (TSA and Mueller Hinton), pour the solution by drigalski and keep them at 33°C for 24 h and count the bacterial growth.

3.3. Mechanical and Physical Properties of the Antifungal Films

3.3.1. Mechanical properties

The film thickness was determined with a micrometer (Pittsburgh, U.S.A.) to the nearest 0.001 mm. Tensile strength (TS) and elongation at break (% E) were tested at 0–14 days of storage using the standard procedure ISO 1924-2-1994 applying Instron Universal Testing 3367 (U.S.A.) and the software Blue Hill 2. (USA: Norwood, Mass).

3.3.2. Water vapor permeability (WVP) of the films

The standard process outlined by the American Society for Testing and Materials (96–80) was used to evaluate a film's water vapor permeability (WVP). The technique with a few changes, [94] film in diameter of 45 cm was sealed on the mouth of the small cups 40 mm in diameter, 25 mm in depth containing 10 g silica gel balls The cups were placed in a conditioning chamber at 85% RH, 37°C. The weight of the cups was observed every two hours up to 12 hours. WVP of the films was calculated using equation (2).

$WVP = (C \times T) \times (A \times \Delta p)^{-1}$ g: mm=$m^2.day^{-1}$: kPa^{-1}

The equation contains following values: A is the exposed film area (0,00126 m 2), T is the film thickness (mm), g/d is the water vapor transmission rate, and p is the vapor partial pressure difference across the film (p: kPa at 85% RH at 37 °C).

3.3.3. Percent water solubility (%WS)

Following the procedures outlined in [95] the film's percent water solubility (% WS) was determined. After being cut into 2×2 cm² squares, the film samples underwent a second drying process at 105±2 °C for 24 hours. Before and following drying, each sample's weight was calculated. After that, the samples were kept at 23±2°C for 24 hours in 50 mL of distilled water by using filter paper (Whatman, Grade 1) to filter the solution, the film's water-insoluble component was obtained. Following a 24-hour drying process at 105±2 °C and weighing, the paper containing the water-soluble component was once again weighed. Utilizing the formula, the % WS was determined.

$$\% \text{ WS} = (m_1 - m_2) \times (m_1) - 1 \times 100 \qquad \text{Equation (2)}$$

In the Primary and last dried weights samples of the whey protein are denoted by m1 and m2 in Equation (2).

3.3.4. . Microbiological analysis of the edible film solutions and dry film samples

The edible film solution (1 ml) or dry film samples (0.1 g) were homogenized in 9 ml or 9.9 ml NaCl solution (0.85%) using a stomacher for 2 min, respectively. After serial dilution, they were plated on TSA and incubated at 30°C for 24 h to investigate the total vegetative cell count of *B. clausii*. To observe spore count in the prepared film solutions or dry film samples, the serial dilutions prepared in a water bath before were heated for 5 minutes at 80° C, cooled immediately, and plated on Casein Pepton Agar. Following incubation at 30°C for 24h, the colonies on the plate were counted.

3.3.5. Antimicrobial of WPC film solution

The film solution, which was left to ferment for 24 h with *B. clausii* (7.2 log CFU/ml) at 33, 35, or 37 °C, was centrifuged at 9000 RPM at 4 ºC for 15 min, then the supernatant (10 ml) was saturated with ammonium sulfate (6 g) and kept at 4 ºC for 24 h. The solutions were then centrifuged at 9000 RPM at 4ºC for 15 min and washed twice with distilled water, after which the pellet was dissolved in 1 ml of 100 mM Tris-HCl buffer (pH 7.0).

In this work, the disk diffusion method was used to investigate the antimicrobial activity of the film [96]. Using a cork borer, the film samples were carefully cut into 6 mm diameter pieces. Then, Muller Hinton Agar was inoculated from the activated culture of *E. coli*, *S. aureus*, *A. niger,* or *P. expansum* (50µl). After applying the disc films to the inoculated agar plates, they were incubated for 24 hours at 35°C on bacteria cultures and 25°C for 5 days for mold cultures. To measure the inhibition zones a ruler was used perpendicular to the discs.

3.3.6. Statistical analysis

For statistical analysis, three replicates were used, utilizing IBM's SPSS software version 26.0. The expression of the values such as mean standard deviation (SD) and evaluation of mean was studied using variance (ANOVA)

4. RESULTS AND DISCUSSION

4.1. The Growth of *B. clausii* in the WPC Film

After 24 h fermentation, the highest number of vegetative and spore forms of *B. clausii* in WPC edible films solution was counted at 8.96 and 6.07 log cfu/ml, respectively. The optimum growth of *B. clausii* (the total of vegetative and spore cells, 7,92 log CFU/ml) was observed when pH of the film solution was 9.5, incubation temperature was 35°C, and protein content in the film was 6 % (Table 2). The most useful parameter was the incubation temperature on the growth of *B. clausii* in the film solution (Figure 2a). In previous studies, unlike our study, optimum growth temperature and pH value for *B. clausii* were reported as 37°C and pH 8 [97]. The nutrition value and low water activity of the film solution might shift its growth requirement. Studies have also shown that *B. clausii* has an alkaliphilic character; this must be why it showed better growth at pH 9.5 in this study compared to low pH conditions [98].

