PHYLOGENETIC ANALYSIS OF BACTERIAL CELLULOLYTIC ETHANOLOGENS FROM *COPTOTERMES HEIMI* (TERMITE)

SHAHEEN, A.[1] – AIHETASHAM, A.[1*] – CHAUDHARY, A.[2] – HUSSAIN, N.[3*] – AZIZ, T.[4] – ALHARBI, M.[5] – ALASMARI, A. F.[5]

[1]*Institute of Zoology, University of the Punjab, Lahore, Pakistan*

[2]*Department of Zoology, Division of Science and Technology, University of Education, Township, Lahore, Pakistan*

[3]*Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan*

[4]*Laboratory of Animal Health, Food Hygiene and Quality, University of Ioannina, Arta, Greece*

[5]*Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia*

[*]*Corresponding authors*
e-mail: ayesha.zool@pu.edu.pk; nageen.mmg@pu.edu.pk

(Received 1st Jun 2024; accepted 18th Oct 2024)

Abstract. Termites are a prospective source of cellulolytic enzymes because they have evolved symbiotic systems that efficiently break down lignocellulosic materials to produce bioethanol. The current study aimed to discover and screen bacteria from the hindgut of Pakistani wood-eating termite species. Characterization of the cellulolytic bacteria, assessment of the cellulolytic and ethanologenic potential were performed. *Coptotermes heimi* (termite) was collected, identified, and dissected for the culturing of gut related cellulolytic bacterial isolates with assigned codes ASC1-ASC25. The cellulolytic potential was assessed by Durham tube test, Congo red staining, and TTC. The highest cellulolytic and ethanologenic potential was detected in ASC21 as 1.83±0.01 µmol/min/mL and 6.70±0.01 g/L respectively. All bacterial isolates exhibited ethanol yield and fermentation efficiency within the range of 0.19-0.40 g/g and 37.35-79.19 % correspondingly. *Bacillus cereus sp.* ASC21 exhibited 0.40 g/g ethanol yield with 79.19% fermentation efficiency. The selected four strains were characterized molecularly as *Enterobacter cloacae sp.* ASC12, *Bacillus paramycoides sp.* ASC13, *Bacillus cereus sp.* ASC21 and *Staphylococcus sciuri sp.* ASC22. Technology for producing bioethanol will focus on properly disposing of fruit waste to lower health concerns address energy emergencies and produce affordable, environment friendly biofuel with financial advantages.
Keywords: *termite gut microbiota, cellulases, enzymatic hydrolysis, ethanologenesis, moleculer characterization*

Introduction

The most prevalent organic polymer in the world is cellulose which plants produce in about 4109 tonnes annually (Irfan et al., 2012; Chatterjee et al., 2015). The breakdown of cellulose is challenging because of its molecular complexity. To transform this natural component into energy resources, the limitations are typically pricy instrument intensive different chemical and physical treatment methods (Sari et al., 2016). It is essential to discover new approaches, strategies, and fields to overcome around all of the obstacles. One method required to increase the effectiveness and understanding of cellulosic bioethanol is the hydrolytic potential for cellulose breakdown. The abundance and enormous biomass production of lignocellulosic also contribute to environmental pollution. However, effective material use could lead to the production of a variety of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

goods including ethanol and biogas (methane) (Vandenbossche et al., 2014). These wastes are viewed as raw materials for competitive bioethanol production in the open market due to their quantity and renewability (Aziz et al., 2023; Mtui, 2009). Bioethanol production proceeded by following different saccharification techniques viz, chemical and enzymatic. Microbes harboring are considered a promising source to fulfill the enzymatic hydrolysis techniques. Termites of different strains were found to harbor cellulolytic bacteria. Among the destructive termites, *Coptotermes heimi* (Termite) is a subterranean termite species that is only found in India and Pakistan (Roonwal, 1959; Roonwal and Chhotani, 1962; Gay, 1967; Chaudhary and Ahmad, 1972). Pakistan's popular, live shisham (Indian rosewood) and morus(mulberry) trees have reportedly been damaged by it. Commonly found in India, *C. heimi* is a polyphagous species that preys on more than 35 distinct plant species (Roonwal, 1970). This species is a serious pest of forest trees and is especially common in Sindh and the Northwest Frontier Province of Pakistan. It also infests buildings and wooden constructions (Akhtar, 1983).

Termite gut exhibited a well-known diverse microbial flora that is involved in cellulose degradation. These termite gut bacteria can produce a variety of cellulolytic and ligninolyticenzymes that can digest the most abundant biological macromolecule. To produce ethanol as a sustainable biofuel, this study finds bacteria that are obtained from termite gut and have the potential of effectively breaking down cellulose into glucose residues. This strategy might make the production of biofuels more effective which would lessen reliance on petroleum and natural gas and help combat climate change (Russell et al., 2008). Three intestinal bacteria were identified in the subterranean termite *Coptotermes curvignathus* hindgut by Ramin et al. (2008). These bacteria were named *Enterobacter aerogenes*, *Clavibacter agropyri*, *E. cloacae* and their involvement in the cellulose breakdown was demonstrated. Microorganisms produce the inducible enzymes cellulases as they grow on cellulose substrates (Lee and Koo, 2001). Termites exhibited a termite-specific distribution pattern for five bacterial genera, including *Enterobacter*, *Citrobacter*, *Acinetobacter*, *Trabulsiella*, and *Kluyvera*. The other bacteria showed no such pattern. Furthermore, to assess the bacterial strains' potential for lignocellulose bioconversion, their lignocellulolytic capacity was assessed using agricultural waste (Shehzadi et al.,2024; Xie et al., 2023).

