GRADUATE SCHOOL
ISTANBUL MEDENIYET UNIVERSITY
DEPARTMENT OF BIOENGINEERING

Investigation of the Use of Different Support Materials in Laccase Enzyme Production by Solid State Fermentation from Waste Coffee

Master's Thesis

Yağmur ÖNER

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THESIS JURY APPROVAL

This Master's thesis was titled "Investigation of the Use of Different Support Materials in Laccase Enzyme Production by Solid State Fermentation from Waste Coffee." written by Yağmur Öner in the Department of Bioengineering was accepted by our jury.

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STATEMENTS

Style and Reference Manual Statement

Having reviewed this thesis written under my supervision, I confirm that it has been written in accordance with APA Manual of Style and used its in text reference format consistently throughout the entire text.

Signature

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Declaration of Originality

I hereby declare that all information in this dissertation has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conducts, I have fully cited and referenced all material and results that are not original to this work.

Signature

Yağmur Öner

GENİŞ ÖZET

Atık Kahveden Katı Kültür Fermantasyonu ile Lakkaz Enzimi Üretiminde Farklı Destek Materyallerinin Kullanımının Araştırılması

Öner, Yağmur

Yüksek Lisans Tezi, Biyomühendislik Anabilim Dalı

Danışman: Dr. Öğr. Üyesi Işık Çoban

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20. yüzyılın ortalarından itibaren dünya nüfusu üç katından fazla artmış olup, 2059 yılına kadar bu sayının 10 milyarı aşması öngörülmektedir. Bu nüfus artışı, küresel düzeyde iklim değişikliği, hava ve çevre kirliliği gibi ciddi sorunları beraberinde getirmiştir. Aynı zamanda, enerji, gıda ve temiz su kaynaklarının etkin yönetimi gibi konular da giderek daha kritik hale gelmiştir. İklim değişikliği, gıda güvenliği, sürdürülebilir ekonomik büyüme, sosyal ve çevresel istikrar gibi büyük küresel zorlukların üstesinden gelmek için bu kaynakların sürdürülebilir yönetimi büyük önem taşımaktadır.

Atıkların tekrar kaynak olarak kullanılabildiği, kaynakların üretim sistemleri içerisinde olabildiğince tutularak atık miktarının düşürüldüğü ve etkin atık yönetiminin yapıldığı döngüsel çözümlere duyulan ihtiyaç giderek artmaktadır. Bu nedenle sistemlerin optimizasyonunun yanı sıra sürdürülebilir atık yönetimi ve döngüsel ekonomi (DE) kavramları üzerinde durulmaktadır. DE kavramı, mevcut ekonomik sistemi sorgulayan çeşitli teorilere dayanır ve doğal kaynakların aşırı kullanımını azaltmayı hedefler. DE, kaynakların geri dönüştürülmesi, azaltılması ve yeniden kullanılması ile kaynak güvenliği ve verimliliğini ön planda tutar.

Bu araştırma, mikroorganizma çoğalmasını teşvik eden biyokömür ve kahve posası gibi farklı destek malzemelerinin mikroorganizmalardan metabolitlerin üretimi ve ekstraksiyonu üzerindeki etkisini incelemektedir. Çalışma, fındık kabuğu ve kahve posası gibi tarımsal yan ürünleri ekonomik olarak değerli kaynaklara dönüştürerek atık kullanımının önemini vurgulamaktadır. Bu amaçla, endüstrilerde döngüsel bir biyoekonomiyi teşvik etmeyi amaçlamaktadır.

Araştırma bulguları, biyokömür ve kahve posasının katı kültürlerde uygun destek malzemeleri olarak kullanılabileceği ve bunların uygulanması için en uygun koşulların belirlenebileceğini göstermektedir.

Kahve, sudan sonra dünya çapında en yaygın tüketilen içecek olup, ham petrolden sonra borsada en çok işlem gören ikinci üründür. Küresel kahve endüstrisi, dünya çapında milyonlarca insana geçim kaynağı sağlayarak ve milyarlarca dolarlık ekonomik güce sahiptir. Ancak bu endüstri aynı zamanda önemli bir çevresel ayak izi de taşımaktadır; kahvenin üretimi, işlenmesi ve tüketimi önemli sera gazı emisyonlarından ve diğer çevresel etkilerden sorumludur. Kritik bir konu da kahve endüstrisi tarafından üretilen önemli miktardaki atığın sadece küçük bir kısmının geri dönüştürülebilir olmasıdır. Günlük yaklaşık iki milyar fincan kahve tüketimi, tahmini 70 milyon ton yıllık emisyona katkıda bulunmaktadır Ayrıca, demlenmiş kahvenin yaklaşık %90'ı kullanılmış Kullanılmış Kahve Telvesine (KKT) dönüştürülmektedir.

Kahve atıklarının yeniden kullanımı, çevresel açıdan karbondioksit ve metan emisyonlarını azaltmada önemli bir adımdır. KKT, kahve ve kahve bazlı içeceklerin hazırlanmasından

kaynaklanan en büyük atık bileşenlerinden biridir ve yıllık yaklaşık 15 milyon ton kahve atığı üretilmektedir

Kahve atıklarının yeniden kullanımına yönelik yenilikçi yaklaşımların ortaya çıkması, bu atıkları değerli bir kaynağa dönüştürmüştür. Kahve atıkları, kahve içeriğine bağlı olarak değişkenlik gösterebilir. KKT'nin düşük maliyeti ve bol bulunabilirliği, onu çeşitli kimyasal ürünlerin üretiminde uygun bir aday yapmaktadır. Örneğin, KKT'den elde edilen aktif karbon, biyodizel gibi yüksek değerli ürünlerin üretiminde kullanılabilir. Ayrıca, KKT'nin karbonhidrat, ham protein, amino asit ve diğer nitrojenler açısından zengin içeriği, onu sürdürülebilir bir yeşil ekonomi için önemli kılmaktadır. KKT'nin kullanım alanlarının arttırılması için kullanılabilecek birkaç teknoloji mevcuttur.

Biyokömür, organik maddelerin termokimyasal dönüşümü ile sınırlı oksijen ortamında oluşturulan gözenekli bir katı malzemedir. Biyokömür, çevre dostu doğası, güvenliği, maliyet etkinliği ve geniş yüzey alanı gibi özellikleri nedeniyle önemli bir çevresel katalizördür. Biyokömür, karbon tutma ve toprak iyileştirme gibi çeşitli çevresel katkıları olan özgül fizikokimyasal özelliklere sahiptir. Biyokömürün özellikleri, piroliz sıcaklığı, ısı transfer hızı, bekleme süresi ve kullanılan hammadde çeşitliliği gibi faktörlerden etkilenir. Biyokömürün kirleticilerin yönetiminde etkinliği, gözenek boyutu dağılımı, toplam yüzey alanı ve iyon değişim kapasitesine bağlıdır.

Biyokömürün gözenekli yapısı, mikroorganizmalar için bir habitat sağlar ve onların büyümesini ve aktivitesini artırır. Biyokömür, çeşitli biyokütle malzemelerinden üretilir ve her biri farklı özelliklere ve kullanım alanlarına sahiptir. Örneğin bunlardan biri olan odun atıklarından üretilen biyokömür, toprak iyileştirme ve sera gazı emisyonlarındaki etkileri gösterebilir. Farklı odun atıkları biyokömür öncülleri olarak kullanıldığında performansta değişikliklere neden olabilir. Bir diğer kaynak ise tarımsal ürün kalıntıları, saman ve pirinç kabuğu gibi tarımsal kalıntılar biyokömür üretiminde kullanılabilir. Bitki artıklarından elde edilen biyokömür, sera gazı emisyonları ve toprak mikrobiyal toplulukları üzerinde farklı etkilere sahip olabilir. Hayvan atıklarından kanalizasyon çamurundan elde edilen hayvansal atıklardan üretilen biyokömür, anaerobik çürütme ve metan üretim süreçlerini etkileyebilir. Alg biyokütlesinden üretilen biyokömür ise, döngüsel biyo-ekonomik uygulamalar için benzersiz özellikler ve potansiyel uygulamalar sunar. Biyokömür kaynağı olarak biyokatıların kullanılmasında ise, toprak iyileştirme, sera gazı emisyonları ve kaynak geri kazanımı ile atık yönetimi için çeşitli fırsatlar sunar.

Biyokömür, tarım ve atık su yönetiminde önemli bir rol oynar. Biyokömür, su ve toprak ortamlarında hem bir kirletici azaltıcı hem de bir gübre olarak çift işlev görür. Toprak verimliliğini, su tutma kapasitesini ve ürün verimliliğini artırırken, atık su arıtımında da filtrasyon ortamı olarak hizmet eder. Ayrıca, kirlenmiş toprakların ve suyun temizlenmesinde ve iklim değişikliğiyle mücadelede karbon tutma yöntemi olarak değerlidir. Organik boyalar, farmasötik aktif bileşikler, fenoller ve çeşitli endüstrilerden salınan kimyasal ara ürünleri etkili bir şekilde elimine eder.

Lakkaz enzimi, polifenol oksidaz (PPO) ailesine ait, hem fenolik hem de fenolik olmayan lignin ile ilişkili bileşikleri okside edebilen bir enzimdir. Lakkaz enzim Japon lak ağacının balçığında tespit edilen ve daha sonra bir mantar enzimi olarak tanımlanan bir enzimdir. Bu enzim, bakır içeren çoklu bakır oksidaz (ÇBO) olarak adlandırılır. Lakkaz enzimi zor kirleticilerin parçalanmasına yardımcı olabilir. Oksijeni nihai elektron alıcısı olarak kullanan lakkaz enzimi, güvenli ve biyouyumlu bir seçenek haline gelir. Lakkaz enzimi ayrıca polifenoller, difenoller, sübstitüe fenoller, aromatik aminler, diaminler ve iyodür gibi çeşitli substratların oksidasyonunu katalize edebilir. Düşük molekül ağırlıklı bileşiklerin varlığında

lakkaz enzimi fenolik olmayan yapıları oksitleyebilir. Bu enzimlerin avantajları arasında kararlı yapı, kofaktör gerektirmeme ve çeşitli substratlar üzerinde etkili olma yeteneği yer alır.

Lakkaz endüstride atık su arıtma, detoksifikasyon ve renk giderme; tekstil endüstrisi atık su renk giderme; boya giderme; çevreye zararlı ksenobiyotiklerin bozunması; şarap ve içecek işleme; kağıt hamuru ağartma ve lignin giderme; biyosensör ve biyoyakıt üretimi; organik sentez; delignifikasyon, biyoremediasyon, etanol üretimi, fenolik bileşiklerin bozunması ve giderilmesi gibi biyolojik işlemler; ilaç endüstrisi, özellikle anti-kanser ilaçlarının geliştirilmesinde; kozmetik, gıda iyileştirme, deterjan ve tıbbi uygulamalarda kullanılmaktadır.

Katı hal fermantasyonu, mikroorganizmaların suda çözünmeyen substratlar veya inert taşıyıcılar içeren nemli katı destek ortamlarında gerçekleştirdiği fermantasyon sürecidir.

Katı faz fermantasyonunda kullanılan katı maddeler iki kategoride sınıflandırılabilir: mikroorganizmanın entegre olduğu tek yer olan inert maddeler ve mikroorganizmaların hem entegre olduğu hem de beslendiği yerler olan inert olmayan destek substratları. Bu substratlar mikroorganizmalar tarafından enerji ve karbon kaynağı olarak kullanılmalı ve büyüme ve gelişmelerine katkıda bulunmak için belirli bir miktarda neme sahip olmalıdır. Substratların nemi, mikroorganizmaların metabolizmasını ve büyümesini desteklemek için çok önemlidir. Bu süreçte kullanılan katı malzemenin seçimi büyük önem taşır ve genellikle tarımsal atık malzemeler substrat olarak kullanılır, bu da bu yöntemi çevre dostu ve ekonomik kılar.

Biyolojik, fizikokimyasal ve çevresel faktörler katı faz fermantasyonu sırasında ürün üretim verimliliğini etkiler. Kullanılacak yöntemin, mikroorganizmaların ve substratların seçimi, kullanılacak substratın kimyasal bileşiminin belirlenmesi, uygun koşulların ve eklenecek karbon ve enerji kaynaklarının seçimi, havalandırma, nem, uygun sıcaklık, pH, karıştırma gibi değişkenlerin optimizasyonu, ürünün elde edilmesi ve geliştirilmesi, izolasyon işlemleri ve oluşan ürünün saflaştırma adımları dikkat edilmesi gereken konulardır. Ayrıca oksijen ve karbondioksit taşınımı için havalandırma, ısı iletimi için karıştırma mekanizmaları ve sıcaklık kontrolü sürecin sorunsuz ilerlemesi için önemli parametrelerdir.

Trametes versicolor, yaygın olarak *Coriolus* olarak bilinen ve lignini parçalama ve fenolik maddeleri metabolize etme kapasitesi ile tanınan yaygın bir beyaz çürük mantarıdır. Beyaz çürükçül mantarlar, organik moleküllerin üretimi için gerekli olan çeşitli enzimler üretir. Bu enzimler sayesinde, parçalanmaya dirençli kirleticilerin parçalanması etkin bir şekilde gerçekleştirilebilir.

Beyaz çürükçül mantarlar, ürettikleri hücre dışı enzimler nedeniyle birçok biyoteknolojik çalışmada kullanılabilmektedir. Kağıt endüstrisinde kağıt hamurundan ligninin uzaklaştırılması, insan sağlığı ve çevre için tehlike oluşturan çeşitli endüstrilerin atık sularındaki pestisit vb. maddelerin uzaklaştırılması, fenolik bileşikler, ksenobiyotikler, polisiklik aromatik bileşikler vb. kimyasalların parçalanması, enzim ve hormon gibi biyolojik ürünlerin elde edilmesi gibi alanlarda kullanılabilir.