Table 4.1. The number of vegetative and spore cells of B. clausii (log10 CFU/g) during WPC film preparation and 14 days of storage

Vegetative cells			**Dry films (log_{10} CFU/g)**		
				4 °C	**25 °C**
Sample Code	**BI (log_{10} CFU/ml)**	**AI (log_{10} CFU/ml)**	**0 day**	**14 days**	
1	7.2 ± 0.13^{a}	7.81 ± 0.12^{a}	8.18 ± 0.18^{a}	7.07 ± 0.44^{a}	7.18 ± 0.31^{b}
2	7.2 ± 0.13^{a}	$7.49 \pm 0,15^{bc}$	8.87 ± 0.13^{a}	6.44 ± 0.11^{b}	4.70 ± 0.14^{ef}
3	7.2 ± 0.13^{a}	$7,70 \pm 0,17^{d}$	8.73 ± 0.20^{a}	6.21 ± 0.30^{b}	5.89 ± 0.16^{def}
4	7.2 ± 0.13^{a}	8.12 ± 0.12^{f}	9.35 ± 0.15^{bc}	7.87 ± 0.12^{b}	6.57 ± 0.12^{f}
5	7.2 ± 0.13^{a}	8.25 ± 0.13^{c}	9.78 ± 0.16^{a}	7.21 ± 0.45^{a}	6.10 ± 0.25^{c}
6	7.2 ± 0.13^{a}	7.59 ± 0.23^{d}	8.60 ± 0.10^{ab}	6.47 ± 0.24^{a}	6.94 ± 0.20^{a}
7	7.2 ± 0.13^{a}	8.52 ± 0.11^{b}	9.74 ± 0.19^{d}	7.50 ± 0.28^{d}	7.54 ± 0.33^{c}
8	7.2 ± 0.13^{a}	8.96 ± 0.08^{a}	9.11 ± 0.14^{c}	7.70 ± 0.14^{cd}	7.25 ± 0.21^{d}
9	7.2 ± 0.13^{a}	8.18 ± 0.16^{e}	9.20 ± 0.12^{c}	6.23 ± 0.11^{c}	6.13 ± 0.18^{de}
Spore cells					
1	6.9±0.11	6.07 ± 0.12^{a}	7.44 ± 0.10^{a}	7.69 ± 0.16^{ab}	7.79 ± 0.15^{ab}
2	6.9±0.11	5.76 ± 0.14^{e}	6.04 ± 0.23^{b}	7.02 ± 0.33^{cd}	6.64 ± 0.21^{c}
3	6.9±0.11	3.37 ± 0.11^{g}	4.92 ± 0.16^{e}	6.34 ± 0.21^{e}	5.53 ± 0.10^{d}
4	6.9±0.11	2.85 ± 0.08^{h}	4.10 ± 0.17^{e}	6.55 ± 0.23^{de}	6.76 ± 0.20^{c}
5	6.9±0.11	4.63 ± 0.09^{e}	5.98 ± 0.14^{b}	7.51 ± 0.16^{bc}	6.82 ± 0.21^{c}
6	6.9±0.11	5.54 ± 0.15^{b}	6.43 ± 0.25^{a}	8.20 ± 0.15^{a}	8.28 ± 0.11^{a}
7	6.9±0.11	4.41 ± 0.13^{d}	5.87 ± 0.28^{bc}	2.66 ± 0.17^{f}	2.36 ± 0.17^{f}
8	6.9±0.11	4.12 ± 0.14^{e}	5.51 ± 0.18^{d}	3.10 ± 0.35^{f}	3.05 ± 0.21^{e}
9	6.9±0.11	3.87 ± 0.09^{f}	4.56 ± 0.07^{cd}	6.51 ± 0.22^{de}	7.23 ± 0.57^{bc}

BI: Before incubation, the concentration of vegetative cells of B. clasuii in the film solutions AI: After 24 h incubation, the concentration of vegetative cells of B. clasuii

in the film solutions. The superscripts of different letters in a column indicate significant differences among the film samples (Duncan test, P<0.05).