The digestive tract of an insect consists of the foregut, midgut, and hindgut although it has undergone numerous alterations due to the adaption of various feeding styles (Chapman et al., 2013; Engel and Moran, 2013). Numerous insects use lignocellulosic material as their main source of food, breaking down the complex polysaccharide with the help of gut bacteria that they already have there and then converting it into a glucose monomer molecule (Sun and Scharf, 2010). Enzymes produced by the cellulolytic bacteria in the termite gut break down cellulose into glucose, which may then be fermented to make ethanol. In addition to being necessary for the termite's sustenance, this bioconversion process may also be used to produce biofuel (Azhar et al., 2024). Termites have two cellulose-digesting mechanisms; one uses endogenous cellulases and the other symbiotic bacteria and protozoa cellulases. Both systems developed the effective digestion of lignocellulose, as do termite morphology and the large number of bacteria found in the gut. Fungi and termites have developed symbiotic interactions (Li et al., 2003). The metabolic need in higher termites is satisfied by endogenous cellulolytic activity. The hindgut of lower termites has significant cellulolytic activity. As a result, the endoglucanase derived from termites can partially break down the ingested cellulose. The unhydrolyzed cellulose then makes its way to the termites' hindgut where the symbiotic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

protists in lower termites endocytose and ferment it. Cellobiohydrolases target the cellulose polymer reducing and EGs hydrolyze the inner portions of the cellulose chain. From the termite gut a diverse range of bacteria and yeasts including hemicellulolytic and cellulolytic ones have been recovered (Prillinger et al., 1996; Schater et al., 1996; Wenzel, 2002). Additionally, it is anticipated that new microbes with a broad range of commercial uses will originate from the termite stomach (Tokuda et al., 2004). These enzymes create cellooligosaccharides that glucosidase further hydrolyzes to glucose (Tokuda et al., 2005). Reliable species-level identification is made possible by 16S rRNA sequencing, and this is essential for comprehending the variety of microbes in termites' guts and their functional involvement in the fermentation and breakdown of cellulose (Johnson et al., 2019). Further characterization was done on the crude cellulases that were generated by the most active cellulolytic bacteria. 32 cellulolytic bacteria were recovered, and their classification was done using the 16S rRNA gene PCR-RFLP method. Ten distinct RFLP patterns representing five different bacterial genera *Acinetobacter, Bacillus, Citrobacter, Paenibacillus,* and *Serratia* were obtained (Boontanom et al., 2021). The potential of bacterial co-cultures, namely *Bacillus cereus* and *Bacillus thuringiensis*, in simultaneous saccharification and co-fermentation (SSCF) processes has been investigated. These microorganisms are efficient at converting complex carbons into sugars that can ferment (Ire et al., 2016). Microbes ferment the sugars that are liberated during hydrolysis to create ethanol. In order to increase the output of ethanol, fermentation requires certain temperatures (usually between 30 and 40°C) and pH levels (between 6-7) (Shah et al., 2023; Promon et al., 2018).

The commercialization of cellulosic ethanol has advanced significantly, but there are still technological issues that need to be resolved. The rate-limiting substrate for the generation of bioethanol is now understood to be cellulose and to overcome cellulose's resistance to biodegradation, new more effective enzymes are needed (Yu et al., 2023). Developing thermophilic microbial groups that can generate powerful enzyme systems with high hydrolytic potential for cellulose degradation is one of the many useful techniques needed to improve the efficiency and comprehension of cellulosic bioethanol (Zambare et al., 2011). The manufacturing of fuel ethanol has gained attention recently as several nations seek to decrease their reliance on foreign oil while also boosting rural economies and air quality. The amount of ethanol produced worldwide has topped 51,000 million liters. The conversion of biomass into fuel ethanol has recently received interest because it is predicted that fossil fuels will run out within the next few decades (Arumugam and Manikandan, 2011).

The objectives of the current study focused on identifying and screening the cellulolytic potential of bacterium from the hindgut of wood-feeding termite species *Coptotermes heimi* in Pakistan. The cellulolytic bacterial characterization, evaluation of cellulase assay, and ethanologenic potential were carried out to understand and confirmation of cellulase application in different bio-applications. *Coptotermes heimi* species of termite prevalent in Pakistan is known for causing significant destruction to both natural and man-made structures. Biofuel manufacturing methods can benefit from the use of termite gut bacteria's cellulases. These enzymes help turn agricultural waste into bioethanol, a renewable energy source, by dissolving lignocellulosic biomass into fermentable sugars. These microorganisms are appropriate for waste management applications because of their capacity to break down complex organic compounds. In order to increase soil health and nutrient recycling, they can be used in composting operations to accelerate the decomposition of plant leftovers and other lignocellulosic wastes.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Materials and methods

Termite sampling for isolation of bacteria

The habitat of *Coptotermes heimi* is rotting wood, tree stumps, and wooden constructions where it frequently builds elaborate tunnel networks that span more than 100 meters. These underground passageways are vital for their foraging activity and protection from environmental extremes. Termites were collected from decomposing logs having tunnels formed in shisham and mulberry trees from different areas of Kasur, Pakistan in July 2021 (31°185′ N, 74°485′ E). Three colonies per site were collected. Twenty-five insects from three colonies were identified as workers and soldiers selected for dissection. The temperature of the site was noted as 45+1.00 C with 52+1% humidity. To ensure proper termite survival and activeness in the laboratory, moderate temperatures (20–30°C) and high humidity (>60%) were maintained which is the basic requirement for termite survival.

Termites eat the bark and soft sections of sugarcane at tree bases in addition to the stems. By baiting termites with the most appetizing wood of *P. euramericana* a hybrid poplar tree together with sugarcane stalks, termites were attracted. The greatest amount of termites possible for the investigation were collected using this technique. Termites are abundant in their native environments and are known to feed on materials high in cellulose such as wood and plant fibers One frequent technique for gathering termites for study is to entice them with wood and sugarcane stalks. The form and size of the mandibles and head enabled the morphological identification of the termite samples. Termites were brought to the lab for identification using a key after being gathered from various sources (Ahmad, 1955; Akhtar, 1975). After sanitizing surfaces with 70% ethyl alcohol 25 termites were sampled and kept in sterile plastic bags at 4°C. For the isolation of bacteria within 48 hours, termites were dissected. Insects will be sterilized by rinsing in 70% ethyl alcohol which will remove the surface microbes.