Bu çalışmada deney tasarımlarının gerçekleştirilerek üretim için belirlenen faktörlere bağlı olarak optimum noktanın bulunması için literatürdeki bilgiler baz alınarak Design Expert Version 13 uygulamasında Yanıt yüzeyi metodolojisi (Response surface methodology (RSM) kullanılarak kurulacak biyoreaktörlerin koşulları belirlenmiştir.

Yanıt yüzeyi metodolojisi (Response surface methodology (RSM)) daha az deneme yaparak ve operasyonel maliyetleri ve harcanan zamanı en aza indirerek üretim süreçlerini iyileştirmek için kullanılır. RSM, daha sonra çalışma sonuçlarını işlemek ve doğru kararı önermek için kullanılan optimum nokta yanıtını elde etmek için deneysel bileşenlerin tasarımını ve istatistiksel yöntemleri kullanır. Süreçlerin genel performansını belirlemek, davranışlarını etkileyen birçok

parametre nedeniyle çok karmaşıktır. Süreç davranış haritalarının oluşturulması, sürecin maksimum, hedeflenen veya minimum performansını elde etmek için önemli parametreleri manipüle eden bir dizi matematiksel modelde ifade edilir. Karmaşık üretim süreçlerinin optimizasyonu, en yüksek veya en iyi performans (verim, verimlilik vb.) için deneysel koşulları belirler.

Üretim optimizasyonunda deneysel tasarım kullanmanın çeşitli avantajları vardır. Bunlar arasında daha kısa işlem süreleri, daha ucuz süreçler ve ekipman, işçilik ve malzeme gibi uygun maliyetli kaynakların daha verimli kullanımı yer alır. Buna ek olarak, optimizasyona girilen faktör seviyelerini tahmin etmek amacıyla verileri matematiksel modellere uydurmak için sayısal regresyon araçlarını kullanır.

RSM yöntemi kullanılarak biyokömür ve kahve telvesi içeren reaktörler için biyokömür ve kahve telvesi miktarı, Destek malzemesinin miktarına bağlı olarak % nem ve reaktör işletim süresi değişken faktör olarak alınarak optimum çalışma ortamı ve faktörler arasında bağlantı bulunması hedeflenmiştir.

Uygulamanın önerdiği şekilde rektörler kurulmuş ve belirlenen günlerde örnekler alınmıştır. Spektrofotometrik yöntem kullanılarak enzim aktivitesi ölçülmüştür.

Lakkaz enzim aktivitesinin belirlenmesi, substrat olarak 2,2-azino-bis(3-etil-benzthiazoline-6 sülfonik asit (ABTS) kullanılarak ve ardından spektrofotometri ile ölçüm yapılarak gerçekleştirilmiştir. Bir aktivite birimi, dakikada 1 μmol ABTS' yi oksitlemek için gereken enzim miktarı olarak tanımlanmıştır ve sonuçlar litre başına birim (U/L) olarak rapor edilmiştir.

Elde edilen sonuçlar hem biyokömürün hem de kahve posasının katı kültürlerde destek malzemesi olarak kullanılabileceğini göstermektedir. Kahve ve biyokömür için elde edilen optimum değerler, 30 g kahve için %70 nem içeriğinde ve 7 günde, 10 g biyokömür için ise %50 nem içeriğinde ve 3 günde elde edilmiştir. Deney tasarımlarının ANOVA değerleri ile modellerin istatistiksel olarak anlamlı olduğu bulunmuştur. Uygulamanın elde edilen sonuçlar karşısında model önermiştir.

Kahve telvesinin destek malzemesi olarak kullanıldığı modelin sonuçlarından yola çıkarak Design Expert Version 13'ün önerdiği reaktör tasarım detayları şöyledir: ortam nemi %65,024, kahve miktarı 14,244g, zamanlama olarak 5,889. gün olarak belirlenmiştir.

Biyokömürün destek malzemesi olarak kullanıldığı biyorektör için Design Expert Version 13'ün önerdiği model; %62,683 nem yüzdesi, 22,620 g biyokömür, 3,763. gün olarak belirlenmiştir.

Ayrıca, incelenen destek malzemeleriyle *Trametes versicolor* kullanılarak katı kültür fermantasyonuna ilişkin literatürün azlığını vurgulamakta ve bu malzemelerin farklı mikrobiyal bağlamlardaki potansiyelini keşfetmek için daha fazla araştırma yapılması gerektiğinin altını çizmektedir. Katı hal fermantasyonu yoluyla lakkaz enzimi üretiminde gözlemlenen umut verici sonuçlar, bu malzemelerin biyoteknolojik süreçlerde anlaşılmasını ve uygulanmasını geliştirmek için gelecekteki çalışmalar için araştırmaya devam edilmesini gerektirmektedir.

Deneyler sırasında karşılaşılan problemler ve gelecekteki çalışmalar için öneriler ise şu şekilde sıralanabilir. Öncelikle, metabolik ısının verimli yönetimi incelenmelidir. Proses sırasında üretilen metabolik ısının etkili bir şekilde yönetilmesi için stratejiler geliştirilmelidir.

İkinci bir zorluk ise çevresel kurutma zorluklarıdır. Ortamın sıcaklığına bağlı olarak ortamın kuruması, daha kontrollü nemlendirme teknikleri gerektiren önemli bir zorluktur. Nem seviyesini sabit tutacak ve prosesin istikrarını sağlayacak sistemler geliştirilmelidir. Yetersiz

yüzey alanı bir diğer zorluktur. Mevcut tasarımda işlemler Erlenmayer flasklarda gerçekleştirilmiştir. Daha geniş yüzeye sahip reaktör tasarımları ile yüzey alanını artırılabilir, metabolik ısının kontrolü ve nemlendirme noktasında kolaylık sağlayabilir. Bu değişim reaktörün performansını ve verimliliğini artırmak için kritik bir faktördür.

Reaktörün test süresinin uzatılması, prosesin uzun vadeli performansı ve güvenilirliği hakkında daha kapsamlı veriler sağlayabilir. Bu, prosesin gerçek dünya uygulamalarına nasıl tepki vereceğini daha iyi anlamak için önemlidir.

Geliştirilmiş analitik tekniklerin kullanılması çalışmanın geliştirilebilir yönü olup, spektrofotometri yerine daha hassas ölçümler sağlayabilecek bir respirometre gibi gelişmiş yöntemler önerilmektedir. Bu, prosesin analizinin doğruluğunu artırabilir ve sonuçların güvenilirliğini sağlayabilir.

Biyokömür kaynaklarında değişkenlik, farklı organik materyallerin biyokömür kaynağı olarak araştırılması, biyokömürün proseslerdeki etkinliğini artırabilir. Bu, daha çeşitli ve optimize edilmiş biyokömür kullanımlarının keşfedilmesine olanak tanır.

Kapsamlı biyokömür içerik analizi bir başka parametre olup, biyokömürün içeriğinin daha detaylı bir şekilde analiz edilmesi, biyokömürün potansiyelini ve kullanımını optimize etmek için önemli bilgiler sağlayabilir. Bu analizler, biyokömürün fizikokimyasal özelliklerini anlamak için temel oluşturabilir.

Örnek hazırlama, numune hazırlama sürecinde biyokömürün yıkanması ve kurutulması, analizlerin netliğini artırabilir ve veri doğruluğunu sağlayabilir. Bu adım, laboratuvar çalışmalarının ve sonuçların güvenilirliğini artırabilir.

Biyokömürün enzimleri tutma kapasitesi, tarımsal ortamlarda biyolojik süreçler için pratik uygulamaları etkileyebilir. Enzimlerin biyokömür üzerinde tutulması, etkili ve uzun süreli biyolojik işlemler için önemli olabilir. Bu dikkate alınması gereken noktalar ve öneriler, biyoproseslerin geliştirilmesi destek malzemelerin daha etkin kullanımı için gelecekteki araştırmaların odak noktalarını belirlemektedir.

Özetle bu çalışma, kahve atıklarının ve biyokömürün çevresel ve ekonomik faydalarını vurgulamaktadır.

Kahve atıklarının yeniden kullanımı, iklim değişikliği ve çevre kirliliği gibi büyük küresel zorlukların üstesinden gelmede önemli bir adım olarak görülmektedir. Ayrıca, biyokömürün kullanımı, toprak iyileştirme, su tutma kapasitesi ve kirletici giderimi gibi çeşitli alanlarda önemli katkılar sağlamaktadır.

Bu bağlamda, döngüsel ekonomi modeli, kaynakların verimli kullanımı ve atıkların yeniden değerlendirilmesi açısından büyük bir potansiyele sahiptir. Gelecekteki çalışmalar, bu alandaki bilgi birikimini artırarak daha sürdürülebilir ve çevre dostu uygulamaların geliştirilmesine katkı sağlayacaktır.

Anahtar Kelimeler: lakkaz, kahve atıkları, biyokömür, katı kültür fermentasyonu, *Trametes versicolor*

ABSTRACT

Investigation of the Use of Different Support Materials in Laccase Enzyme Production by Solid State Fermentation from Waste Coffee

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Rising urbanization and industrialization have transformed soil and water pollution into pressing global issues that endanger both ecological stability and public health. Exposure to various pollutants, including organic toxins and heavy metals, puts water bodies and soil at risk. To address this problem, a combination of physical, chemical, and biological techniques has been used for environmental remediation. One such technique, bioremediation, employs organisms to remove or neutralize contaminants and offers a cost-effective and eco-friendly solution. However, a suitable immobilization carrier is essential for the sorption of enhanced contaminants and the promotion of microorganism growth. In this study, the following hypotheses were tested;

The use of biochar to stimulate microorganism proliferation,

The increase and harvest of metabolites produced by thriving microorganisms,

Resilience of microorganisms to stress conditions without genetic editing, such as metabolic heat and the absence or deficiency of essential nutrients.

Based on the experiment results, the highest response value of 0.0333333 was achieved using 10 grams of biochar with 50% moisture content on the third day. Likewise, the maximum reaction value of 0.0138889 was recorded with 30 grams of coffee pulp at 70% moisture content on the seventh day, according to the experimental data.

Keywords: laccase, coffee waste, biochar, solid state fermentation, *Trametes versicolor*

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1 INTRODUCTION

Today, the world population has more than tripled compared to that in the mid-20th century. From 1950 to 1987, the population doubled to more than 5 billion. The next doubling of the population is projected to take 70 years and will exceed 10 billion by 2059 (United Nations Department of Economic and Social Affairs 2022). Globally, climate change, air and environmental pollution, and population growth have led to a variety of food, clean water, and energy challenges. Effective resource management of energy, food, and clean water is essential in confronting significant worldwide issues, which encompass climate change, food security, sustainable economic growth, as well as social and environmental stability (Pokharel et al., 2020).

In the future, we will need to reduce pollution, increase food production and clean resources, and explore alternative energy source options with minimal resource use while bringing the environmental impacts to acceptable levels.

Bioengineering-based circular solutions can provide global solutions to the problems we face today and in the future.

Biomass abundance can play a substantial role in decreasing the use of fossil fuels and creating a positive impact on sustainable development. Pyrolysis is a technological approach used to obtain energy from biomass. The distinctive feature of pyrolysis compared to the methods used in biomass energy extraction is the production of biochar, a carbon-rich by-product of pyrolysis (Lehmann, 2007).

2 LITERATURE

2.1 COFFEE

Coffee is the second most popular beverage worldwide, followed by water, and it ranks as the second most traded commodity in the stock market after crude oil (Girotto et al., 2018).

The coffee industry is a substantial contributor to the global economy, providing employment to over 100 million individuals across 80 countries. As per the International Coffee Organization (ICO), the anticipated global coffee consumption for the 2022/23 season is 168.2 million bags.

Coffee's discovery can be traced back to the 6th century, and by the 16th century, it firmly established itself as one of the three most popular beverages globally. The coffee industry produces substantial waste owing to its widespread consumption, with coffee waste representing the most substantial portion of this waste stream. Each year, approximately 15 million tons of coffee waste are produced globally, the majority of which is generated as litter. The disposal of coffee waste as garbage causes the formation of methane, a greenhouse gas that is 30 times more powerful than carbon dioxide (Pujol et al., 2013). While certain coffee wastes are repurposed for animal feed, fertilizer, and other uses, more innovative techniques and strategies are needed to transform coffee waste into a valuable source material. From an environmental standpoint, the reuse of waste can help reduce carbon dioxide and methane emissions (Keleş & Yazar, 2023).

According to these statistics, a significant amount of waste coffee grounds (SCG), resulting from the preparation of coffee and coffee-based beverages, is released into the environment as municipal or industrial waste, causing environmental concerns. SCG is categorized as a toxic waste owing to its polyphenol, tannin, and caffeine contents. The low cost and abundant availability of SCG make it a suitable candidate for reuse in the production of various chemical products, such as activated carbon, as well as high-value items such as biomass energy (biodiesel) (Cerino-Córdova et al., 2020).

2.2 REVALORIZATION OF COFFEE WASTE

Various forms of coffee waste (e.g., coffee silver shells, coffee bean shells, coffee husks, and coffee grounds) have been used to remove inorganic compounds and organics from aqueous solutions for more than 20 years (Cerino-Córdova et al., 2020).