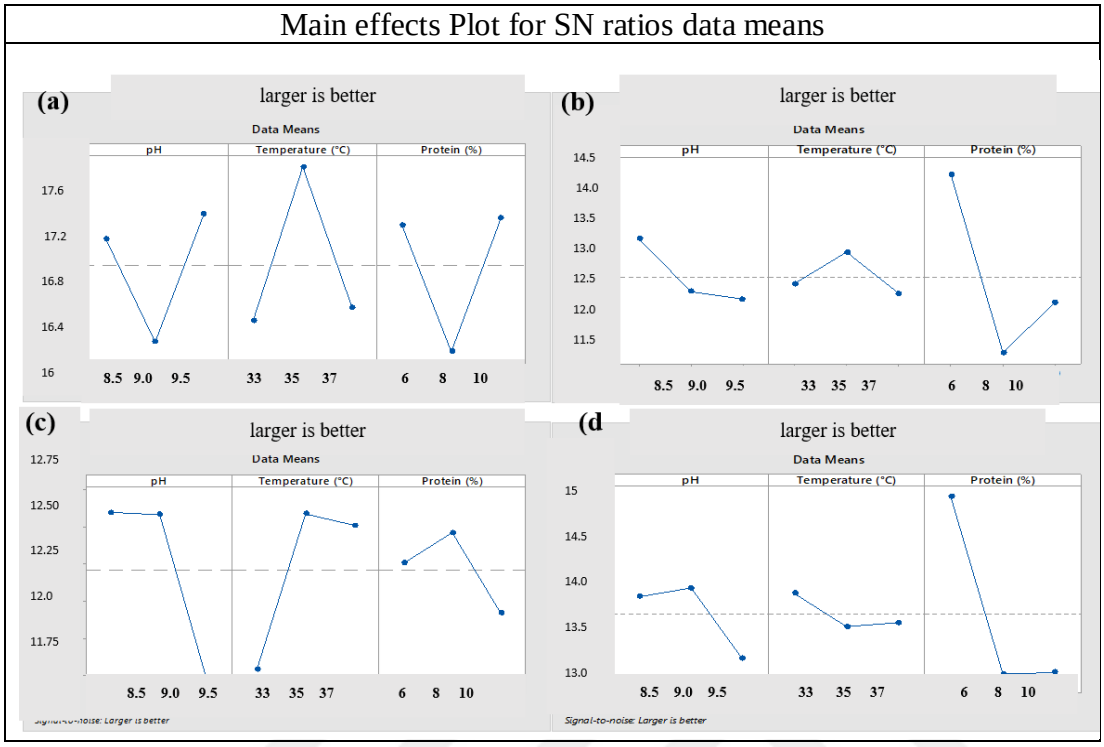

Figure 4.1. (a) The growth of B. clausii vegetative cells in the film solution, (b) The growth of B. clausii spore cells in the film solution, (c) The number of B. clausii vegetative cells in the dry film, (d) The number of B. clausii spore cells in the dry film i

4.2. The Survival of B. clausii in the Films

After drying the films, vegetative and spore cells on the first day of storage were reported as 8.18 and 7.44 log cfu/g, respectively. The best survival ratio of vegetative and spore cells of *B. clausii* in the dry film at 25°C during 14 days was observed as 87 and 164%, respectively (Table 3). The survival of *B. clausii* in vegetative form during 14 days was supported by increasing the temperature of storage from 4 to 25ºC. The highest number of vegetative *B. clausii* cells was reported when pH of the film was 9.5 and protein content was 6% (Figure 3a, 3b). According to Taguchi test trial, pH and protein content in the film formula were the most effective parameters on the survival of *B. clausii* vegetative cells at initial stages as well as 4 and 25°C, respectively. On the other hand, changes in pH most effectively change the survival ability of the spores at both temperatures (Figure 3c, 3d).

Table 4.2. The survival of vegetative and spore cells of B. clausii in the WPC films during 14 days.

Sample Code	Survival of vegetative cells (%)		Survival of spore cells (%)	
	4°C	25°C	4°C	25°C
1	86.43	87.78	103,36	104,70
2	72.60	52.99	116,23	109,93
3	71.13	67.47	128,86	112,40
4	84.17	70.27	159,76	164,88
5	73.72	62.37	125,59	114,05
6	75.23	80.70	127,53	128,77
7	77.00	77.41	45,32	40,20
8	84.52	79.58	56,26	55,35
9	67.72	66.63	142,76	158,55

Significant considered at P≤0.05, variables at same letters on column indicates non-significant differences, N=3.