Twenty-five termite guts were removed using sterile tweezers and sanitized for thirty seconds in 70% alcohol. After that, it dried for one minute after rinsing with sterile distilled water. When the termite was dissected the whole digestive tract was removed and crushed to create a suspension. Suspension was made by crushing the gut in sterilized 0.9% Phosphate Buffer Saline (PBS). Phosphate Buffer Saline (PBS) was prepared by mixing (g/L) Na_2HPO_4 1.44, KCl 0.2, NaCl 8, KH_2PO_4 0.24 by adjusting pH at 7.4 gut homogenized in 2 ml sterilized 0.9% buffer (PBS). For one day the suspension (0.1 ml) was plated on Carboxymethyl cellulose (CMC) enriched media at 37 ±1°C. From the gut contents of termites spread plate method. A Spread plate was adopted for bacterial strain isolation. The suspension (0.1 mL) was taken and spread on CMC (Carboxymethyl cellulose) agar media for 2 days at 37°C. Agar media was prepared by mixing different chemicals. The composition of media involves cellulose 2 g, peptone 1.5 g, NH_4SO_4 0.1 g, KH_2 PO_4 0.1 g, $MgSO_4$ 0.05 g, Yeast extract 1 g, Agar 2 g, $CaCl_2$ 0.1 g by dissolving in of distilled water (100 ml) (Zhang et al., 2014a). It could be considered that CMC, peptone, and yeast extract are the main sources of carbon for proficient fermentation (Pan et al., 2025). The medium was taken in a 250 ml flask and sterilized at 120°C for 15 minutes and then incubated for 24 hours to check contamination by pouring in petri plates. For 24-hours petri plates were incubated at 37°C. On the plate many bacterial colonies emerged. Various bacterial isolates were chosen according to their colony form, size, and color. To get pure culture the chosen colonies were cultured on solid media.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

Analyzing sugar breakdown biochemically

Fermentation efficiency and ability to degrade sugar were evaluated by various biochemical tests. The gas generation in Durham tubes suggests that the isolated bacterium is most likely a lactose fermenter based on the information supplied. Following congo red staining, the clear zones show that the bacteria can degrade the dye suggesting the existence of active enzymes. Since TTC is a redox indicator and changes color when it comes into contact with metabolically active cells its maroon hue lends even more credence to the concept of active metabolism. The liquid media was prepared by mixing the chemicals. The composition of media involved peptone 1.5 g, Yeast extract 1 g, NH_4SO_4 0.1 g, KH_2PO_4 0.1 g, $MgSO_4$ 0.05 g, and cellulose 2 g and dissolved in 100 ml distilled water. In the test tubes (10 ml) and Durham tubes (2 mL) medium was dispensed. Then in a medium containing test tubes (filled with liquid media followed by inoculation of bacterial isolates, Durham tubes were placed inversely. The same medium without bacterial inoculation served as control. No bubble formation was noted in a control whereas gas generation in bacterial inoculated tubes was evident as a positive test by bubble formation in Durham tubes. In order to detect gas generation from bacterial fermentation this procedure most likely aimed to sterilize the medium and produce an anaerobic condition inside the Durham tube. Durham tubes are frequently used to measure the gas that bacteria produce during fermentation. In this instance, several bacterial strains were added to test tubes holding Durham tubes and the tubes were then cultured for 10 days at 37°C without being shaken. Every day gas development was monitored by searching the Durham tubes for bubbles (Yarrow, 1998).

Congo red dye can be used to assess bacterial strains capacity to degrade carbohydrate polymers like cellulose. Under a microscope, the dye Congo red appears as a reddish-orange complex after binding to cellulose (Zhang et al., 2006). The medium containing peptone 1.5 g, cellulose 2 g, NH_4 SO_4 0.1 g, KH $_2PO_4$ 0.1 g, $MgSO_4$ 0.05 g, Yeast extract 1 g, Agar 2 g, $CaCl_2$ 0.1 g was dissolved distilled water (100 ml) (Zhang et al., 2014b). The medium was taken in a 250 ml flask sterilized for 15 minutes at 121℃ and poured into sterilized petri plates. Different selected bacteria were inoculated on sterilized petri plates and were incubated at 37℃ for 16 hours. To make the Congo red stain 1.0 g congo red o dissolve in 100 ml of distilled water in a reagent bottle and shake it for 15 minutes. After flooding 1% Congo red stain onto petri plates containing bacterial growth they were incubated for 30 minutes at 37°C. Extra stain was drained off after incubation and destained with 1% NaCl solution. To stop the unbound region, the petri plates were incubated for a further 30 minutes at 37°C. For distinct results, the same procedure was performed three to four times. The breakdown of cellulose was shown by the clear zones surrounding the yeast isolates. The diameters of clear zones were measured and the cellulolytic index was computed by

Following expression (*Eq. 1*) (Demissie et al., 2024).

$$cellulytic\ index = \frac{Diameter\ of\ zone - diameter\ of\ colony}{Diameter\ of\ bacterial\ colony} \quad \text{(Eq.1)}$$

A sample of the same substrate (agar) without microbial inoculation was prepared as a control. No cellulose breakdown was noted in the control whereas cellulose breakdown occured in bacterial inoculated petri plates was evident as a positive test by Congo red stain.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

The composition of media involved cellulose 2 g, peptone 1.5 g, peptone 1.5 g, NH_4 SO_4 0.1 g, KH $_2PO_4$ 0.1 g, $MgSO_4$ 0.05 g, Yeast extract 1 g, Agar 2 g, $CaCl_2$ 0.1 g and was dissolved in distilled water (100 ml). The medium was placed in a 250 ml flask and allowed to sterilize for 15 minutes at 121℃. To make the TTC solution 10 ml of distilled water was autoclaved at 121℃ for 15 minutes. After that, 10 ml of distilled water that had been sanitized was mixed with 0.05 g of TTC. After mixing the media and TTC solution the mixture was added to sterile petri plates. Petri plates containing TTC medium was streaked with one colony and the plates were then incubated at 37°C for 24 hours. The colony color was noted to be maroon to pink. Tetrazolium salt solution without microbial inoculation was prepared. No change in colony color was noted in control color change in inoculated petri plates evident as a positive test by TTC.