Coffee waste accounts for approximately 2/3 of the mass of the dried coffee beans. They are the main inedible and inevitable wastes of roasted and ground coffee. These coffee wastes are generated from three different consumption sources: in the coffee industry, in households, and at points of sale. As a kind of biomass waste consisting mainly of 45.3% carbohydrates, 13.6% crude protein, 3.7% amino acids and other nitrogens, and thus containing abundant raw materials, the reuse of coffee waste is an important step towards a sustainable green economy (Keleş & Yazar, 2023).

Table 2.1. Chemical composition of coffee pulp (Yucesen,2012).

Component	**Dry mass (g/100g)**
Mannan	21.2
Galactan	13.8
Protein	13.6
Cellulose (Glucans)	8.6
Hemicellulose	6.7
Acetyl groups	2.2
Arabinan	1.7
Ash	1.6

According to the literature, several technologies can be used to upgrade spent coffee grounds (SCG). One such technology involves solid state fermentation with the fungal strain Neurospora Crassa, which can be used to produce α-amylase enzymes from SCG. These enzymes are capable of breaking down the glycosidic bonds in starch, resulting in the production of glucose, maltose, maltotriose, and dextrin. This process has many applications in various industries such as food, paper, textile, and detergent manufacturing (Murthy et al., 2009).

Microorganisms such as yeast and fungi can be grown using either submerged or solid state fermentation methods. Spent coffee grounds (SCG), which contain fermentable sugars, are considered suitable materials for microbial cultivation and the synthesis of valuable products. This approach leads to the generation of enzymes, such as pectinase, caffeinase, and tannase, along with flavor compounds utilized in the food sector. Moreover, it facilitates enzymatic detoxification of coffee husks and the synthesis of organic acids, flavors, and flavoring agents (Pandey et al., 2000).

Fungi are grown in solid state environments to harvest phenolic compounds from the leftover coffee grounds. This method involves the generation and retrieval of phenolic compounds from coffee pulp remnants, which are widely used in the food and pharmaceutical sectors (Machado et al., 2012).

2.3 BIOCHAR

Biochar is a carbonaceous solid material characterized by its porous structure that is created through the thermochemical transformation of organic substances in an environment with limited oxygen. This material possesses specific physicochemical characteristics that make it well suited for extended carbon sequestration and soil improvement(Shackley et al., 2012)

Biochar's porous structure provides a habitat for microorganisms, enabling them to thrive in a sheltered environment. Its high surface area also increases microorganism colonization, promoting their growth and activity.

Biochar enhances the water-retaining capacity of the growth media by acting as a sponge that absorbs and retains water, which is essential for microbial survival and activity. Adequate moisture levels created by biochar foster favorable conditions for microorganism growth and enzyme production.

Several studies have demonstrated that biochar increases enzyme production by providing microorganisms with organic matter and essential elements, such as nitrogen and phosphorus, which serve as essential elements for their growth and enzyme formation. Additionally, biochar can be used as a food source for microorganisms (Ajeng et al. 2020).

2.3.1 Sources of Biochar Production

Biochar can be generated from a wide range of biomass materials, each possessing distinct characteristics and potential uses.

1. Wood Waste: Biochar derived from wood waste, such as decayed spruce and pine trees, exhibits specific characteristics that may influence its application in soil remediation and greenhouse gas emissions. Different wood types used as biochar precursors can cause variations in performance during anaerobic digestion processes.

2. Agricultural Crop Residues: Agricultural residues, including straw and rice husks, can be utilized as raw materials for biochar production. Biochar derived from plant residues may vary in its impact on greenhouse gas emissions and soil microbial communities.

3. Animal Waste: Biochar produced from animal waste such as sewage sludge can affect processes such as anaerobic digestion and methane production.

4. Algal Biomass: Biochar derived from algal biomass offers unique properties and potential applications, contributing to the diversity of biochar sources for circular bio-economic applications.

5. Biosolids: Utilizing biosolids as a source of biochar presents opportunities for resource recovery and waste management, with potential impacts on soil remediation and greenhouse gas emissions.

The variations in biochar originating from these sources may impact their physical, chemical, and biological characteristics, as well as their efficacy in various applications, such as soil remediation, greenhouse gas emission mitigation, and interactions with microbial communities. Understanding these differences is crucial for optimizing the selection and use of biochar from different sources in the context of circular bioeconomy and resource recovery (Liu et al., 2021).

2.3.2 Biochar applications

Biochar plays a vital role in agriculture and wastewater management. It enhances soil fertility, water retention, and crop productivity when used as soil amendment. Additionally, it serves as a filtration medium for wastewater treatment, reducing pathogen levels and aiding in nutrient recovery. Biochar is also valuable for remediating polluted soils and water, and as a carbon sequestration method to combat climate change (Werner et al., 2018).

The characteristics of biochar that are critical to its properties are influenced by factors such as pyrolysis temperature, heat transfer rate, residence time, and the variety of feedstocks used. The efficacy of biochar in pollutant management is contingent on its pore size distribution, overall surface area, and ion-exchange capacity, which are critical components of its molecular

composition and physical configuration. The following elements are essential for the effective utilization of biochar in both soil and aquatic ecosystems (Ahmad et al. 2014).

2.3.3 The Role of Biochar in Pollution Abatement

Biochar serves a crucial dual function as a pollution mitigator and a fertilizer in both water and soil environments. In terms of pollution reduction, biochar effectively eliminates organic dyes, pharmaceutically active compounds, phenols, and chemical intermediates released by various industries. Organic dyes are among the most prevalent pollutants resulting from the production of paper, textiles, rubber, and paints. Despite their good solubility and chemical stability in water due to their complex aromatic structures, these organic dyes do not easily decompose in nature. When these dyes contaminate water bodies, they limit the availability of light and oxygen in the water. Techniques such as catalysis, membrane separation, adsorption, and photocatalysis have been used to remove organic dyes, with biochar serving as an adsorbent in the adsorption process. Chemical intermediates, such as pentachlorophenol (PCP), bisphenol A (BPA), phenol, and para-nitrophenol (PNP) are among the most well-known organic contaminants released from organic matter, paper industry products, plastics, and agricultural production. These compounds also exhibit high stability, which makes biodegradation difficult. Biochar-derived materials are attractive options for phenol removal during organic paint production (Karthik et al. 2021).

2.3.4 Biochar for Soil Treatment

Research on the incorporation of biochar has shown that it can improve the physical, chemical, and biological characteristics of degraded soils (Lehmann et al., 2011). Soils amended with biochar exhibit improvements in organic carbon content, nutrients, pH buffering, water retention capacity, and biological indicators, all of which contribute to increased crop yields (Bi et al., 2022). Research has demonstrated that the incorporation of biochar into the soil can have a beneficial impact on various soil properties and processes. These include enhancing aggregate stability, creating adequate pore spaces for water retention, influencing soil pH, improving water-use efficiency, reducing bulk density, increasing cation exchange capacity, enhancing water-retention capacity, promoting carbon sequestration, and promoting plant growth. Moreover, biochar has been suggested as a possible method to modify nutrient cycling, reduce soil nitrous oxide emissions, and improve carbon sequestration in the soil (Wojewódzki et al., 2023).

2.3.5 Biochar for Wastewater Treatment

Biochar has several applications in water and wastewater treatment owing to its distinctive properties, such as unique surface area, adsorption capacity, ion exchange capacity, and microporosity. The physicochemical characteristics of biochar, such as polarity, surface area, atomic ratio, elemental composition, and pH, are significantly influenced by the feedstock type and pyrolysis conditions employed in its production. These properties affect the efficacy of biochar in wastewater treatment. The mechanisms by which pollutants are removed by biochar depend on interactions influenced by the pyrolysis temperature and feedstock type. Increased pyrolysis temperatures are known to yield biochar with a greater carbon content, hydrophobicity, aromaticity, microporosity, and surface area (Pokharel et al., 2020).

2.3.6 Biochar for Enhancing Microorganism Metabolites

Biochar has a range of potential uses such as carbon sequestration, enhancement of soil fertility, pollution control, utilization of agricultural by-products, and recycling of waste materials (Werner et al., 2018). One of the fundamental mechanisms by which biochar enhances

microorganism retention is its porous structure. Biochar contains a network of pores that can serve as a habitat for microorganisms and provide a sheltered environment for their development. The high surface area of biochar also promotes microorganism colonization and enhances its growth and activity.

The use of biochar in growth media increases its water retention capacity. Its porous structure functions as a sponge, absorbing and retaining water, which is essential for the survival and activity of microorganisms. By maintaining adequate moisture levels, biochar creates favorable conditions for microbial growth and enzyme production.

Biochar has been shown to boost enzyme production in several ways. It comprises organic matter and essential elements, such as phosphorus and nitrogen, which can serve as nutrients for microbial growth and stimulate enzyme synthesis. Furthermore, biochar can modify the physical and chemical characteristics of the growth medium, such as increasing the cation exchange capacity and soil pH, thereby creating a more suitable environment for microbial activity. Alterations in soil properties can affect enzyme production by microorganisms, because some enzymes have specific pH ranges for their activity (Liu et al., 2021).

Furthermore, biochar has been shown to enhance microbial diversity in growth media. Biochar can attract a broad range of microorganisms with diverse metabolic capabilities. The expansion of microbial diversity can result in the generation of a broader range of enzymes, facilitating effective degradation of organic substances and promoting nutrient circulation.

The change in the conversion of bacteria to fungi at higher pyrolysis temperatures could be influenced by several factors and warrants further investigation. This finding implies that fungi may be more suited to the surface conditions, as shown in Table 2.2 (Brewer et al.2012).

Table 2.2. Microorganism populations in control and amended soils based on bulk plate counts (Brewer et al., 2012).

Soil Treatment	**Fungi colonies g^{-1} soil**	**Bacteria colonies g^{-1} soil**	**Bacteria-Fungi ratio colonies g^{-1} soil**
Control	$(6.8 \pm 1.0) \times 10^4$	$(9.9 \pm 1.9) \times 10^6$	148 ± 36
Stover(Stem of plant)	$(31.3 \pm 8.8) \times 10^4$	$(14.0 \pm 2.6) \times 10^6$	47 ± 16
Biochar #1	$(6.8 \pm 1.0) \times 10^4$	$(9.8 \pm 1.3) \times 10^6$	145 ± 30
Biochar #2	$(7.0 \pm 0.9) \times 10^4$	$(9.1 \pm 1.0) \times 10^6$	130 ± 10
Biochar #3	$(7.7 \pm 1.2) \times 10^4$	$(8.1 \pm 0.6) \times 10^6$	107 ± 11

Biochar displays varying compositions and levels of aromaticity that are influenced by the extent of pyrolysis. Biochar#1 contained the highest concentrations of aliphatic and oxygenated carbon functional groups. In contrast, biochar #3 exhibited the highest levels of aromatic carbon and the largest proportion of non-protonated carbon. Biochar#2, on the other hand, displayed a composition that was considered average in comparison.

2.3.7 Future Perspectives of Biochar in Microbial Metabolite Production

The addition of biochar to the growth medium enhances microorganism retention and enzyme production. The porous structure provides habitats for microorganisms, and its water retention capacity ensures optimal moisture levels. Biochar also serves as a nutrient source and alters soil

properties by promoting microbial growth and enzyme synthesis. The increased microbial diversity resulting from biochar addition further contributed to enhanced enzyme production.

Owing to its significantly porous composition, organic nutrient content sourced from biomass, and capacity to retain nutrients conducive to elevated water and microbial proliferation, biochar is presently regarded as a favorable candidate for use as a carrier material (Ajeng et al., 2020). In order to improve characteristics and efficiency, biochar may serve as a bio-carrier for the purpose of amalgamating the capabilities of microorganisms and nanoparticles (Yu et al., 2020).

2.4 LACCASE ENZYME

Laccases (benzenediol: oxygen oxidoreductases; EC1.10.3.2) have been studied for the past ten years. This enzyme, which was initially identified in the ooze of the Japanese lacquer tree (*Rhus vernicifera*) and later identified as a fungal enzyme, is part of the copper-containing polyphenol oxidase (PPO) family. Laccase enzymes, commonly called multicopper oxidase (MCO), can oxidize both phenolic and nonphenolic lignin-related compounds, as well as pollutants that are difficult to degrade. This is because oxygen is used as the final electron acceptor, making them a safe and biocompatible option for various applications (Mayer, 1986). Moreover, laccases exhibit the ability to catalyze the oxidation of a diverse array of substrates, encompassing polyphenols, diphenols, substituted phenols, aromatic amines, diamines, and inorganic compounds like iodide (Christopher et al., 2014). Furthermore, in the presence of low-molecular-weight compounds called redox mediators, laccases can oxidize nonphenolic structures. Unlike peroxidases, which use only molecular oxygen as a co-substrate, laccases are considered promising enzymes for various biotechnological applications (Mayer, 1986; Sheikhi et al., 2012).

Laccase has several advantages. These are;

- It is an enzyme with a stable structure,
- No need for additional cofactors during use,
- Ability to act on a wide variety of substrates,
- Unlike other enzymes used for oxidation, dissolved oxygen can be used instead of hydrogen peroxide as the final electron acceptor (Kocyigit and Toker, 2021; Gedikli et al., 2010).

Despite its many advantages, there are some limitations to the use of laccases. These are

- Very large amounts of enzyme are needed for use in industrial areas, but the enzyme cannot be produced easily and cheaply using these methods.
- Enzyme stocks are insufficient because they cannot be produced in large quantities.
- Raw enzyme preparations are difficult to obtain and the cost of redox mediators is very high.
- Laccases can be strongly inhibited by cyanide, fluoride, thiocinate and many other anions as a result of their interaction with copper sites, regardless of their source (Ozsolen, 2010; Demiralp et al., 2015; Gedikli et al., 2010).