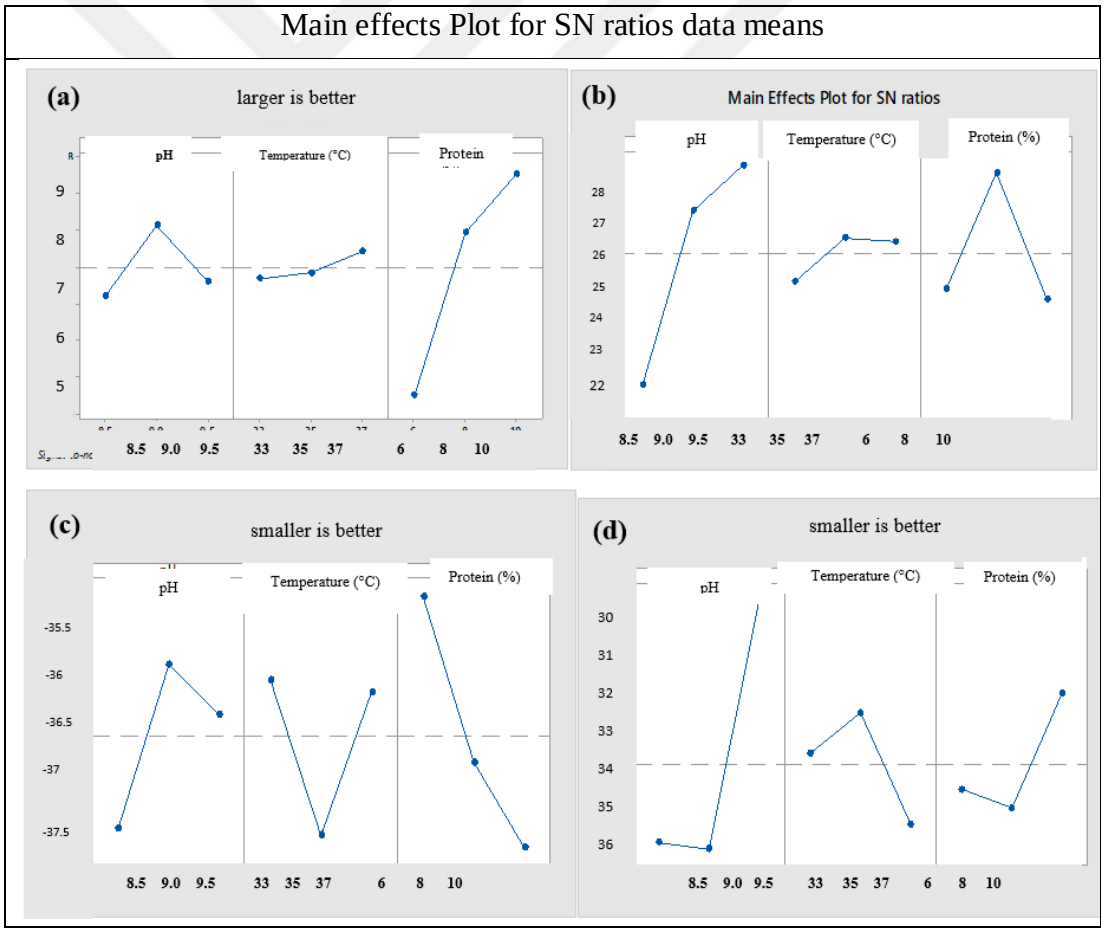

Figure 4.2. (a) Signal/noise ratio results of tensile strength, (b) percent elongation, (c) water vapor permeability, (d) percent water solubility of the film containing B. clausii in Taguchi experimental design.

Encapsulation of microorganisms by polysaccharide and protein-based films generally enhanced the capability of microorganisms both in drying and storage [99]. Likewise, the survival of the microorganism is the most important factor in available water content in the film. When the protein content in the film was 6% compared with 10%, a better survival ability for *B. cluasii* was observed. The most important reason for this might be that the amount of free water decreases indirectly when the amount of whey protein increases, Whey protein as a water-soluble protein has a high binding affinity towards water molecules. Increasing the amount of protein in the film formula could directly affect the survival ratio of *B. clausii*. After bacteria cells were not able to grow in the environment with aw lower than 0.76 and water activity of the WPC film changed from 0.64 to 0.61 [6]. However, some deep-sea halophilic eubacteria like *B. clausii* can resist in a dry film environment by producing compatible solutes like ectoine [96]. In a high osmatic pressure, *B. clausii* can maintain internal osmotic pressure using ectoine [97]. The survival ratio of *B. clausii* in the WPC film was similar to that of *B. coagulans* in the milk protein-based film (87%) at a similar condition [98].

4.3. Mechanical Properties of WPC Films

The thickness of WPC film containing *B. clausii* was not changed significantly by parameters such as the film's pH, temperature, and concentration of protein ($p>0.05$). (Table 2, Figure 3a). The film optimum TS (3.14 MPa) and %E (27.63%) were observed at pH 9.5, 37°C, and 8% WPC and pH 9, 35°C, and 10% WPC, respectively.

Although increasing in pH of the film improved TS, rising in incubation temperature did significantly affect TS. According to the Taguchi program, TS and %E were affected mostly by pH and protein content, respectively ($p<0.05$) (Figure 3a, 3b) (Table 5). The combination effect of pH and temperature, protein concentration and temperature, and all three parameters on TS were also found significant ($p<0.05$). However, only a combination of pH and temperature significantly affected %E of the film ($p<0.05$).

In our previous work, when *Williopsis saturnus* yeast was added to the whey protein-based film, the TS was measured as 5.6 MPa and %E 23% [6]. However, an increase in the number of yeasts added did not significantly affect the TS but decreased percent elongation is seen. While the TS of the film measured in this study was reported to be smaller than in the previous study, E% of the film was similar. The reason for its small

size is that the protease enzyme synthesized during the fermentation of *B. clausii* in the film solution for 24 hours should have reduced the strength of the film as a result of the formation of peptides from the protein chains. Thus, shortening the length of the protein chains that make up the structure of the film would allow also a strength reduction and elasticity improvement. The presence of bacteria also might support % E and reduce the strength by probably acting as a plasticizer [99]

In the production of whey protein film, denaturation is carried out by breaking the hydrogen bonds in the protein structure by heating. This process also allows the release of hydrophobic and internal SH groups. The film is formed by the formation of new hydrogen and hydrophobic bonds as well as disulfide bonds between protein chains during drying [100][101]; Studies have shown that disulfide bond formations between protein chains increase above pH 8 [102]. In this study, the high pH 9.5 should have increased the TS property while supporting the formation of disulfide bonds.

4.4. Water Vapor Permeability (WVP) of the Films

The optimum WVP of the film sample is 45.89 g.mm. m^{-2}. $d^{-1}kPa^{-1}$ was reported at the condition with pH 9, 37° C, and 6% WPC (Table 4) According to Taguchi program, protein contents in the film formula had the highest effect on WVP of the film (Figure 3c). WVP was significantly increased by increasing protein amount in the film ($p<0.05$) (Table 5). Moreover, pH of the film was the second effective parameter on WVP after protein content. [106] also reported that increasing pH from 7 to 10 in the film formulation based on WPC and chia mucilage improved WVP properties.