Bacterial isolates screening based on morphology

Different bacteria showed different attributes for growth on media. The attributes were evident in the form of colony characteristics. Colony characteristics not only helped to identify different bacterial isolates but also facilitated the understanding of microbial functions or potential applications. The color of the colony manifested different characteristics i.e., the production of pigments, texture in the form of granular or agranular, and optical features as opaque/transparent. In the current study, the variety of shape, color, and texture of well-isolated colonies were found on cellulose supplemented medium which preferentially helped to identify different strains by correlating morphological differences observed with functional differences (metabolic capabilities) and by allowing to exploration a broader range of biochemical activities among the isolates. Sterilized petri plates were filled with the medium (used to screen bacterial isolates) which was prepared into 100 ml of distilled water and autoclaved. Bacterial Strains were streaked after incubation at 37°C for 24 hours and several colonial properties such as size, color, texture, and optical aspects of the colony were studied. The colonial and microscopic characteristics were studied based on protocols mentioned in Benson, 1984. Gram's reaction was performed for cellular characteristics. Using ocular and stage micrometers the size of the stained bacterial cells was determined by micrometry. A thorough investigation of microbial variety and potential functional (gas generation or enzymatic activity) applications is made easier by the selection of 25 different strains.

Fermentation experiments

The synthetic medium was prepared with little modification by mixing different minerals. The composition involved 2% CMC, enriched medium. Synthetic medium contained (g/L) yeast extract 6.5 g, CMC 20 $(NH_4)_2SO_4$ 2.6 g, KH_2 PO_4 2.72 g, $MgSO_4.7H_2O$ g, $CaCl_2$ 0.3 g, $ZnCl_2$ 0.00042 g, citric acid 1.5 g, sodium citrate 6 g by dissolving in 1 liter of distilled water (Bonicus et al., 2010). The mixture was dispensed in narrow-necked bottle (250 ml) and sterilized for 15 minutes at 120℃.

To start the growth of bacteria an inoculum was created which also served as a culture medium. The inoculum involved cellulose 1 g peptone 0.7 g, NH_4 SO_4 0.05 g, KH_2 PO_4 0.005 g, $MgSO_4$ 0.0.25 g, Yeast extract 0.5 g, $CaCl_2$ 0.05 g in distilled water (500 ml). The inoculum was sterilized at 121℃ for 15 minutes. Following sterilization, a selection of bacterial strains were added to the medium and it was shaken at 37°C for an entire night to produce new inoculum. A bacterial culture containing 1 ml was added to the synthetic medium along with cellulose. For ten days the medium was incubated at 30°C in a shaking incubator. By taking samples every day the decreasing and ethanol levels

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

were estimated. The ethanol contents were used to calculate the ethanol yield and fermentation efficiency (the ability of yeast strain to respond over stress conditions leading to the end product) in the fermentation medium. Spectrophotometrically bacterial growth was measured at 600 nm. Ethanol and fermentation efficiency were computed by following expressions (*Eq. 2, Eq. 3*) (Azhar et al., 2024).

$$\text{Ethanol yield (g/g)} = \frac{Ethanol\ produced\ (\frac{g}{L})}{Reducing\ sugar\ consumed\ (\frac{g}{L})} \quad \text{(Eq.2)}$$

$$\text{Fermentation efficiency} = \frac{Practical\ ethanol\ yield}{theoretical\ ethanol\ yield} \times 100 \quad \text{(Eq.3)}$$

Practical yield refers to the actual amount of ethanol contents produced during fermentation under specific conditions. Theoreticle yield was calculated following the concept that one mole of glucose (180 g) produces two moles of ethanol (92 g) and was referred to as 0.51 g/g (92 g/180 g).

The acid dichromate test which usually consists of sulfuric acid and potassium dichromate is used to oxidize ethanol to acetic acid which is subsequently converted to carbon dioxide and water. To create the acid dichromate solution 5M sulfuric acid is mixed with 7.5 grams of potassium dichromate. Deionized water is then added to the mixture to dilute it until the volume is 250 ml. After that, the mixture is well-mixed to guarantee that all of the potassium dichromate has dissolved and that the mixture is uniform. A sample of fermented media (0.3 ml) is usually obtained and utilized in the test to quantify the amount of ethanol present in the sample. Afterward ethanol solutions with various concentrations were used to generate a standard curve (300 µl, 200 µl, 150 µl, 100 µl, 50 µl, 0 µl) in test tubes, and acid dichromate (3 ml) solution was added. After sealing them with aluminum foil they are usually left to rest for a while for 30 minutes at room temperature. This gives the color change response enough time to complete and stabilize before the measuring absorbance. At 590 nm, the absorbance was determined using a spectrophotometer.

Using the DNS method, the sugar contents were estimated (Miller, 1959). Three test tubes are usually set up for the test experimental sample, the control sample, and the blank. In each test tube 1 ml of distilled water and 0.5ml of culture media are added to each tube, individually and then 1ml of DNS reagent was added. For five minutes, the mixture was heated to 100°C to create the DNS color. Usually, 2 M NaOH solution (25 mL) is used to dissolve 1.5 g of dinitrosalicylic acid in order to make the DNS reagent. To prepare DNS in 75 mL distilled water 45 g of sodium potassium tartrate was dissolved in a different flask. Following this mixing the two solutions combine to generate the DNS reagent. The absorbance was measured after cooling in a cold water bath with a spectrophotometer at 540 nm. Preparing of sample dilutions precisely six dilutions with concentrations between 100 and 600 µg/ml. The control solution in the blank tube is devoid of any sample material. All the tubes including blank were proceeded in the same way as described previously.