Laccase is used in industry for wastewater treatment, detoxification and decolorization; textile industry wastewater decolorization; dye removal; degradation of environmentally harmful xenobiotics; wine and beverage processing; pulp bleaching and lignin removal; biosensor and biofuel production; organic synthesis; biological processes such as delignification, bioremediation, ethanol production, degradation and removal of phenolic compounds; pharmaceutical industry, especially in the development of anti-cancer drugs; cosmetics, food

improvement, detergent and medical applications (Sheikhi et al., 2012; Altınok, 2019; Birhanlı, 2008; Demiralp et al., 2015; Birhanlı and Yesilada, 2017; Kocyigit and Toker, 2021; Gedikli et al., 2010; Eren et al., 2009).

In a study by Altınok (2019), laccase obtained from *Trametes versicolor* was immobilized on poly(N-isopropylacrylamide)-calcium alginate beads. The immobilized enzyme was used for enzymatic decolorization, and its properties were compared with those of the free enzyme. The decolorization percentages of immobilized and free laccase enzymes used for methyl orange decolorization were found to be 70% and 73%, respectively.

Eren et al. (2009) evaluated the effect of laccase enzyme in combination with ozone on bleaching, the activity of the enzyme and the degree of whiteness. Although the use of laccase alone did not increase whitening, the concurrent use of laccase and ozone increased the degree of whiteness from 61% to 69%.

In a study conducted by Cançelik et al. (2017), bioleaching of Direct Black 22 azo dye with Pleurotus ostreatus mushrooms and treatment of synthetic dyes in textile wastewater were investigated. In addition, the activities of these enzymes were investigated. As a result of this study, it was observed that various other peroxidase enzymes, especially laccase, were effective in the removal of azo dyes, and it was concluded that these enzymes can be used for biodegradation.

For the laccase enzyme to be used in various fields, it needs to be obtained in larger quantities and in a cost-effective manner. First, it is necessary to determine the most effective laccase-producing species to be used in future studies. For this, fungal species should be selected correctly, economic isolation methods should be developed, and production conditions should be optimized. In order to produce more effective laccase enzymes in sufficient quantities with the information obtained, it is necessary to select the fungal species to be used in the study and the medium in which the enzyme production will be carried out, to determine the incubation time of the inoculated medium, to add substances that increase enzyme production to the medium and to find more effective enzyme production methods (Birhanlı and Yesilada, 2017; Gedikli et al., 2010; Gedikli, 2008).

The factors affecting the activity of laccase enzymes are temperature, pH, and media composition. The effect of temperature on laccase activity is limited, and the optimum temperature of the enzyme varies greatly between strains. The optimum temperature for laccase enzyme production is between 25-30ºC. The enzyme activity decreased when the fungus was grown at higher temperatures. The effect of initial pH on laccase activity is limited, and the optimum initial pH varies with the type of substrate because different substrates may cause different reactions for laccase. Research has indicated that the optimal pH range for laccase enzyme production typically falls within the range of pH 4.5 to 6, despite the fact that fungi cultivated in pH 5.0 media tend to yield an excess of laccase. Regarding consequence of medium composition on laccase activity, production of the enzyme is triggered by nitrogen exhaustion, but some nitrogen sources do not affect enzyme activity. Some studies have shown that high laccase activity is achieved using a low carbon/nitrogen ratio, whereas others have shown that it is achieved at a high carbon/nitrogen ratio. Some studies have shown that laccase enzymes are produced earlier when fungi are grown in nitrogen-rich media than in nitrogen-limited media. Although glucose concentration increases laccase production to a certain level, excessive glucose concentration inhibits laccase production in various fungal species (Shraddha et al., 2011; Brijwani et al., 2010).

2.5 SOLID STATE FERMENTATION (SSF)

Fermentation carried out by microorganisms in moist solid support media consisting of water-insoluble materials or inert carriers under conditions where there is little or no water in the environment is known as solid state fermentation (Ozsolen, 2010; Serin, 2009).

Solid materials used in solid state fermentation can be classified into two categories: inert materials, which are the only places where the microorganism is integrated, and non-inert support materials, which are the places where the microorganisms are both integrated and fed. These materials should be used as a source of energy and carbon by microorganisms, and should have a certain amount of moisture to contribute to their growth and development. The humidity of the materials is very important for supporting the metabolism and growth of microorganisms (Ozsolen, 2010; Serin, 2009).

The success of the process in solid state fermentation depends on the choice of solid material. The low cost and availability of solid materials are of great importance in the choice of solid state fermentation. Generally, agricultural waste materials are used as support materials, which makes this method environmentally friendly and economical (Ozsolen, 2010; Serin, 2009).

Biological, physicochemical, and environmental factors affect product production efficiency during solid state fermentation. The selection of the method, microorganisms, and support materials to be used, determination of the chemical composition of the support material to be used, selection of appropriate conditions and carbon and energy sources to be added, optimization of variables such as aeration, humidity, appropriate temperature, pH, mixing, obtaining and improving the product, isolation processes, and purification steps of the formed product are the issues to be considered. In addition, aeration for oxygen and carbon dioxide transport, mixing mechanisms for heat conduction, and temperature control are important parameters for the process to proceed smoothly (Konak et al., 2012).

Advantages

Because it is a stationary process, the amount of energy used is low, the cost is low, the efficiency is high, and it is a practical method.

- The amount of water used in the process and the amount of wastewater generated as a result of the process are low.
- Agricultural waste is generally used as a support material.
- The product capacity is high, and the product has high stability and concentration.
- With some exceptions, all enzymes can be produced by solid state fermentation.
- Some support materials cannot be used in submerged Fermentation, but can be used in solid state fermentation.
- Because it is a method similar to the natural living environment of microorganisms, it stands out as a very environmentally friendly technique with features such as adaptability to many studies (Ozsolen, 2010; Serin, 2009).

Table 2.3. Advantages and implications of solid state fermentation.

Advantages of SSF	Implications
Low water demand	Reduced wastewater generation
High concentration of end product is formed	Cost reduction

Tablo 2.3. (Continued)

Solid materials are used	High concentration ensures growth
Very similar to the natural environment of fungi	Fungi work at higher efficiency
High volume production can be achieved	Smaller fermenter volume required
Consume unused carbon resources	Cost-effective and very carbon-efficient
No chemical or mechanical antifoam is used	They do not cause loss of microorganisms in fermentation

Disadvantages:

It is difficult to control variables such as pH, oxygen content, humidity, and temperature during incubation.

- It is difficult to remove the heat released during the process.
- High heat is generated during the process, which can damage the product structure.

This method poses several challenges in the scale-up processes used for application to larger processes.

The product produced as a result of the process is of low purity and must be purified using various methods to improve the product. This increases the cost of this method and limits its industrial use. (Ozsolen, 2010; Serin, 2009)

Uses:

- Production of biologically important secondary metabolites,
- To render wastes, toxic and hazardous substances harmful to human health and the environment harmless or less harmful through detoxification, biological remediation and destruction,
- It is used in the production processes of valuable and costly products (e.g., substances used in biopharmaceuticals). (Ozsolen, 2010; Serin, 2009)

In solid state fermentation, the appropriate bioreactor must be selected for fermentation to take place. Various bioreactor types, such as gas-solid fluidized bed reactors, packed bed rotary reactors, immersion bioreactors, rotating cylinder bioreactors, and tray bioreactors, are used in solid state fermentation processes. Currently, among these reactors, packed bed reactors are preferred because they are economical, controllable, and allow for intervention. (Ozsolen, 2010; Serin, 2009)

Because the solid state fermentation technique is suitable for the natural habitat of white rot fungi, which are members of *Basidiomycetes*, it allows efficient production of these fungi and increases their activity. Thus, white-rot fungi have become very important in solid state fermentation processes. (Ozsolen, 2010; Serin, 2009)

Industrial processes require large amounts of enzymes; however, raw enzymes can be obtained at very high costs. Therefore, the economical supply of enzymes by biological processes, such as solid state fermentation, has gained great importance.

Ozsolen et al. (2010), evaluated white rot fungi such as *Pleurotus sajorcaju*, *Phanerochaete chrysospsorium*, *Trametes versicolor*, barley, corn, rice or wheat bran, oats, dried tea, *P. nigra*

cones, sawdust, soybean meal, canola, sunflower stalks, alfalfa, and dried distillers' seeds, and the production of lignin peroxidase, manganese peroxidase, and laccase enzymes in solid state fermentation. Among the solid support materials, the highest laccase activity was obtained with alfalfa (22.36 U/ml), and the highest MnP activity was obtained with oat (0.36 U/ml) (Ozsolen et al., 2010).

2.6 LACCASE FROM *TRAMETES VERSICOLOR*

White rot fungi are heterogeneous fungi belonging to the *Basidiomycetes* group that use hemicellulose, cellulose, and lignin components in woody structures as a food source and degrade them, forming white and fibrous residues on wood whose structure is degraded (Ozan et al., 2014; Ozsolen, 2010; Birhanlı, 2008; Gedikli, 2008). When nitrogen and carbon sources become limiting factors, they degrade the wood structure with extracellular enzymes such as lignin peroxidase, manganese peroxidase and laccase (Cancelik et al.,2017). These eukaryotic organisms, which usually develop in dead tree trunks, have the ability to depolymerize and mineralize lignin and are the most accurate organisms that can be used for lignin removal because they can break down lignin into CO_2 and H_2O components (Goksel Dizge, 2007; Birhanlı, 2008). *Phanerochaete crysosporium*, *Chrysosporium lignorium*, *Agaricus bisporus*, *Phellinus nigrolimitatus*, *Funalia trogii*, *Pleurotus ostreatus*, and *Trametes Versicolor* (Ozsolen, 2010; Gedikli, 2008).

The most important feature of white rot fungi that makes their use in biotechnological fields indispensable is their ability to secrete lignin-degrading (lignolytic) enzymes to eliminate environmental pollution (Birhanlı, 2008). These enzymes are laccase, manganese peroxidase and lignin peroxidase. Laccase is a key enzyme in the ligninolytic system of white-rot fungi. Almost all these extracellular enzymes, which can be used in many areas, especially in lignin degradation, have the ability to oxidize polyphenols and destroy woody structures. The extracellular enzymes laccase, manganese peroxidase and lignin peroxidase are secreted by the fungus *Trametes versicolor* (Goksel Dizge, 2007; Ozan et al., 2014; Birhanlı, 2008; Fidan et al., 2012).

White rot fungi can be used in many biotechnological studies because of the extracellular enzymes they produce. White-rot fungi produce various enzymes that are essential for the production of organic molecules. Through these enzymes, the breakdown of pollutants resistant to degradation can be effectively carried out. The areas of use include; removal of lignin from pulp in the paper industry, removal of pesticides, etc. in the wastewater of various industries that pose a danger to human health and the environment, degradation of chemicals such as phenolic compounds, xenobiotics, polycyclic aromatic compounds, etc., obtaining biological products such as enzymes and hormones, color of dyes (Ozsolen, 2010; Birhanlı, 2008).

In a study by Birhanlı and Yesilada (2017), laccase production from *Funalia trogii* and *Trametes versicolor* fungi, which have high laccase production potential as enzyme producers, was investigated in order to identify the organisms producing high amounts of laccase enzyme, suitable enzyme production media and methods, and to produce the enzyme at low cost. Copper-free and copper-containing distilled water, whey, Malt Extract Broth and Sabouraud Dextrose Broth were used as the enzyme production media. A repeated batch process was used to produce laccase. The highest laccase activity was obtained as 17.68 and 6.27 U mL^{-1} for *Funalia trogii* and *Trametes versicolor* in copper added Malt Extract Broth media, respectively. Both fungal species were able to produce laccase in all media, and the amount of laccase produced increased significantly in the copper media.

Ozan et al. (2014) aimed to investigate the activity of laccase enzyme in 6 different white rot fungi collected from soil and tree trunk in a certain area. Samples were collected from four different parts of the collected mushrooms: outer, inner, above the cap, and below the cap. The growth conditions of the mushrooms were optimized by determining the most active and contamination-free growth medium of the mushrooms planted in two different solid media with different glucose concentrations, containing and without trace elements, and the activity of the laccase enzyme was determined. The highest laccase activity was observed in both media. In the medium containing 5 gL^{-1} glucose concentration and without trace elements, the highest laccase activity was 0.06 U mL^{-1}, in the medium containing 10 gL^{-1} glucose concentration and containing trace elements, the highest laccase activity was 0.25 U mL^{-1}.

Trametes versicolor, commonly referred to as Coriolus, is a prevalent white-rot fungus that is widely recognized for its exceptional capacity to degrade wood, particularly lignin, and to metabolize phenolic substances. This fungus is highly sought after in the field of biotechnology owing to its noteworthy attributes. This fungus has significant potential as a biocatalyst in oxidative biological processes owing to its production of extracellular oxidative enzymes such as lignin peroxidase, manganese peroxidase, and laccase, which initiate the breakdown of lignin, a complex aromatic polymer Laccase is a multicopper enzyme that functions as a benzenediol: oxygen oxidoreductase, with the EC number 1.10.3.2. It can oxidize a wide range of aromatic substrates, such as polyphenols, aromatic amines, and methoxy-substituted phenols. The oxidation process carried out by laccases involves a single-electron reaction that converts oxygen into water. Fungal laccase can be applied in wood delignification, bioremediation, wine clarification, and dye decolorization. Large quantities of enzymes are required for industrial processes, and their production can be enhanced through novel enzymatic production strategies such as the use of inducers and substrate limitation conditions. Optimization of fermentative processes is necessary to maximize productivity and minimize costs. Statistical experimental design can be utilized to evaluate the effects and interactions of different parameters governing a biological process (Tavares et al., 2006).