Due to its hydrophobic feature, films made with WPC have high water vapor permeability. Compared to similar studies that studied WPC-based film, it was determined that the WVP of the film was approximately 2 times higher in this study [6]. Since the protein chain's interactions within the film structure may be weakened by the incorporated *B. clausii* cells and the peptides formed because of fermentation, the WVP of the film was higher compared to similar studies[107].

4.5. Percent Water Solubility (%WS)

WS % of the WPC film containing *B. clausii* ranged from 24.49 to 80.02% with the lowest %WS measured when the WPC in the film was 10% (wt/v), incubation

temperature was 35°C and pH of the film before fermentation was 9.5 (Table 4, and Figure 3d). The pH or protein increase in the film formula significantly improved the %WS of the films ($p<0.05$); however, higher or lower than 35°C incubation temperature decreased %WS properties. The pH and protein content of edible film were the most effective parameters on %WS of the film ($p<0.05$) (Table 5). Compared to other similar studies, the water solubility of the films produced in this study was quite higher (Karabulut and Cagri-Mehmetoglu 2018). One of the reasons might be that the studies showed that incorporating bacteria or yeast into protein film formula could significantly increase solubility since the addition of the cells weakens the interaction between intermolecular protein chains. For example, [6] reported that incorporating *Willopsis Saturnus* yeast cells into WPC-based film significantly increased the solubility of the film from 23 to 36%. The other reason may be that hydrolyzing of proteins of the film by protease enzymes produced by *B. clausii* during 24 h incubation leading an increase in the amount of low molecular weight proteins and peptides in the film texture. This will increase the number of film-to-water transitions and solubility.

Table 4.3. The properties of WPC-based edible film containing B. clausii

Samples	Thickness (mm)	Tensile Strength (MPa)	Elongation at Break (%)	Water Vapour Permeability (WVP) (g.mm. $m^{-2}d^{-1}kPa^{-1}$)	Water Solubility (%)
1	0.22 ± 0.01^{a}	1.25 ± 0.17^{c}	10.87 ± 0.88^{c}	61.44 ± 2.95^{d}	77.76 ± 1.93^{ab}
2	0.20 ± 0.01^{a}	2.63 ± 0.10^{ab}	18.65 ± 1.20^{bc}	71.45 ± 3.92^{bc}	60.27 ± 30.08^{ab}
3	0.22 ± 0.01^{a}	1.51 ± 0.06^{c}	21.06 ± 4.52^{ab}	95.80 ± 5.08^{a}	51.47 ± 12.22^{abc}
4	0.19 ± 0.04^{a}	2.85 ± 0.91^{ab}	20.08 ± 2.33^{ab}	74.96 ± 7.43^{bc}	59.31 ± 26.53^{ab}
5	0.22 ± 0.02^{a}	2.08 ± 0.17^{bc}	27.63 ± 5.40^{a}	77.12 ± 7.49^{b}	53.80 ± 6.82^{ab}
6	0.18 ± 0.03^{a}	3.01 ± 0.39^{ab}	14.94 ± 5.16^{bc}	45.89 ± 3.52^{e}	80.02 ± 5.63^{a}
7	0.21 ± 0.01^{a}	2.97 ± 0.30^{ab}	23.05 ± 3.61^{ab}	60.16 ± 5.59^{d}	24.49 ± 6.98^{c}
8	0.19 ± 0.04^{a}	2.67 ± 0.09^{ab}	10.32 ± 0.08^{c}	77.85 ± 7.51^{b}	24.67 ± 6.21^{c}
9	0.18 ± 0.02^{a}	3.14 ± 0.37^{a}	20.66 ± 3.71^{ab}	65.22 ± 2.06^{cd}	$50.27 \quad 12.35^{bc}$

The superscripts of different letters in a column indicate significant differences among the film samples (Duncan test, $P<0.05$).

Table 4.4. Analysis of variance on the effect of different parameters and their interactions on the properties of the film (p-values for independent variables and interactions).

Dependent Variable: WVP						
Source	Type III Sum of Squares	df	Mean Square	F	P-Sig.	Partial Eta Squared
Corrected Model	2150,231[a]	6	358,372	2,299	,075	,657
Intercept	132,251,005	1	132,251,005	848,449	,000	1,000
pH	539,856	2	269,928	9,150	,002	,949
temperature	459,774	2	229,887	7,792	,004	,910
protein	1,150,601	2	575,301	19,501	,000	1,000
pH * temperature	3,737,051	4	934,263	31,668	,000	1,000
pH * protein	3,046,224	4	761,556	25,814	,000	1,000
temperature * protein	3,126,306	4	781,577	26,493	,000	1,000
pH * protein * temperature	2,586,450	2	1,293,225	43,836	,000	1,000
Error	3,117,476	20	155,874			
Total	137,518,711	27				
Corrected Total	5,267,707	26				
Dependent Variable: Water solubility						
Corrected Model	9177,425[a]	8	1,147,178	4,964	,002	,688
Intercept	77,461,686	1	77,461,686	335,167	,000	,949
pH	5,635,974	2	2,817,987	12,193	,000	,575
temp	927,101	2	463,551	2,006	,164	,182
protein	1,513,946	2	756,973	3,275	,061	,267
pH * temperature	2,614,349	4	653,587	2,828	,056	,386
pH * protein	2,027,505	4	506,876	2,193	,111	,328
temperature * protein	6,736,377	4	1,684,094	7,287	,001	,618
pH * protein * temperature	1,100,403	2	550,202	2,381	,121	,209
Error	4,160,050	18	231,114			
Total	90,799,161	27				
Corrected Total	13,337,475	26				
Dependent Variable: Tensile strength						
Corrected Model	7,518[a]	8	,940	6,574	,005	,854
Intercept	108,437	1	108,437	758,539	,000	,988
pH	4,152	2	2,076	14,521	,002	,763
temperature	,114	2	,057	,399	,682	,082
protein	1,611	2	,806	5,635	,026	,556