Activity of cellulolysis

The base medium for the production of crude enzymes includes (g/L) CMC 2 and maintained pH 7.0 using the following parameters: sodium citrate 0.05 g/L, yeast extract 0.1, magnesium sulfate 0.01, disodium hydrogen phosphate 0.7, potassium dihydrogen

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

phosphate 0.2 (Bai et al., 2012). The inoculated basal medium was agitated at 200 rpm 37 ±1°C for 72 hours (Abu-Gharbiya et al., 2018). Incubated cultural media centrifugation (15 min) was done at 1000 rpm. After centrifugation (1000 rpm) for 15 minutes supernatants served as a crude enzyme. A Substrate buffer for the cellulolytic test was made by combining 2% CMC with 0.2 M acetate buffer (pH 5.0). The components of 0.2 M acetate buffer were sodium acetate trihydrate 54.43 g/L and glacial acetic acid 12 ml. For cellulolysis assay substrate buffer (1.0 mL) was mixed with a crude enzymes (0.5). Kept the enzyme mixture at 50 °C for 30 minutes. The process of measuring reducing sugars involved adding 3 mL of DNS reagent and heating it for 5 minutes in a water bath. At 640 nm in color change was detected using spectrophotometry. The transformation of cellulose into monomeric sugars was revealed by the change in hue of the DNS reagent (Miller, 1960). The following (*Eq. 4*) was used to compute the cellulolytic potential (Azhar et al., 2024).

$$Enzymatic\ activity\ (\mu mol//mL) = \frac{Sample\ O.D. \times\ Standard\ factor\ (10.64) \times\ 1000\ \times\ Reaction\ volume\ (1.5\ mL)}{Moleculer\ weight\ (150.13) \times Total\ crude\ enzyme\ (0.5\ mL) \times Incubation\ time\ (30\ min)} \quad \text{(Eq.4)}$$

Characterization of specific bacterial isolates using molecular techniques

Four isolates ASC12, ASC13, ASC21, and ASC22 were selected for molecular characterization based on significant ethanol titer and cellulolytic efficiency. The Range of ethanolic contents and celluloytic potential were noted as 6.05±0.02-6.70±0.01 g/L, 1.52±0.02-1.83±0.01 umol. The high cellulytic potential of selected bacterial isolates is displayed in the form of increased reducing sugars as the isolates can convert the polymeric cellulose into glucose. Similarly, the released reducing sugars are converted into ethanol as an end product that is manifested as a high ethanol titer. A phylogenetic study of bacterial isolates ASC12, ASC13, ASC21, and ASC22 in light of 16rRNA gene sequence similarity this makes it possible to identify the isolate's nearest relatives or phylogenetic neighbors. Selected isolates were *Enterobacter cloacae sp.* ASC12, *Bacillus paramycoides sp.* ASC13, *Bacillus cereus sp.* ASC21, *Staphylococcus sciuri sp.* ASC22. The sequenced genes were added as Accession No. SUB14105933 ASC12, SUB14105933 ASC13, SUB14105933 ASC21, and SUB14128643 ASC22 to GenBank in the NCBI database.

Phylogenetic characterization of efficient bacterial isolates

Bacterial isolates ASC12, ASC13, ASC21 and ASC22 were selected for 16S rRNA based molecular analysis. Gene JET Genomic DNA Purification Kit (catalog number K0721) is used for the extraction of DNA Nanodrop quantification of DNA was performed by a Multiscan microplate reader by ThermoFisher Scientific to assess purity. Targeted amplification of amplicon (s) was achieved by utilizing target-specific primers with AmpliTaq Gold Mater Mix (Catalogue number: 4390939) to PCR product and the quality of PCR product was determined by agarose gel electrophoresis. The product of PCR was then purified by Exo SAP-IT™ PCR Product Clean-up Reagent (Catalogue number: 78200.200). Cycle sequencing was performed by using BigDye™ Terminator v3.1 Cycle Sequencing Kit for 25 cycles by using a single primer followed by Capillary Electrophoresis. The cycle sequencing product was later purified by Ethanol/EDTA precipitation to remove unincorporated terminators and finally suspended into HiDi

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Formamide to be resolved by Capillary Electrophoresis on Seq Studio Genetic Analyzer 3200 by Applied Biosystems. From bacterial isolates, the amplified gene was sequenced and its BLAST homology query was made by Lab Genetix, Lahore, Pakistan, using the Genbank database. (http://www.ncbi.nlm.nih.gov/blast). Accession numbers for four isolates were obtained.

Statistical evaluation of data

A general linear model using Tukey's post-hoc test with a 95.0% confidence level was executed by Minitab 16 for evaluation of significance differences from the outcomes of different bacterial isolates for ethanologensis. The effect of these factors was declared significant if $P<0.05$. Tukey's post-hoc test was used if there were more than two groups to compare for their significant differences at $P<0.05$.

Results

Collection of sample

On 13th July 20121 Sample was collected from multiple areas of Kasur, Pakistan on 13th July 2021 from wood destructive logs (*Figure 1*). The termites were collected and identified as *Coptotermes heimi* due to red brown colored mandibles and pale brown oval heads. The labrum is subtriangular with a pointed apex.