In a study by Goksel Dizge (2007), the optimum production conditions for laccase enzyme, an industrial enzyme obtained from *Trametes versicolor*, a white rot fungus, were determined, purified, and partially characterized. *Trametes versicolor* was incubated in a liquid medium at 125 rpm and 32˚ C for 12 days. Laccase activity was monitored in the medium, and it was observed that the maximum activity was observed at pH 5 on day 4, after which the activity decreased rapidly. Enzyme activity increased proportionally up to 60˚ C and the maximum activity was observed at 65°C. Above 65˚ C, enzyme activity decreased sharply, and the enzyme was denatured at 70°C (Goksel Dizge, 2007).

2.7 CIRCULAR ECONOMY: AN ENGINEERING PERSPECTIVE

The concept of a circular economy (CE) is grounded in various theories that challenge the prevailing economic system, which is characterized by the excessive exploitation of natural resources.

In recent years, the circular economy has garnered increasing global attention because of its recognition of resource security and efficiency, which are crucial to the well-being of economies and businesses (Rizos et al., 2017).

Compared to the linear economy, which is widely accepted worldwide, the circular economy presents opportunities to implement circular design and production strategies, promote

economic growth, reduce environmental impacts, stimulate job markets, and enhance brand image for companies while benefiting society at large (Dumée, 2022).

Industrial ecology is a research field that uses systematic methods to examine human economic activities and their relationship with sustainability. Achieving a sustainable industrial economy requires economic, cultural, structural, and technological transformations to enhance energy and material utilization. This includes minimizing non-recyclable waste and reducing the consumption of scarce materials and energy sources through improved production methods and product design. Implementing the principles of industrial ecology within a company can encourage firms to cooperate in exchanging resources and by-products regardless of their geographical proximity. Such cooperation can result in the creation of networks that promote knowledge sharing and eco-innovation (Rizos et al.2017).

The circular economy is primarily focused on physical and material resources within the economy, emphasizing recycling, reducing, and reusing resources as well as utilizing waste as a resource to decrease the consumption of primary resources. The goal of this model is to extend the use of resources and extract as much value as possible by recovering and reusing the products and materials (Rizos et al., 2017).

2.7.1 Impact of Biochar Production and using on Economy

Biochar is a crucial environmental catalyst due to its eco-friendly nature, safety, cost-effectiveness, porous structure, extensive surface area, and remarkable capacity for absorbing pollutants, which makes it a significant contributor to pollution control (Karthik et al., 2021).

Biochar can aid the recovery of resources in a circular economy. It can be produced from different types of organic wastes, such as agricultural residues, forestry waste, and municipal solid waste, and can be converted into valuable resources. Additionally, biochar can be used as a soil amendment to improve soil health, retain water, and cycle nutrients, reducing the need for chemical fertilizers and encouraging sustainable farming. Furthermore, biochar can serve as feedstock for energy production through gasification or pyrolysis, generating renewable energy and decreasing greenhouse gas emissions. Lastly, biochar can be used as a replacement for fossil fuels in various industrial processes, such as cement production, which reduces the dependence on non-renewable resources and supports a circular economy (Liu et al., 2021).

2.7.2 Economic Value of Biochar Industry

The Global Biochar Market Report 2023 revealed that the industry has experienced significant economic growth over the past few years. The market has expanded rapidly, with revenues surpassing USD 600 million by 2023, representing a compound annual growth rate (CAGR) of 97% compared to the growth rate in 2021. According to the report, revenue from biochar producers, distributors, value-added producers, and equipment manufacturers is expected to continue to grow, reaching $3.3 billion by 2025, which is approximately to 5-5.5 times its current capacity.

Table 2.4. Global revenue generated by the biochar industry.

Industry Members	**2021**	**2023**
Biochar Produces	54,750,000$	330,130,000$
Distributors And Value-Add Producer	7,250,000$	38,880,000$
Equipment Manufacturers	94,380,000$	241,250,000$
Total Revenue	156,380,00$	610,260,000$

2.7.3 Growth in the Biochar Industry

The future of the biochar industry is promising, as it plays a significant function in carbon removal and climate change mitigation. According to a global biochar market report, biochar has led to carbon dioxide removal, representing more than 90% of global deliveries by 2023. This dominance highlights the industry's potential to contribute significantly to global carbon reduction.

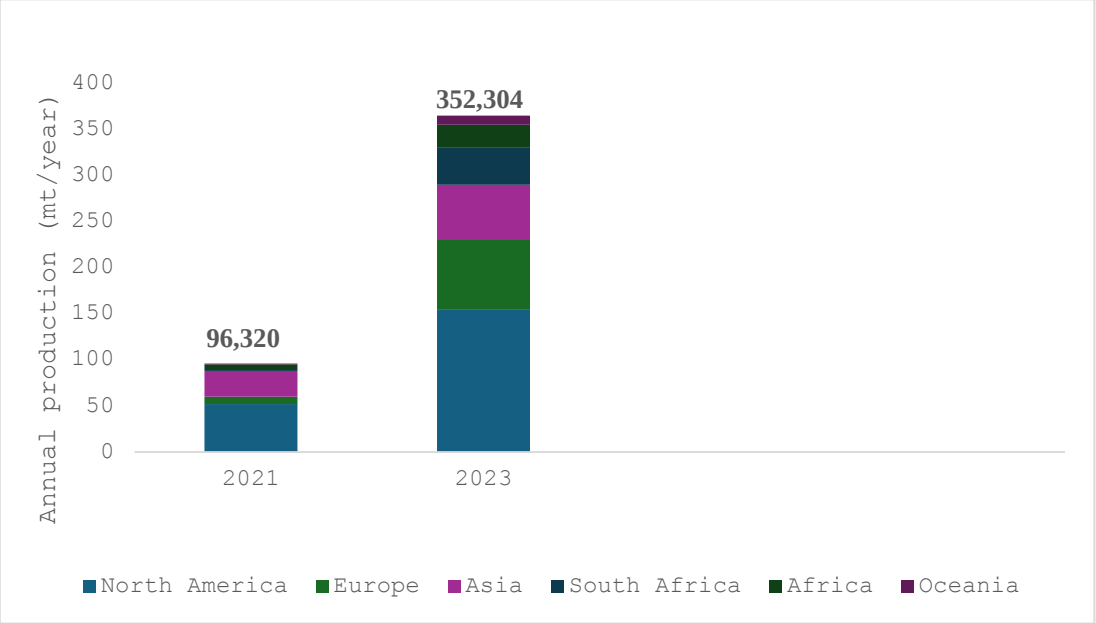

Figure 2.1. Estimated global biochar production in metric tons (mt) by year based on values reported by respondents in the Biochar Producer category**.**

2.7.4 The Economics of Coffee

The global coffee industry has long been a significant contributor to the economic landscape, providing livelihoods for millions of people worldwide and generating billions of dollars of revenue annually. However, this industry also carries a substantial environmental footprint, with the production, processing, and consumption of coffee responsible for significant greenhouse gas emissions and other environmental impacts (Humbert et al., 2009).

A critical issue is the substantial quantity of waste generated by the coffee industry, with only a small portion being currently recyclable. The daily consumption of approximately two billion cups of coffee contributes to an estimated 70 million tons of annual emissions (Reay, 2019). Furthermore, approximately 90% of brewed coffee is converted into Spent Coffee Grounds (SCG). The emergence of innovative approaches for repurposing coffee waste has transformed it into a valuable resource and supported a circular economy (Kourmentza et al., 2018). Coffee waste can be generated based on coffee content. Biofuels can also be indirectly used in areas such as biodiesel production, electric biosygas, soil amendment, and additional biogas production, as the potential of coffee waste as a source of biofuels and other valuable compounds is being increasingly recognized (Hernández-Varela & Medina, 2023).

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microorganism

In this study, the pure culture white rot fungus *Trametes versicolor* obtained from a mixed culture was used as a laccase-producing organism. For continuity of the culture, it was periodically transferred to malt extract agar medium. The culture was stored in tubes on malt extract agar (MEA) at +4° C.

3.1.2 Media, solutions and chemicals used

All media, solutions, and chemicals used in the development of *Trametes* isolates and spore formation, preparation of the developed spores as inoculum, and production by solid culture fermentation in Erlenmayer flasks were sterilized by autoclaving at 121°C for 15 min.

3.1.2.1 Malt extract agar (MEA)

Commercially available Malt Extract Agar (Biolife) medium was used for storage, growth, and sporulation of *Trametes versicolor* strains.

Table 3.1. Malt extract agar components.

Component Name	Amount
Malt Extract Agar	16.0 g
Distilled Water	1000 ml

3.1.2.2 Solid state production medium coffee pulp

The coffee pulp was mixed with the humidification liquid, placed into flasks, sterilized in an autoclave at 121° C for 15 min, and then used. The coffee pulp used was obtained from a coffee brand in the Üsküdar District of Istanbul.

3.1.2.3 Solid state production medium biochar

Biochars derived from hazelnut shells of the Biorfe brand were used in the trials. The analyzed data are presented in Table 3.2. The production method involved initial processing at 400-600°C for 2 h, followed by pyrolysis, physical activation, and exposure to water vapor at 900-1000 degrees, tailored for the company's specific application area.

Tablo 3.2. Analysis values of biochar obtained from Biorfe hazelnut shell.

State of Sample	Unit	Dry Value in Air	Dry value	moisture ash free (MAF) value	As Received	Method of Test
Total Moisture	%	-	-	-	5,39	IS0589
Inherent Moisture	%	2,54	-	-	-	ASTM 03173
Ash	%	2,89	2,97	-	2,81	ISO 1171
Volatile Matter	%	3,19	3,27	3,37	3,09	ISO 562

Tablo 3.2. (Continued)

Gross Calori Value	kcal/kg	7430	7624	7857	7213	ISO 1928
Net calori Value	kcal/kg	7299	7489	7718	7056	ISO 1928
Total Sulphur	%	0,03	0,03	-	0,03	ASTM 04239
Fixed Carbon	%	91,38	93,76	-	88,71	ASTM 03112

During the experiment, the biochars were ground and homogenized.

3.1.2.4 Preparation Of Buffer Solution

To prepare a buffer solution, sodium acetate ($C_2H_3NaO_2$) weighing 0.68 grams was added to a 1-liter flask, followed by the addition of distilled water to the top of the flask. Subsequently, 0.287 mL of acetic acid (CH_3COOH) was introduced into the flask, and the pH was adjusted to 4.7 to create the buffer solution.

3.1.2.5 Preparation of Medium

Analytical grade chemicals were used for the preparation of the solid medium, liquid medium, buffer solution, and reaction medium.

Table 3.3 Liquid medium prepared for *Trametes versicolor*.

Chemical	**Amount (gL^{-1})**
Malt Extract	10
$MgSO_4$ x $7H_2O$	1
KH_2PO_4	2
$FeSO_4$ x $7H_2O$	0,001
$CaCl_2$ x $7H_2O$	0,1

3.1.2.6 Tween 80 Solution (0.1% v/v)

In the agar medium used for spore inoculation in bioreactors, 1 mL of Tween 80 was dissolved in 1 liter of distilled water, and the solution was aliquoted into tubes for serial dilutions to collect amplified spores from the medium.

3.1.2.7 Solutions used to regulate the initial pH value of solid state fermentation media

5 N Hydrochloric Acid (HCl) Solution

To prepare 100 ml of 5 N HCl solution to be used for adjusting the initial medium pH in solid culture production optimization studies, 41.4 ml of 37% HCl was added to 58.6 ml of pure water and completed to 100 ml.

5 N Sodium Hydroxide (NaOH) Solution

To prepare 100 ml of 5 N NaOH solution to adjust the initial medium pH in solid culture production optimization studies, 20 g NaOH was weighed, and 100 ml was completed with pure water.

1 N Sodium Hydroxide (NaOH) Solution

To prepare 100 ml of 1 N NaOH solution to adjust the initial medium pH in liquid culture production optimization studies, 4 g of NaOH was weighed and added to 100 ml of distilled water.

3.1.3 Instruments used

3.1.3.1 Precision balance

A RADWAG AS 220.R2 precision balance was used to prepare the media and mixtures.

3.1.3.2 pH meter

WTW pH3110 pH meter was used to check and adjust the pH values of the prepared medium and other mixtures.

3.1.3.3 Heated magnetic stirrer

DLAB MS-H380 magnetic stirrer with heater was used for homogenization of the medium and other mixtures to ensure that the medium and other mixtures used were in the desired conditions and with the desired properties.

3.1.3.4 Otoclave

Ildam brand 23L autoclave was used to ensure sterilization in the preparation of the necessary environments and to make the microbiologically contaminated material resulting from the processes reusable and to kill all microorganisms.

3.1.3.5 Incubator

Microtest brand incubator was used for the cultivation of *Trametes* strains at 28° C temperature.

3.1.3.6 Microscope

SOIF brand XDS1 model tri-ocular microscope, which can be attached to a digital camera, was used for microscopic examinations and counting *Trametes* spores in inoculum culture.

3.1.3.7 Centrifuge

Nüve NF400 brand centrifuge was used in solid liquid separation processes at 4100 rpm for 10 minutes.