Table 4.4. (Continued) Analysis of variance on the effect of different parameters and their interactions on the properties of the film (p-values for independent variables and interactions).

pH * temperature	3,252	4	,813	5,687	,015	,717
pH * protein	1,755	4	,439	3,069	,075	,577
temperature * protein	5,793	4	1,448	10,130	,002	,818
pH * temperature * protein	1,641	2	,820	5,739	,025	,560
Error	1,287	9	,143			
Total	117,242	18				
Corrected Total	8,804	17				
Dependent Variable: % Elongation						
Corrected Model	511,158[a]	8	63,895	5,199	,012	,822
Intercept	6,215,753	1	6,215,753	505,750	,000	,983
pH	51,532	2	25,766	2,096	,179	,318
temperature	3,075	2	1,538	,125	,884	,027
protein	435,916	2	217,958	17,734	,001	,798
pH * temperature	456,552	4	114,138	9,287	,003	,805
pH * protein	23,711	4	5,928	,482	,749	,177
temperature * protein	72,167	4	18,042	1,468	,290	,395
pH * temperature * protein	20,635	2	10,318	,840	,463	,157
Error	110,612	9	12,290			
Total	6,837,523	18				
Corrected Total	621,770	17				
a. R Squared = ,822 (Adjusted R Squared = ,664)						
b. Computed using alpha = ,05						

4.6. The Antimicrobial Effect of the WPC Film Solution

The film solution based on WPC contains *B. clausii* showed the inhibition effects on the molds (*Aspergillus niger* MRC 200806, *Penicillium expansum* MRC 200806) and the bacteria (*Escherichia coli* O157:H7 NCTC 12900 *and Staphylococcus aureus* ATCC 25923). The highest diameter of inhibition zones against the growth of *A. niger MRC 200806* and *P. expansum MRC 200806* was reported by 13.63 and 16.88 mm, respectively (Table 6). The optimum inhibition against mold was observed when the incubation temperature was 35ºC, the pH of the film solution was 8.5, and protein content in the film formula was 8% respectively (w/v). The pH was the most effective

parameter on the antifungal properties of the film solution (Figure 4a, 4b). While the diameter of inhibition zones against the growth of *E. coli* O157:H7 NCTC 12900 ranged from 7.38 to 17.25 mm, the optimum antimicrobial properties were received against *E. coli* when the incubation temperature was 33ºC and protein content was 10% (Figure 4c, 5D). Furthermore, the growth of gram-positive *S. aureus ATCC 25923* was inhibited by WPC film solution with inhibition zones changing from 8.88 to 11.38 mm (Figure 5C) According to the Taguchi test trial, first one is pH and second protein contents were the most effective parameters on the antimicrobial properties of the film against *S. aureus* (Figure4d).