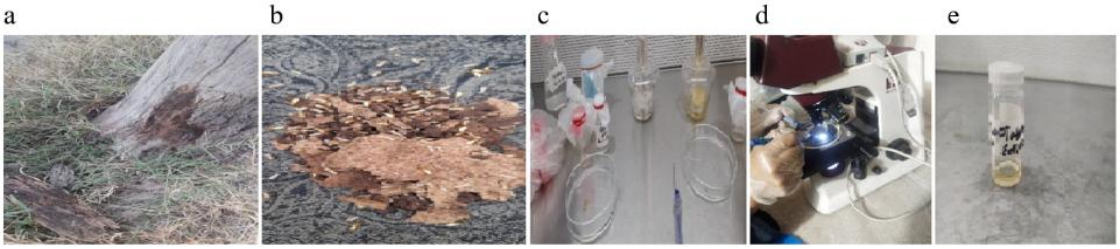


Figure 1. *Coptotermes heimi, Collection of termite (a) Termite isolation (b, c), dissection of termite for gut isolation (d), gut extract (e)*

Termite sample collection for microbial screening

Using the morphological codes ASC1–ASC25 the prefix twenty-five bacterial strains were identified from termite gut extract.

From the termite gut extract 25 microbial isolates were selected for pure culture having the capacity to degrade the cellulose substrate as a sole source of carbon. To get pure cultures all extracted bacteria were cultivated on a medium supplemented with 2% cellulose. The resulting glycerol stock was then stored for future research.

Colonial characterization morphologically

On cellulose-enriched media, the colony characteristics of the bacterial isolates were noted, including their color, size, elevation, texture, margin, optical features, consistency, and pigmentation (*Table 1*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Table 1. *Colonial characteristics of microbial isolates after streaking on agar media enriched with cellulose*

Sr. No.	Isolated Bacteria	Size	Color of Colony	Margin	Surface Texture	Elevation	Optical feature	Pigmentation	Consistency
1	ASC1	Pinpoint	White	Entire	Smooth	Dome	Opaque	No	Buttery
2	ASC2	1 mm	White	Entire	Granules	Flat	Opaque	No	Brittle
3	ASC3	Pinpoint	Off white	Entire	Granules	Flat	Opaque	No	Viscous
4	ASC4	Pinpoint	White	Undulate	Granules	Spreading	Opaque	Yes	Brittle
5	ASC5	2mm	White	Entire	Smooth	Flat	Opaque	No	Buttery
6	ASC6	Pinpoint	White	Undulate	Granules	Dome	Opaque	No	Brittle
7	ASC7	2mm	Creamy	Entire	Granules	Raised	Opaque	No	Brittle
8	ASC8	3mm	Off white	Entire	Smooth	Flat	No	No	Viscous
9	ASC9	Pinpoint	Off white	Entire	Smooth	Flat	No	No	Brittle
10	ASC10	3mm	Creamy white	Undulate	Granules	Dome	No	No	Viscous
11	ASC11	Pinpoint	Off white	Entire	Smooth	Dome	No	No	Buttery
12	ASC12	2mm	Creamy white	Entire	Granules	Convex	No	No	Brittle
13	ASC13	3mm	White	Undulate	Granules	Concave	No	Yes	Viscous
14	ASC14	2mm	White	Entire	Smooth	Dome	Transparent	Yes	Brittle
15	ASC15	2.5mm	Off white	Entire	Smooth	Convex	No	Yes	Brittle
16	ASC16	1mm	White	Entire	Granules	Dome	No	No	Brittle
17	ASC17	Pinpoint	White	Entire	Smooth	Dome	No	No	Viscous
18	ASC18	2mm	Off white	Undulate	Smooth	Concave	No	No	Buttery
19	ASC19	Pinpoint	Creamy white	Undulate	Granules	Dome	No	No	Brittle
20	ASC20	2mm	White	Entire	Smooth	Flat	No	No	Brittle
21	ASC21	1mm	Off white	Irregular	Granules	Spreading	No	No	Brittle
22	ASC22	Pinpoint	White	Entire	Granules	Flat	No	No	Brittle
23	ASC23	2mm	Creamy white	Entire	Smooth	Dome	No	Yes	Viscous
24	ASC24	1.5mm	Off white	Undulate	Smooth	Flat	No	No	Viscous
25	ASC25	Pinpoint	White	Entire	Granules	Flat	No	No	Buttery

Bacterium isolated colonies from termite gut extract (ASC1, ASC3, ASC4, ASC6, ASC9, ASC11, ASC17, ASC19, ASC22, ASC25) was pinpoint. The colonies of ASC2, ASC16, ASC21 were 1 mm in size and ASC5, ASC7, ASC12, ASC14, ASC18, ASC20, ASC23, ASC8, ASC10, ASC13 were 2 mm, ASC24 1.5 mm and ASC15 was 2.5 in size. The colors of colonies were white (ASC1, ASC2, ASC4, ASC5, ASC6, ASC13, ASC14, ASC16, ASC17, ASC20, ASC22, ASC2), off white (ASC3, ASC8, ASC9, ASC11, ASC15, ASC18, ASC21, ASC24). The ASC10, ASC12, ASC19, ASC23 was creamy white and ASC7 creamy color. Margin of ASC1, ASC2, ASC3, ASC5, ASC7, ASC8, ASC9, ASC11, ASC14, ASC15, ASC16, ASC17, ASC20, ASC22, ASC23, ASC25 and ASC12 was (entire). ASC4, ASC6, ASC10, ASC13, ASC18, ASC19, ASC24 (undulate) and ASC21 (irregular). Texture of ASC1, ASC5, ASC8, ASC9, ASC11, ASC14, ASC15, ASC17, ASC18, ASC20 were smooth and others granules. Elevation of ASC18, ASC13 and ASC12, ASC15 were convex. While ASC7 raised. ASC1, ASC6, ASC10, ASC11, ASC14, ASC16, ASC17, ASC19, ASC23 have dome like elevation while ASC4, ASC21 were spreading others flat. Isolates of ASC1 to ASC7 are opaque while ASC14 was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

transparent. ASC4, ASC13, ASC14, ASC15 secret pigmentation and others have no pigmentation. Consistency of ASC1, ASC5, ASC11, ASC18, ASC25 were buttery while ASC3, ASC8, ASC10, ASC13, ASC17, ASC23, ASC24 viscous others brittle.