3.2 METHODS

3.2.1 Experimental Design to Process Optimization

Response surface methodology (RSM) is used to improve manufacturing processes by performing fewer trials and minimizing operational costs and time spent. RSM utilizes the design of experimental components and statistical methods to obtain the optimum point response, which is then used to process the study results and recommend the correct decision. Determining the overall performance of processes is very complex because of the many parameters that affect their behavior. The generation of process behavior maps expressed in a set of mathematical models that manipulate important parameters to achieve the maximum, targeted, or minimum performance of the process. The optimization of complex manufacturing processes determines the experimental conditions for the highest or best performance (yield, efficiency, etc.). There are several advantages to using an experimental design in production optimization. These include reduced processing times, cheaper processes, and more efficient use of cost-effective resources, such as equipment, labor, and materials. In addition, it uses

numerical regression tools to fit the data into mathematical models to predict the factor levels entered in the optimization (J. Cerino-Córdova et al., 2020).

Tablo 3.4. Experimental Design Factors.

Factor	**Name**	**Units**	**Type**	**SubType**
A	Time	Day	Numeric	Continuous
B	Amont of support material	g	Numeric	Continuous
C	Moisture (depending on the amount of support material)	Percentage	Numeric	Continuous

From this point forward, the term "% moisture" refers to the percentage moisture content based on the amount of solid support material.

Table 3.5. Experimental design results.

Number Of Experiment	**Support material (g)**	**% Moisture**	**Day**
1	30	70	3
2	10	70	3
3	10	50	3
4	30	50	3
5	20	60	3
6	20	60	5
7	20	70	5
8	20	60	5
9	20	60	5
10	20	50	5
11	20	60	5
12	10	60	5
13	20	60	5
14	20	60	5
15	30	60	5
16	20	60	7
17	30	50	7
18	10	70	7
19	10	50	7
20	30	70	7

Table 3.6. The moisture content of the experiment and the amount of Tween-80 to be added.

Number of Experiment	Support material (g)	% Moisture	Day	Coffee Solution (ml)	Tween-80 (V/W %10)
1	30	70	3	21	5,1
2	10	70	3	7	1,7
3	10	50	3	5	1,5
4	30	50	3	15	4,5
5	20	60	3	12	3,2
6	20	60	5	12	3,2
7	20	70	5	14	3,4
8	20	60	5	12	3,2
9	20	60	5	12	3,2
10	20	50	5	10	3
11	20	60	5	12	3,2
12	10	60	5	6	1,6
13	20	60	5	12	3,2
14	20	60	5	12	3,2
15	30	60	5	18	4,8
16	20	60	7	12	3,2
17	30	50	7	15	4,5
18	10	70	7	7	1,7
19	10	50	7	5	1,5
20	30	70	7	21	5,1

3.2.2 The Bioreactor Installation

Before the experiment, the biochar was ground to increase its surface area, preventing the humidification liquid from pooling in one area because of the shape of the nutshell, and ensuring a homogeneous distribution. Coffee medium (Table 3.3) was prepared and adjusted for use. A Tween-80 solution was also prepared to aid the passage of microorganism spores into the liquid. Tween-80, a nonionic surfactant, serves as an emulsifying and dispersing agent in biological and chemical processes. Its properties help break down the cell walls of microorganisms, facilitating the passage of spores into liquid.

All the prepared solutions were sterilized by autoclaving, and the bioreactors were set up according to the experimental design (Table 3.5) and placed in an incubator at 28°C.

3.2.3 Downstream processes

After incubation, sodium acetate buffer (pH 4.7) was added to the solid mass (biochar) in a 1:1 ratio. The mixture was stirred and allowed to stand for 30 min. Subsequently, the samples were filtered through muslin cloth. The initial filtrate was further filtered using a tea filter, while the remaining sample, filtered through a muslin cloth, was centrifuged. To prevent denaturation during centrifugation, samples were placed in an ice bath for 5 min every 5 min and centrifuged for 15 min. The filtered filtrates were stored in the laboratory for 30 min.

Both centrifuged and twice-filtered samples were added to spectrophotometer cuvettes with 800 µL of sodium acetate buffer, 100 µL of substrate, and 100 µL of sample, following the ABTS method. The absorbance values over time (ΔA) were measured immediately in the kinetic mode of the spectrophotometer at a wavelength of 420 nm with a measurement duration of 60 second. The laccase activity was determined based on the obtained ΔA values.

3.2.3.1 Determination of laccase enzyme activity

The determination of laccase activity was accomplished by employing 2,2-azino-bis (3-ethyl-benzthiazoline-6 sulfonic acid (ABTS) as the substrate, followed by a measurement through spectrophotometry. One activity unit was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute, and the results were reported in units per liter (U/L) (Osma et al., 2010).

Sodium acetate buffer (pH 4.7) was prepared as the buffer solution for laccase enzyme activity determination. 0.68 g of Sodium Acetate.$3H_2O$ was dissolved in pure water. Subsequently, 0.286 mL of 100% acetic acid was added. The total volume was 100 mL. Subsequently, 100 mg of commercially available laccase (Sigma-Aldrich) was measured and transferred to a Falcon tube. The enzyme was then dissolved in sodium acetate buffer and the total volume was adjusted to 10 mL using the same buffer solution. 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich), 54.9 mg was weighed and transferred to Falcon tubes. The substrate was dissolved in sodium acetate buffer (pH 4.7), and the final volume was adjusted to 10 mL with sodium acetate buffer (pH 4.7). All solubilizations were performed using a magnetic stirrer.

The falcon tubes containing the enzyme and substrate solutions, which are sensitive to light, were covered with aluminium foil as soon as possible to prevent them from being affected by light.

In the spectrophotometer, the modes were selected to measure the kinetic change, the wavelength of the light was set to 420 nm, and the change in absorbance with respect to time was measured using the device with a total measurement time of 60 second.

Amount of enzyme = Amount of sample from reactors

Reactors were constructed using the values in Table 3.5 and left in the incubator at the appropriate temperatures to be sampled on the days recommended by the design.

Sodium acetate buffer (pH 4:7) was added at a ratio of 1:1 w/v based on the amount of biochar added to the reactors on the sample days and mixed with a clean mixer. Waiting for 30 minutes. The reactors were treated for 30 min in sodium acetate buffer and filtered through a clean (muslin cloth) filter. After coarse filtration, the samples were placed in an ice bath at 4100 rpm every 5 min to prevent denaturation during centrifugation, and centrifuged for a total of 15 min. The centrifuged samples were added to the spectrophotometer cuvettes as 800 µl sodium acetate buffer, 100 µl substrate, and 100 µl sample, in accordance with the ABTS method, and the absorbance value ΔA against time was obtained at 420 nm wavelength in the kinetic mode of the spectrophotometer with a measurement time of 60 s. The ΔA values were used to calculate laccase enzyme activity.

Laccase activity was calculated using the following formula:

$$U = \frac{V * \Delta A * dilution\ coefficient}{\vartheta * \varepsilon * d} \quad (3.1)$$

V: total reaction volume (ml)

v: enzyme volume (ml) (sample)

ε: Molecular extinction coefficient (36 mM^{-1} cm^{-1} at 420 nm for ABTS)

d: beam distance (cm) (1 cm)

ΔA: absorbance change

3.2.4 Total Sugar Determination

Sugar content was determined using the phenol-sulfuric acid method, which is a widely accepted and accurate method for determining the total sugar content.

Total Sugar Determination Reagents and Solutions

- Glucose Standard Solution (0.01% w/v)

0.01 g of d-glucose (Merck) was weighed, dissolved in a small amount of distilled water, and added to a volume of 100 ml with distilled water. Stock solutions were stored at +4°C.

- Phenol Solution (%5.0 wt/vol)

5.0 g of phenol (Riedel de Häen) was weighed and dissolved in a beaker containing distilled water by heating. After the complete dissolution of phenol, the solution was made up to a volume of 100 ml volume with distilled water. Stock solutions were stored at +4°C.

- Concentrated Sulfuric Acid: 95-98% purity, H_2SO_4 Merck) was used.

The glucose solution was used as a standard by dilution, as shown in (Table 3.7).

Table 3.7. Standard Concentrations of total sugar determination method.

	%0.01 Glucose (ml)	Distilled Water (ml)	Glucose Concentration (mg/ml)
Std 1	0	1.0	0
Std 2	0.2	0.8	20
Std 3	0.4	0.6	40
Std 4	0.6	0.4	60
Std 5	0.8	0.2	80
Std 6	1.0	0	100

The tubes were mixed using a vortex tube mixer (Table 3.7), and left at room temperature for 10 minutes.

After waiting, the samples were again mixed using a tube mixer. Subsequently, samples were incubated in a water bath at 25-30°C for 20 min.

Afterwards, the absorbance value of the samples was read at a wavelength of 490 nm compared to the blank sample, and a standard graph (Figure 4.7) was constructed based on the absorbance values and concentrations of the standard samples (Table 4.14).

3.2.5 Using the Thoma Plate for Cell Counting

Cell counting is a fundamental technique used in various fields of biology and biomedical research. The Thoma plate, also known as a hemocytometer, is commonly used for this purpose and is a microscope-based device that enables the enumeration of cells, such as blood cells, algae, and other microscopic organisms, with a high degree of accuracy and precision. This method is widely utilized in the life sciences (Inchur et al., 2020).

The Thoma plate consists of a thick glass slide with a grid-like pattern etched onto its surface, creating a defined volume in which the cells can be counted(Lund et al., 1958).This grid pattern was typically divided into nine large squares, each of which was further subdivided into smaller squares. By counting the cells within a specific number of these smaller squares, researchers can extrapolate the total cell count of the sample (Agarwal et al., 2020).

In this study, cell counting was performed using a Thoma plate. A sample taken from a pure culture of *Trametes versicolor* was used as the study material. Initially, 10 ml of Tween-80 was added to *Trametes versicolor* colonies grown on agar. After this process, the sample was incubated for 30 min and diluted 10-fold. The diluted sample was placed in a clean Thoma chamber and examined under a microscope.

4 RESULTS AND DISCUSSION

4.1.1 Analysis of Reactor Design Using Biochar as Support Material

The experimental design utilizing biochar was based on the variables outlined in Table 4.1 and the results are presented in Table 4.2.

Tablo 4.1. Ranges of Variation for Three Independent Variables in a Central Composite Design (CCD) Used in Solid Culture Fermentation Experiments.

Factor	Name	Units	Type	SubType	Min.	Max.	Coded Low	Coded High	Mean	Std. Dev.
A	biochar	G	Numeric	Continuous	10	30	-1 ↔ 10,00	+1 ↔ 30,00	20,00	7,25
B	moisture	%	Numeric	Continuous	50	70	-1 ↔ 50,00	+1 ↔ 70,00	60,00	7,25
C	day	day	Numeric	Continuous	3	7	-1 ↔ 3,00	+1 ↔ 7,00	5,00	1,45

Table 4.2. Responses of the Three Independent Variables in Solid Stade Fermentation Experiments Using Central Composite Design (CCD).

Std	Run	Factor 1 A: Biochar (g)	Factor 2 B: % moisture	Factor 3 C: time (day)	Response (U)
13	1	20	60	3	0,0055556
2	2	30	50	3	0,0088889
3	3	10	70	3	0,0116667
4	4	30	70	3	0,0061111
1	5	10	50	3	0,0333333
18	6	20	60	5	0,0094444
16	7	20	60	5	0,0111111
20	8	20	60	5	0,0077778
19	9	20	60	5	0,0172222
10	10	30	60	5	0,0111111
15	11	20	60	5	0,0205556
9	12	10	60	5	0,0133333

Tablo 4.2. (Continued)

12	13	20	70	5	0,0222222
17	14	20	60	5	0,0144444
11	15	20	50	5	0,0222222
5	16	10	50	7	0,0055556
14	17	20	60	7	0,0105556
7	18	10	70	7	0,0116667
8	19	30	70	7	0,015
6	20	30	50	7	0,015

According to the experimental results, the highest response value, a reaction value of 0.0333333, was achieved using 10 g of biochar and 50% moisture and was conducted on the 3rd day. Regression analysis was subsequently performed to elucidate the relationship between the response function and experimental data, as shown in Table 4.3.

4.1.1.1 ANOVA Results

The ANOVA results of the experimental results and model were found to be statistically significant. The interactions between moisture and biochar amount between the factors were statistically significant ($p < 0.05$).

Table 4.3. ANOVA Results for Response Function in Solid Culture Fermentation Experiments Using Central Composite Design (CCD).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0,0007	9	0,0001	3,08	0,0473	Significant
A-biochar	0	1	0	1,6	0,235	
B-moisture	0	1	0	1,42	0,2609	
C-day	6,05E-06	1	6,05E-06	0,2556	0,6241	
AB	0	1	0	0,8622	0,375	
AC	0,0002	1	0,0002	9,66	0,0111	
BC	0,0001	1	0,0001	4,93	0,0507	
A^2	0	1	0	0,4741	0,5068	
B^2	0,0002	1	0,0002	7,4	0,0216	
C^2	0,0001	1	0,0001	4,45	0,0612	
Residual	0,0002	10	0			

Tablo 4.3. (Continued)

Lack of Fit	0,0001	5	0	0,9827	0,5074	not significant
Pure Error	0,0001	5	0			
Cor Total	0,0009	19				

4.1.1.2 Coefficients in Terms of Coded Factors

The coefficient estimate signifies the anticipated alteration in the response variable for each unit change in factor value, assuming that all other factors remain constant.

Table 4.4. Coefficients in Terms of Coded Factors.

Factor	Coefficient Estimate	Df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0,0138	1	0,0017	0,01	0,018	
A-biochar	-0,0019	1	0,0015	-0,005	0,002	1
B-moisture	-0,0018	1	0,0015	-0,005	0,002	1
C-day	-0,0008	1	0,0015	-0,004	0,003	1
AB	0,0016	1	0,0017	-0,002	0,005	1
AC	0,0053	1	0,0017	0,0015	0,009	1
BC	0,0038	1	0,0017	0	0,008	1
A^2	-0,002	1	0,0029	-0,009	0,005	1,8
B^2	0,008	1	0,0029	0,0014	0,015	1,8
C^2	-0,0062	1	0,0029	-0,013	4E-04	1,8

Table 4.5. statistical results of the experiment design.