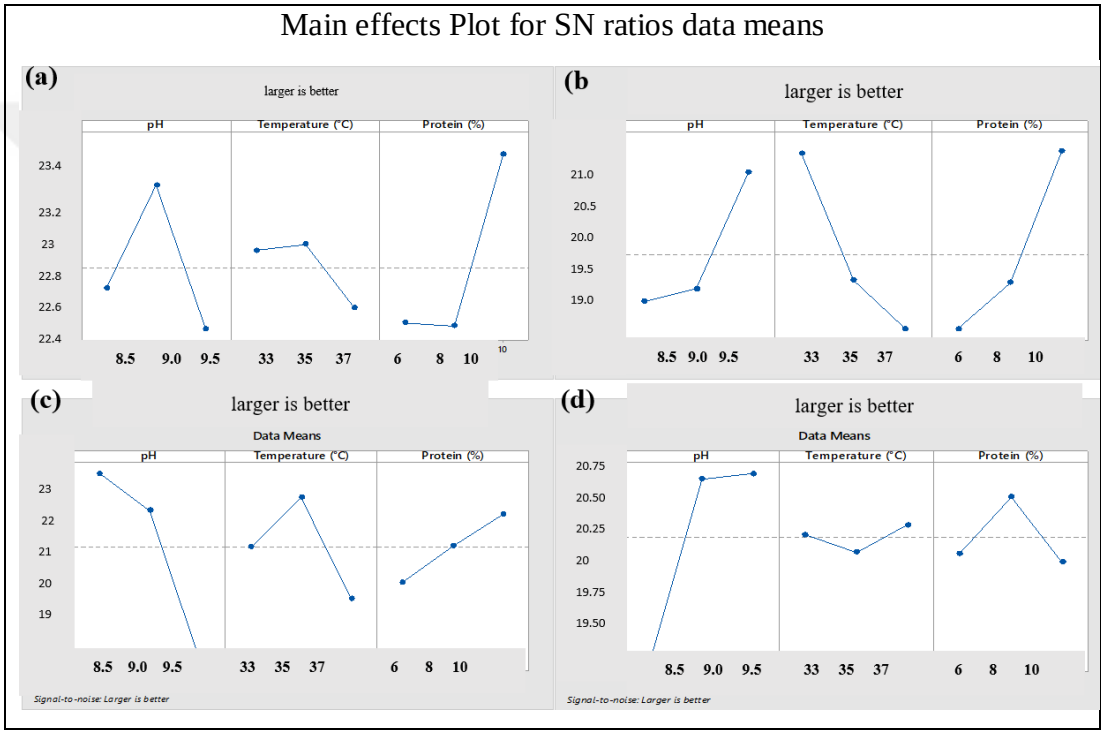

Figure 4.3. (a) Inhibition of the film containing B. clausii against A. niger, (b) P. expansum, (c) E. coli, (d) S. aureus in Taguchi experimental design.

In this study, *B. clausii* was allowed to grow and produce bacteriocins in the film solution for 24 hours. Studies have reported that *clausin*, one of these bacteriocins produced by *B. clausii*, inhibits peptidoglycan synthesis and inhibits gram-positive bacteria (Banerji et al., 2022). Essential amino acids can be found in whey proteins, which are a significant nutritional source. [108]. As shown in the previous studies, *B. clausii* antimicrobial agent production was achieved by using whey as a medium [109]. Other than bacteriocin *B. clausii* synthesized, some antimicrobial peptides were also produced from hydrolyzing whey protein by protease enzymes during fermentation. There are antibacterial effects of these hydrolyzed antimicrobial peptides on both

gram-positive and gram-negative bacteria such as *Salmonella typhimurium, Shigella flexneri, Escherichia coli, Listeria monocytogenes, Enterococcus faecalis* and *Staphylococcus aureus.*

Table 4.5. Inhibition zones of the WPC film solutions and dry films containing B. clausii

	Inhibition zone (mm)			
	Film Solutions			
Sample Code	***A. niger***	***P. expansum***	***E. coli***	***S. aureus***
1	11.38 ± 0.48^{bc}	13.88 ± 2.14^{b}	9.75 ± 0.29^{c}	8.88 ± 0.25^{c}
2	13.63 ± 1.93^{a}	16.63 ± 1.03^{a}	11.00 ± 0.71^{b}	10.00 ± 0.41^{b}
3	9.88 ± 1.25^{cd}	12.50 ± 0.82^{bc}	7.38 ± 0.48^{d}	9.00 ± 0.71^{c}
4	11.00 ± 1.87^{bc}	12.00 ± 1.29^{c}	7.88 ± 0.25^{d}	11.00 ± 0.91^{ab}
5	12.38 ± 0.85^{ab}	16.88 ± 1.25^{a}	10.50 ± 0.41^{bc}	10.13 ± 0.25^{b}
6	9.88 ± 0.75^{cd}	10.88 ± 0.95^{cd}	10.00 ± 0.41^{c}	11.38 ± 0.85^{a}
7	8.13 ± 0.48^{de}	10.13 ± 0.75^{de}	17.25 ± 0.87^{a}	11.25 ± 0.65^{a}
8	6.88 ± 0.48^{e}	8.63 ± 0.75^{e}	7.38 ± 0.48^{d}	10.38 ± 0.75^{ab}
9	10.50 ± 2.16^{bc}	8.50 ± 0.00^{e}	9.75 ± 0.50^{c}	11.00 ± 0.58^{ab}

The superscripts of different letters in a column indicate significant differences among the film samples (Duncan test, $P<0.05$)

4.7. Antimicrobial Activity of WPC Nanocomposite Films

Inhibition zones of the dry film samples only observed growth oppositely of *A. niger* on the agar plate with 12-15 mm (Figure 5A). However, the film could not inhibit the growth of *P. expansum* (Figure 5B). The optimum inhibition against *A. niger* was observed when pH was 8.5 or 9.0, and protein concentration was 8 or 10% (Figure 2a, 2c). The change in incubation temperature did not affect antifungal properties of the film (Figure 2c). Moreover, the inhibition effect of the film against the growth of bacteria could not be measured since *B. clausii* cells in the film diffused and prevented observing the inhibition zone. Therefore, the antimicrobial measurement against bacteria was carried out with the film solution after separating *B. clausii* cells.