Bacterial isolates microscopic characteristics

By cultivating on a medium enriched with cellulose the microscopic cell properties of several bacterial strains were examined. These attributes include the kind, size, and shape of the cell as demonstrated (*Table 2*).

Table 2. *Microscopic features of the bacteria incubated (37°C) over a cellulose enriched medium*

Sr. No.	Isolated bacteria	Gram staining	Length (µm)	Diameter (µm)	Shape
1	ASC1	Gram-positive	4.2	2	Diplococci
2	ASC2	Gram-negative	5	2 .7	Rods with pointed ends
3	ASC3	Gram-positive	2	1.5	Short rod
4	ASC4	Gram-negative	5	2.5	Long rod
5	ASC5	Gram-negative	2	1	Diplococcic
6	ASC6	Gram-positive	2.3	1.5	Signal rod
7	ASC7	Gram-positive	3	1.5	Spiral
8	ASC8	Gram-negative	2	1	Single rod
9	ASC9	Gram-positive	3	2	Coccus
10	ASC10	Gram-positive	2	0.5	Single rod
11	ASC11	Gram-negative	4	2.3	Cluster coccus
12	ASC12	Gram-negative	2	1.5	Diplococci
13	ASC13	Gram-positive	2.2	0.56	Coccus
14	ASC14	Gram-negative	3	1.49	Cluster coccus
15	ASC15	Gram-Negative	4	2	Rods with oval shape
16	ASC16	Gram-positive	2	1	Spiral
17	ASC17	Gram-positive	5	2	Long rods
18	ASC18	Gram-negative	5	2.9	Rods with pointed ends
19	ASC19	Gram-negative	5	1.89	Long rods
20	ASC20	Gram-positive	3	1	Spiral
21	ASC21	Gram-positive	5	3	Slightly smaller and curved rod
22	ASC22	Gram-positive	3.5	1.2	Signal rod
23	ASC23	Gram-positive	3.2	2.1	Coccus
24	ASC24	Gram-positive	5.2	2.5	Long rods
25	ASC25	Gram-positive	3.8	2.2	Short rods

Every bacterial strain were rod like shaped including short rods with pointed or oval ends), long rods with the exception of ASC1, ASC5, (diplococcic) ASC9, ASC13, ASC23, (Coccus), and ASC7, ASC16, ASC20 (spiral). Short rods and long rods with pointed or oval ends were also present. Spiral bacteria range in size from 2×0.5 µm to 5×3 µm. ASC21 had the largest size measured, 5 x 3 µm, while ASC10 had the smallest size, 2 x 0.5 µm. The size of spiral bacteria ranged from 2×1 µm, with one or two twists. While ASC2, ASC4, ASC5, ASC8, ASC11, ASC12, ASASC14, ASC15, ASC18, and ASC19 were Gram-negative bacterial strains, all other strains were Gram-positive.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Analysis of cellulose hydrolyzing potential for bacterial isolates

In this work, a range of biochemical assays, including gas generation in Durham tubes and hallow formation using Congo red staining and color development on TTC, were used to evaluate the utilization of cellulose by bacterial strains. Results of the cellulose degradation were noted in *Table 3*. On days 2-4, bacterial isolates viz. ASC1, ASC2, ASC10, ASC12, ASC13, ASC14, ASC19, ASC21, ASC22 and ASC25 produced varying-sized bubbles, measuring 0.3–4 cm. While ASC3 and ASC4 created a light pink color indicated by TTC, all bacteria produced a maroon color to represent the maximum degradation of cellulose. Clear zones were formed by bacterial isolates using Congo red staining. Congo red staining revealed a distinct zone as a positive reaction in six bacterial isolates and a negative response in 19. The results showed that the highest and lowest cellulolytic indices were 3.0 (ASC21) and 0.17 (ASC18).

Table 3. *Evaluation of cellulolytic activity by various bacterial strains biochemically*

Sr. No.	Isolated bacteria	Bubbles' formation in Durham tubes	Color development (TTC)	Clear zones by Congo red staining (mm)	Bacterial colonies diameter	Cellulolytic Index
1	ASC1	2nd day (0.1cm)	Maroon	0.71	0.4	0.77
2	ASC2	3rd day	Maroon	0.58	0.32	0.81
3	ASC3	No	Light pink	-	0.2	0.0
4	ASC4	No	Light pink	0.4	0.3	0.33
5	ASC5	No	Maroon	-	2.3	0.0
6	ASC6	No	Maroon	-	4.2	0.0
7	ASC7	4rth day	Maroon	0.52	0.41	0.26
8	ASC8	No	Maroon	-	2.0	0.0
9	ASC9	No	Maroon	0.43	0.22	0.21
10	ASC10	2nd day (0.1cm)	Maroon	1.25	1.0	0.25
11	ASC11	2nd day (1.5cm)	Maroon	-	3.5	0.0
12	ASC12	2nd day (2.5cm)	Maroon	3cm	2.0	0.5
13	ASC13	3rd day(1.0cm)	Maroon	2cm	0.6	2.33
14	ASC14	2nd day (0.1cm)	Maroon	0.3	0.2	0.5
15	ASC15	No	Maroon	0.62	0.3	1.06
16	ASC16	No	Maroon	0.43	0.26	0.65
17	ASC17	No	Maroon	0.82	0.43	0.91
18	ASC18	No	Maroon	0.67	0.57	0.17
19	ASC19	2nd day (0.1cm)	Maroon	0.2	0.1	1.0
20	ASC20	No	Maroon	0.5	0.2	1.5
21	ASC21	2nd day (1cm)	Maroon	4	1.0	3.0
22	ASC22	2nd day (2cm)	Maroon	3	1.5	1.0
23	ASC23	No	Maroon	0.4cm	0.2	1.0
24	ASC24	No	Maroon	-	0.1	0.0
25	ASC25	2nd day (0.2cm)	Maroon	0.4cm	0.2	1.0