Std. Dev.	0,005	**R^2**	0,7347
Mean	0,014	**Adjusted R^2**	0,4959
C.V. %	35,67	**Predicted R^2**	-1,551
		Adeq Precision	7,5251

According to the R^2 values in Table 4.5, 73.47% of the data variables could be expressed by the current model.

Adeq Precision assesses the signal-to-noise ratio (SNR), with a ratio exceeding four considered favorable. A design ratio of 7,525 signifies sufficient signal strength. This particular model is applicable to the exploration of the design parameters.

4.1.1.3 Suggested Optimized Experimental Set

The optimized experimental reactor factors for biochar proposed by Design Expert 13 are listed in Table 4.6.

Table 4.6. Variable factor details of the experimental set proposed by Design Expert 13 software.

Number	biochar	moisture	day	R1	Desirability	
1	***22,620***	***62,683***	***3,763***	**0,010**	**1,000**	**Selected**
2	*20,000*	*60,000*	*5,000*	0,014	1,000	
3	*10,000*	*70,000*	*7,000*	0,010	1,000	
4	*10,000*	*50,000*	*3,000*	0,029	1,000	
5	*20,000*	*60,000*	*7,000*	0,007	1,000	
6	*20,000*	*70,000*	*5,000*	0,020	1,000	
7	*30,000*	*50,000*	*3,000*	0,011	1,000	
8	*10,000*	*50,000*	*7,000*	0,009	1,000	

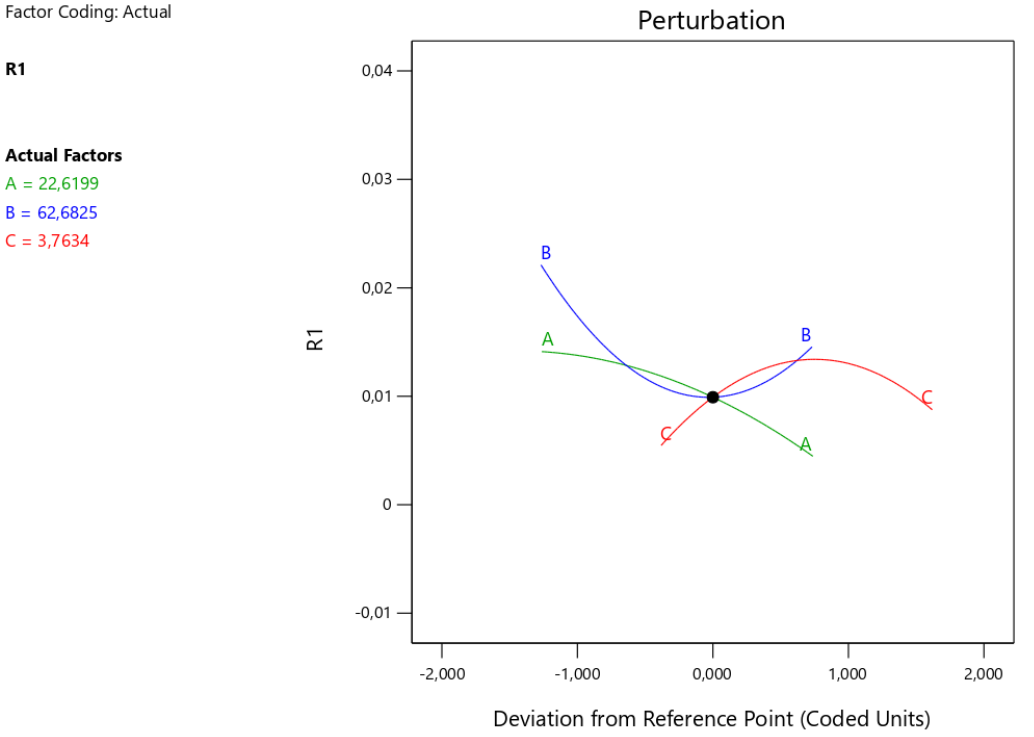

Figure 4.1. Graph of the optimal design proposal.

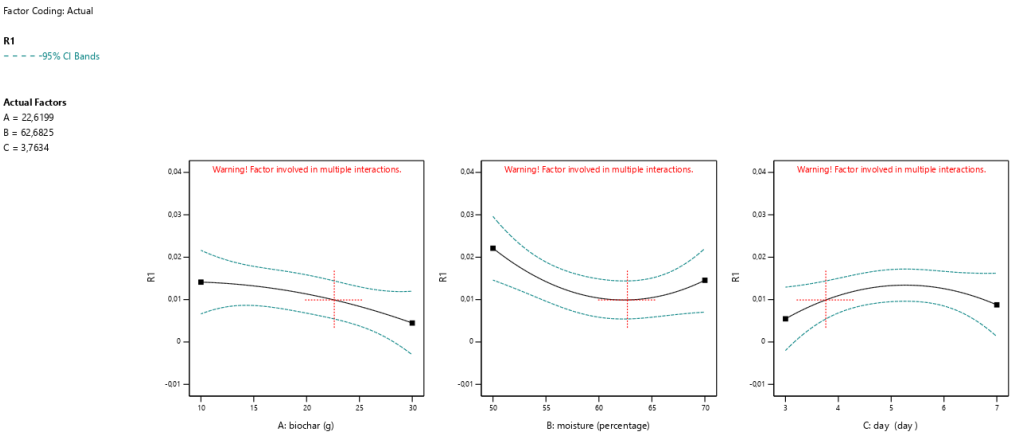

Figure 4.2. Optimal value proposition graphs for variables.

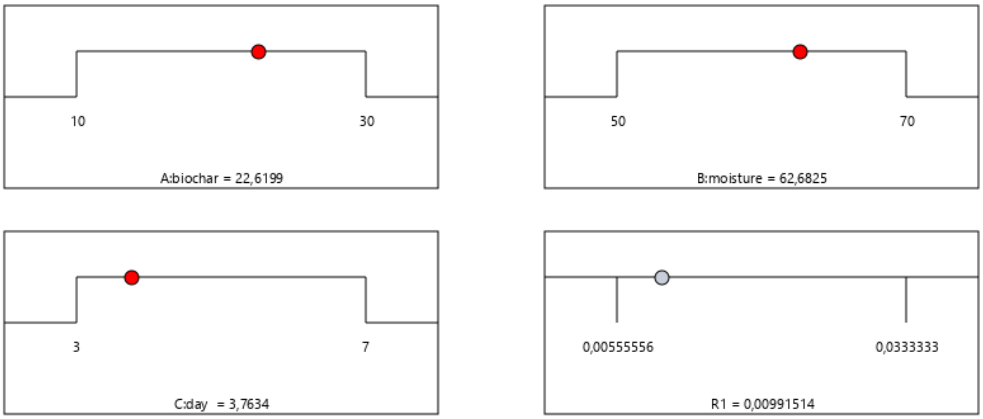

Figure 4.3. Stamps of the optimal value of variables.

4.1.2 Analysis of Reactor Design Using Coffe Pulp as Support Material

The coffee pulp experiment was conducted based on the specific variables outlined in Table 4.7, and the results are listed in Table 4.8.

Table 4.7. Ranges of Variation for Three Independent Variables in the Central Composite Design (CCD) Employed in Solid state Fermentation Trials.

Factor	Name	Units	Type	SubType	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	coffee	Gram	Numeric	Continuous	10	30	-1 ↔ 10,00	+1 ↔ 30,00	20,00	7,25
B	moisture	Percentage	Numeric	Continuous	50	70	-1 ↔ 50,00	+1 ↔ 70,00	60,00	7,25
C	day	Day	Numeric	Continuous	3	7	-1 ↔ 3,00	+1 ↔ 7,00	5,00	1,45

Table 4.8. Responses of the Three Independent Variables in Solid Culture Fermentation Experiments Using Central Composite Design (CCD).

Std	Run	Factor 1 A: Coffee pulp (g)	Factor 2 B: %moisture	Factor 3 C: day	Response(U)
3	1	10	70	3	0,00166667
14	2	20	60	7	0,005
2	883	30	50	3	0,00055556
4	4	30	70	3	0,00111111
7	5	10	70	7	0,0111111
15	6	20	60	5	0,00444444
11	7	20	50	5	0,00388889
8	8	30	70	7	0,0138889
9	9	10	60	5	0,00555556
20	10	20	60	5	0,00777778
12	11	20	70	5	0,0133333
13	12	20	60	3	0,00111111
5	13	10	50	7	0,00555556
18	14	20	60	5	0,00555556
6	15	30	50	7	0,005
16	16	20	60	5	0,00555556
10	17	30	60	5	0,00611111
19	18	20	60	5	0,005
17	19	20	60	5	0,00555556
1	20	10	50	3	0,00055556

Based on the experimental results, the highest reaction value of 0.0138889 was achieved with 30 g of coffee pulp at %70 moisture percent on Day 7. Subsequently, a regression analysis was performed to clarify the correlation between the response function and the experimental data, as shown in Table 4.8.

4.1.2.1 ANOVA Results

The ANOVA results of the experimental results and model were statistically significant (Table 4.8), and the interactions between moisture and biochar amount between the factors were statistically significant ($p < 0.05$).

Table 4.9. Analysis of variance (ANOVA) findings of the experimental model.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0,0002	9	0	10,11	0,0006	Significant
A-coffee	4,94E-07	1	4,94E-07	0,1811	0,6794	
B-moisture	0,0001	1	0,0001	23,96	0,0006	
C-day	0,0001	1	0,0001	46,37	< 0.0001	
AB	9,65E-07	1	9,65E-07	0,3538	0,5652	
AC	9,65E-07	1	9,65E-07	0,3538	0,5652	
BC	0	1	0	7,49	0,021	
A^2	2,12E-07	1	2,12E-07	0,0778	0,7859	
B^2	0	1	0	6,3	0,0309	
C^2	0	1	0	9,42	0,0119	
Residual	0	10	2,73E-06			
Lack of Fit	0	5	4,17E-06	3,24	0,1115	not significant
Pure Error	6,43E-06	5	1,29E-06			
Cor Total	0,0003	19				

P-values below 0.050 indicate statistical significance of the model terms. In this instance, the terms related to percentage moisture, day, and moisture-day configurations were statistically significant.

4.1.2.2 Coefficients in Terms of Coded Factors

Table 4.10. Coefficients in Terms of factors.

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0,0058	1	0,0006	0,0046	0,0071	
A-coffee	0,0002	1	0,0005	-0,0009	0,0014	1

Tablo 4.10. (Continued)

B-moisture	0,0026	1	0,0005	0,0014	0,0037	1
C-day	0,0036	1	0,0005	0,0024	0,0047	1
AB	0,0003	1	0,0006	-0,001	0,0016	1
AC	0,0003	1	0,0006	-0,001	0,0016	1
BC	0,0016	1	0,0006	0,0003	0,0029	1
A^2	-0,0003	1	0,001	-0,0025	0,0019	1,82
B^2	0,0025	1	0,001	0,0003	0,0047	1,82
C^2	-0,0031	1	0,001	-0,0053	-0,0008	1,82

The coefficient estimate represents the expected change in response per unit change in the factor value when all remaining factors were held constant.

Table 4.11. Statistical Results of The Experiment Design.

Std. Dev.	0,002	**R^2**	0,901
Mean	0,005	**Adjusted R^2**	0,8118
C.V. %	30,48	**Predicted R^2**	0,2844
		Adeq Precision	12,337

According to the R^2 value in Table 4.11, it is seen that 90,10% of the data variables can be expressed by the current model.

The Adeq Precision assesses the signal-to-noise ratio (SNR), with a preference for ratios exceeding 4. A design ratio of 12,336 was deemed to be sufficient to indicate a strong signal. This particular model holds potential for guiding exploration within the design space.

4.1.2.3 Suggested Optimized Experimental Set

The optimized experimental reactor factors for coffee proposed by Design Expert 13 are listed in Table 4.12.

Table 4.12. Variable factor details of the experimental set proposed by Design Expert 13 software.

Number	coffee	moisture	day	R1	Desirability	
1	***14,244***	***65,024***	***5,889***	**0,009**	**1,000**	**Selected**
2	20,000	60,000	5,000	0,006	1,000	
3	20,000	60,000	3,000	-0,001	1,000	
4	10,000	50,000	3,000	0,001	1,000	
5	10,000	70,000	7,000	0,012	1,000	

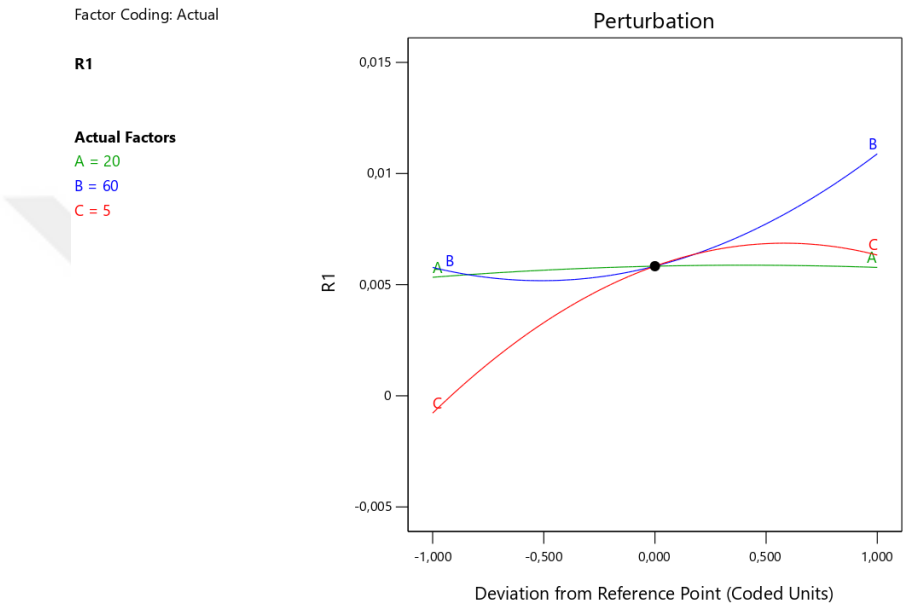

Figure 4.4. Graph of the optimal design proposal.