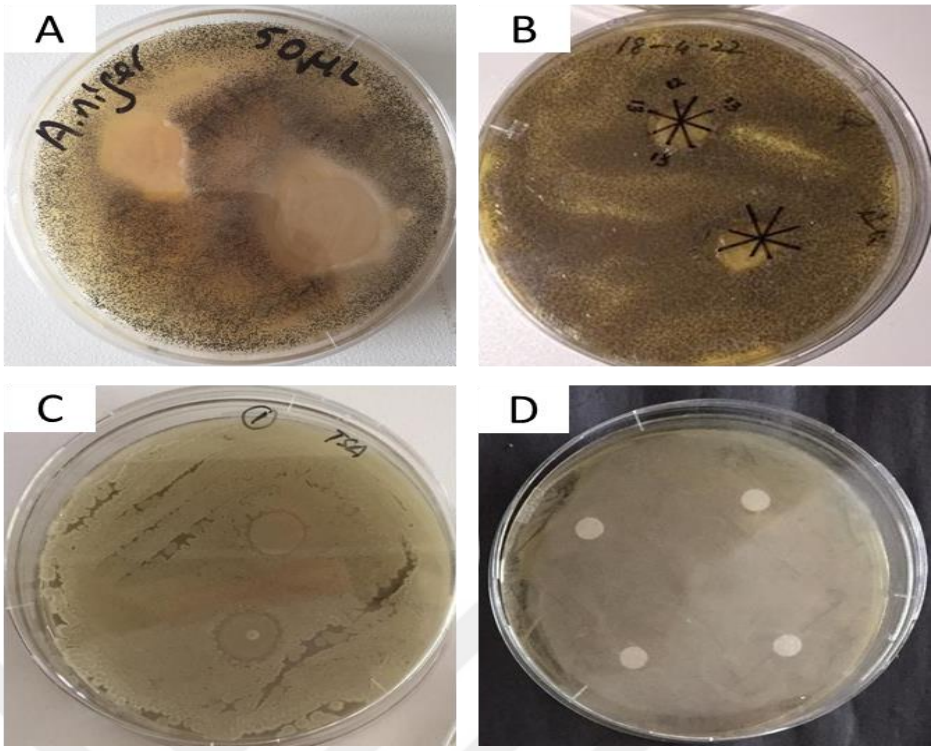

Figure 4.4. (A, B) A. niger, P.expansum inhibition zones of the molds, clear area is the in bacteria (C, D) S.aureus, E.coli the E.coli have extra growth with the .presence of B. cluasii on TSAYE.

Main effects Plot for SN ratios data means

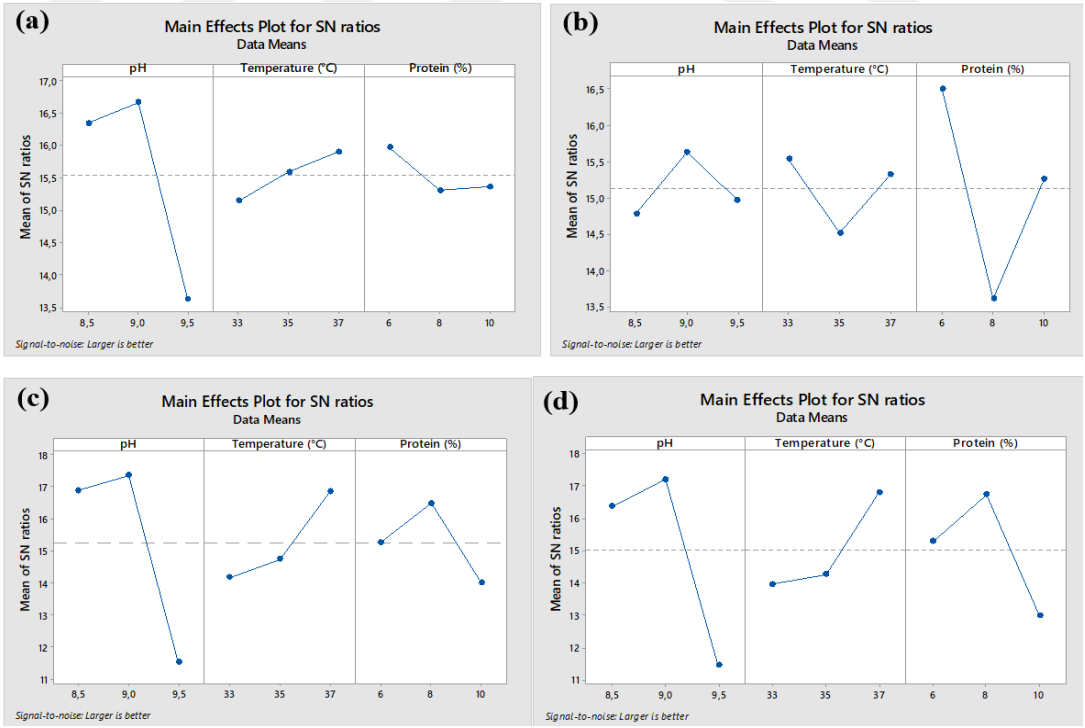

Figure 4.5. Signal/noise ratio results of the number of B. clausii vegetative cells in the dry film after 14 days of storage at 4 (a) and 25ºC (b), the number of B. clausii spore cells in the dry film after 14 days of storage at 4 (c) and 25ºC (d)

5. CONCLUSION

In conclusion, a suitable substrate for adding antagonist bacteria, such as *B. clausii*, was given by WPC-based films, which also sustained the survivability of the bacteria. The inhibition of *A. niger, P. expansum, E. coli,* and *S. aureus* on TSAYE at pH 8.5, 9.0, and 9.5 was achieved by incorporating *B. clausii* into WPC films. The survival percentage of *B. cluasii* cells in the dry film was found to be around 87% during 14 days of storage at 4 and 25 °C. Mechanical properties of the film, such as TS and %E, were best measured when the film contained 8% protein. Increasing the pH of the film resulted in the improvement of both TS and WVP. The WVP and %WS properties of the film also improved as the protein decreased and increased, respectively. This study showed that it is possible to produce a film solution containing antimicrobial substances by fermenting *B. clausii*. For future studies, it may be suggested to associate the antimicrobial substances produced by the fermentation of *B. clausii* in the film solution with the changes in the properties of the film.

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