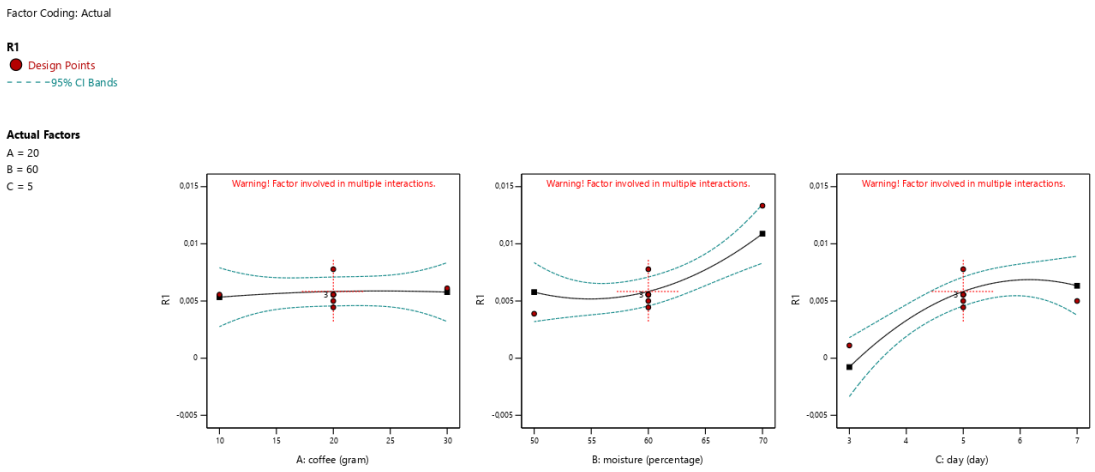

Figure 4.5. Optimal value proposition graphs for variables.

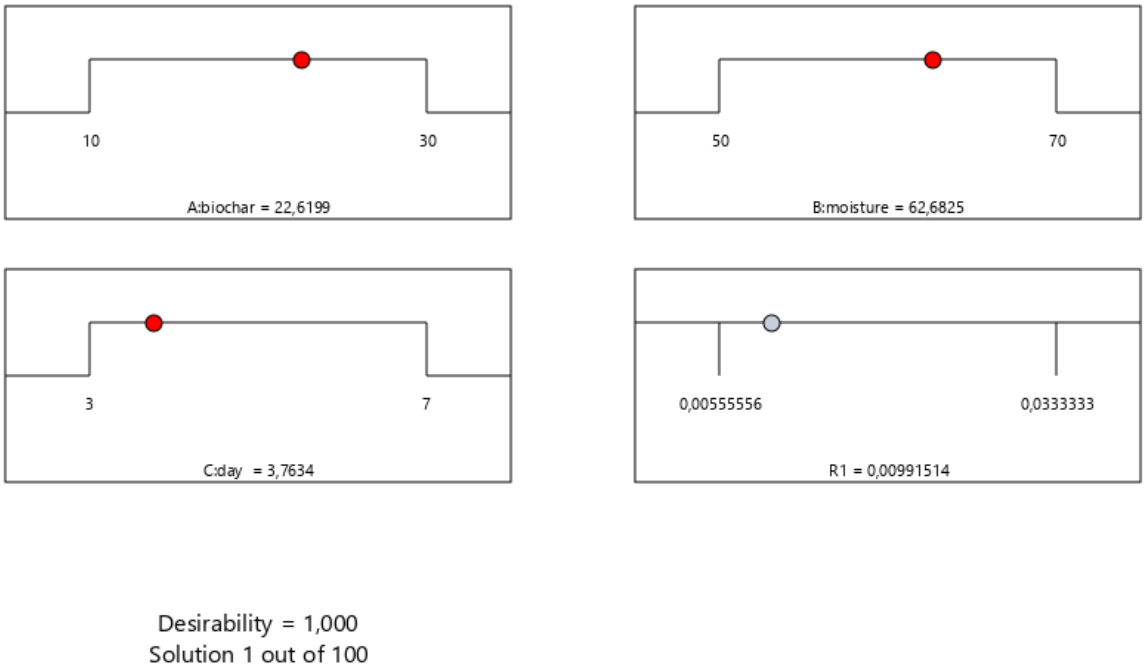

Figure 4.6. Stamps of the optimal value of variables.

4.1.3 Total Sugar Determination

The phenol-sulfuric acid method was used to determine total sugar content.

The absorbance value of the samples was read at a wavelength of 490 nm compared to the blank sample, and a standard graph was constructed based on the absorbance values and concentrations of the standard samples.

Table 4.14. Sample and standard contents and absorbance values of total sugar determination method.

	%0.01 Glucose (ml)	**Sample**	**Phenol (%5)(ml)**	**Concentrated H_2SO_4 (ml)**	**ΔA (490nm)**
Blank	-	-	1	5	
Std 1	1	-	1	5	0,029
Std 2	1	-	1	5	0,36
Std 3	1	-	1	5	0,478
Std 4	1	-	1	5	0,453
Std 5	1	-	1	5	0,537
Std 6	1	-	1	5	0,97
10kX diluted sample	-	1	1	5	0,607
100kX diluted sample	-	1	1	5	0,054

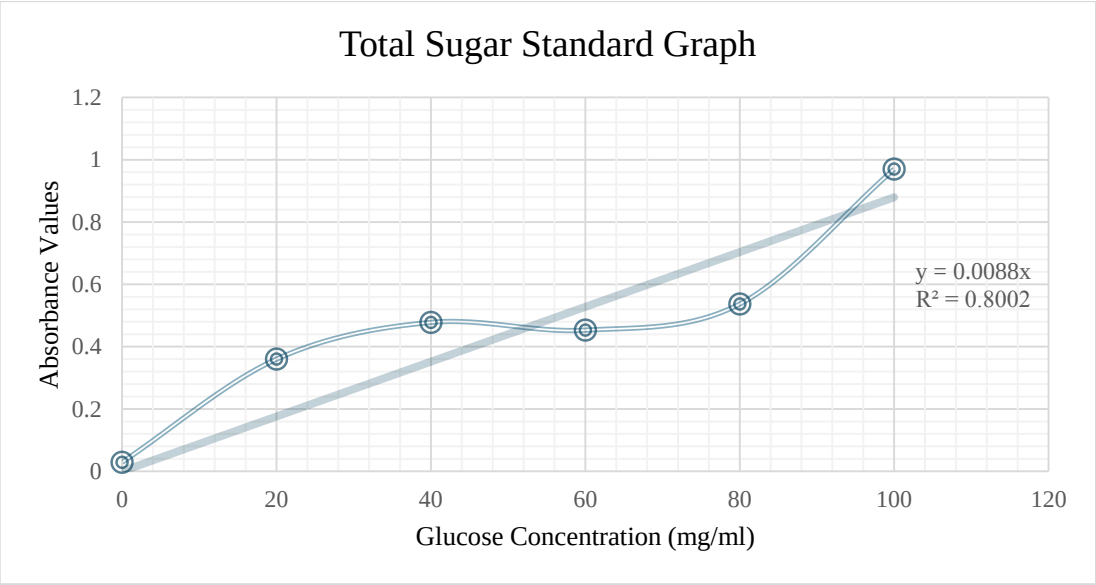

Figure 4.7. Glucose Standard Curve.

According to the equation of the standard graph (Figure 4.7), the sample concentration diluted 10k times: 69 mg/ml, 100k diluted sample concentration: 6,1 mg/ml.

4.1.4 Using the Thoma Plate for Cell Counting

As a result of the microscopic analysis, 164 cells were counted.

The result of the count on the Thoma plate was calculated using the following formula:

Colony Forming Units (CFU) / ml = A x SF x 10.000 (4.1)

where A represents the number of cells counted in 16 large squares, and SF represents the dilution factor. In this study, the count result was 164 and the dilution factor (DF) was determined to be 10. When these values are substituted into the formula:

164*16*10,000* 10 = 2.624*10^7 Colony Forming Units / ml (4.2)

Thus, the colony forming units per milliliter (CFU/ml) of the inoculum was determined.

4.2 DISCUSSIONS

Urbanization and industrialization increase soil and water pollution, which threatens ecological security and public health. Water bodies and soil are contaminated with heavy metals and organic materials. Various physical, chemical, and biological techniques have been used for environmental remediation. Bioremediation, which uses organisms to remove or neutralize pollutants, is cost-effective and environmentally friendly. An ideal immobilization carrier promotes microorganism colonization and enhances contaminant adsorption. In this study

The effects of different support materials on the extraction of metabolites from microorganisms, biochar, and coffee pulp induced microorganism proliferation. The microorganisms used were focused on providing resistance to stressful conditions (metabolic heat and nutrient deficiency), without genetic regulation.

At the same time, the utilization of waste by transforming waste into economically valuable products is an important study of waste utilization. The transformation of hazelnut shell, an agricultural product waste, into an economically valuable product, the use of the waste content of coffee pulp by integrating it into production systems, provides a circular bioeconomy in industries, while multiple gains have been achieved by using waste as a resource.

The results of this study indicate that both biochar and coffee pulp can be used as support materials in solid culture. The best values obtained for coffee and biochar were obtained in 30 g of coffee at 70% moisture content and in 7 day for coffee and 10 g of biochar at 50% moisture content and for 3 day for biochar.

There is limited information in the literature on the production of solid culture fermentation using *Trametes versicolor* with the support materials employed in this study. Owing to the commercial importance of this topic, details of large-scale production are scarce in scientific literature. Therefore, this is an important initial study. Future research can provide more detailed information on the use of these materials through experiments using different microorganisms, inducers, and humidification liquids. Similar results to those reported in the literature on laccase enzyme production by solid state fermentation were observed, which is promising for future studies.

5 CONCLUSION AND SUGGESTIONS FOR FUTURE RESEARCH

5.1 CONCLUSION

In this study, muslin cloth was used for the first filtration, and the color of the samples appeared dark owing to both biochar and coffee. The samples were centrifuged to remove this color and precipitate suspended biochar particles. However, no activation was observed at the expected levels after centrifugation. To explain this, it was thought that an increase in temperature during centrifugation could lead to denaturation of the samples.

To minimize the effects of the temperature increase, the samples were placed in an ice bath before centrifugation, the total centrifugation time was divided into three, and the samples were placed in an ice bath at the end of each step. In the measurements obtained as a result of these procedures, a slight activation was observed. This result shows that an increase in temperature during the centrifugation process has a negative effect on activation, and this effect can be reduced by an ice bath.

Another reason for the low activation was the settling of the removal enzymes to the bottom, along with the biochar. To remedy this situation, a second filtration step using a herbal tea filter was used. After filtration, the samples were allowed to stabilize for a certain period of time, and measurements were taken. The results showed that higher activation levels were obtained in the filtration with the herbal tea filter, although not as much as decolorization by centrifugation.

These findings suggest that ice bath and intermittent centrifugation have positive effects on activation, reducing the negative effects of heating during the centrifugation process. It was also concluded that filtration using a herbal tea filter improved the activation efficiency by preventing spores from settling to the bottom. The combination of these methods resulted in more effective results for the removal and activation processes of biochar and coffee particles.

5.2 SUGGESTIONS FOR FUTURE RESEARCH

Several challenges were encountered during the course of these studies. To address these challenges, potential solutions have been hypothesized, which could inform and guide future research efforts. Recommendations based on the difficulties experienced are as follows:

•Removal of Metabolic Heat: Effective strategies are required to manage the metabolic heat generated during this process.

•Environmental Drying: Temperature-dependent drying of the environment poses a significant challenge and requires more controlled moisturisation methods.

•Insufficient Surface Area: The current design does not provide adequate surface area for these processes. The use of trays has been proposed to increase the surface area.

•Extended Testing: Testing the reactor over a longer period can yield more comprehensive data on its performance and reliability.

•Alternative Analytical Methods: The analytical method can be improved using a respirometer instead of spectrophotometry for more accurate measurements.

•Biochar Source Variability: Exploring different organic materials as biochar sources may enhance its effectiveness.

•Biochar Content Analysis: Conducting a more thorough analysis of biochar content can provide insights for optimizing its use.

•Sample Preparation: Washing and drying the biochar to remove the sample color can improve the clarity of the results.

•Adjusting Reactor Conditions: Modifying the reactor conditions, such as using different humidification methods, may improve the process outcomes.

•Biochar Pore Size: The age and storage conditions of biochar affect its pore size (Liu et al., 2021)Biochars with larger pores (0.5-5 micrometers, 500-50,000 nanometers) may retain enzyme molecules, complicating their extraction. Separation and purification methods can be used to address this issue.

• Enzyme Retention: The ability of biochar to retain enzymes can facilitate direct application, storage, and transfer, especially in agricultural applications, to improve soil phenolics.

These challenges and recommendations highlight the need for continued research and optimization of the reactor design to enhance the efficiency and effectiveness of bioprocesses.

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