Cellulolytic index = (clear zone diameter−bacterial colonies diameters)/ Bacterial colonies diameter

The study used qualitative analysis for efficient bacterial strains according to the activity of their cellulase. A total of 12 isolates were selected for further analysis. The potential of these strains to break down cellulose into glucose was used to evaluate their cellulolytic activity in *Table 4*. The amount of glucose generated per minute per milliliter

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

(µmol/min/ml) of culture supernatant was used for assessing this activity. The isolates cellulolytic activity varied between 1.30±0.03 µmol/min/ml (isolate ASC8) and 1.83±0.01 µmol/min/ml (isolate ASC21). ASC12 and ASC21 showed the best potential for cellulolysis with isolate ASC21 displaying the highest level of activity. This indicates that these isolates have the potential for more investigation and possible use in the mechanisms involved in the breakdown of cellulose.

Table 4. *Different bacterial isolates cellulolytic potential estimation to hydrolyze cellulose with their standard error of means (SEM) and significance of different groups*

Sr. No.	Bacterial isolates	Enzymes activity (µmol/min/ml)
1	ASC1	1.65 ± 0.0^{a}
2	ASC2	1.54 ± 0.02^{hi}
3	ASC3	1.66 ± 0.01^{a}
4	ASC4	1.79 ± 0.02^{h}
5	ASC5	1.56 ± 0.01^{ef}
6	ASC6	$1.56\pm0.01^{h-j}$
7	ASC7	1.67 ± 0.02^{c}
8	ASC8	1.30 ± 0.03^{fg}
9	ASC9	$1.55\pm0.01^{h-j}$
10	ASC10	1.52 ± 0.02^{ab}
11	ASC11	$1.41\pm0.01^{h-j}$
12	ASC12	1.83 ± 0.01^{e}
13	ASC13	1.65 ± 0.01^{c}
14	ASC14	1.54 ± 0.02^{bc}
15	ASC15	1.55 ± 0.01^{bc}
16	ASC16	$1.79\pm0.02^{h-j}$
17	ASC17	$1.56\pm0.01^{h-j}$
18	ASC18	1.56 ± 0.01^{e}
19	ASC19	1.67 ± 0.02^{c}
20	ASC20	1.30 ± 0.03^{k}
21	ASC21	1.83 ± 0.01^{d}
22	ASC22	1.52 ± 0.02^{bc}
23	ASC23	1.41 ± 0.01^{d}
24	ASC24	1.44 ± 0.01^{k}
25	ASC25	1.44 ± 0.01^{k}

A significant difference was recorded by different letters at $p\leq 0.05$ by single factor ANOVA

Ethanol production by cellulose

Information connected with ethanol titer after choosing isolates was kept in *Table 5*, *Figure 2*. Greatest ethanol was delivered in various days by various isolates. Most elevated ethanol (g/L) 6.70 ± 0.01^{a} was created by ASC21 on seventh day of brooding followed by decline in contents. In different strains, the greatest qualities were seen between days 7-8 and that was the sign of ethanol bearance by different bacteria.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Table 5. *Ethanol titer (g/L) from Cellulose by different bacterial isolates*

Days	ASC1	ASC2	ASC10	ASC12	ASC13	ASC14	ASC19	ASC21	ASC22	ASC25
1	1.42±0.04^{at-au}	1.47±0.04^{at-au}	2.12±0.23^{ao-as}	2.13±0.27^{an-ar}	1.89±0.04^{aq-au}	1.40±0.06^{au}	1.53±0.06^{as-au}	1.96±0.17^{ap-au}	1.73±0.04^{ar-au}	1.50±0.06^{at-au}
2	1.98±0.05^{ap-au}	2.37±0.18^{aj-aq}	2.76±0.03^{ag-ah}	2.64±0.10^{ag-ao}	2.52±0.05^{ah-ap}	2.15±0.03^{an-ar}	1.69±0.04^{ar-au}	2.00±0.07^{ae-am}	2.32±0.03^{ap-at}	2.84±0.05^{ac-ak}
3	2.18±0.07^{am-ar}	2.99±0.03^{aa-ai}	3.18±0.03^{w-ag}	2.98±0.14^{aa-ai}	2.94±0.08^{aa-aj}	2.72±0.18^{af-an}	2.21±0.03^{am-ar}	2.24±0.06^{al-ar}	2.82±0.03^{ac-al}	3.39±0.16^{v-ac}
4	2.88±0.05^{ab-ak}	3.08±0.07^{z-ah}	3.34±0.15^{v-ae}	3.31±0.02^{v-af}	3.00±0.07^{aa-ai}	3.38±0.12^{v-ac}	3.11±0.12^{x-ag}	4.18±0.03^{l-s}	4.13±0.06^{m-s}	3.60±0.05^{s-z}
5	3.07±0.02^{z-ai}	3.79±0.11^{q-v}	4.61±0.14^{i-m}	3.79±0.13^{q-v}	4.76±0.08^{i-l}	3.70±0.01^{r-x}	3.15±0.06^{x-ag}	5.46±0.14^{efg}	4.63±0.10^{i-m}	3.68±0.07^{z-ad}
6	3.09±0.07^{y-ah}	4.80±0.06^{h-k}	5.08±0.25^{g-hi}	4.84±0.08^{H-K}	5.39±0.11^{e-g}	4.14±0.02^{m-s}	3.53±0.10^{t-aa}	6.34±0.03^{abc}	4.67±0.11^{i-m}	4.19±0.09^{l-r}
7	3.35±0.01^{v-ad}	4.39±0.10^{j-p}	5.16±0.06^{ghi}	6.08±0.04^{bcd}	6.42±0.05^{ab}	5.82±0.05^{c-f}	4.60±0.25^{i-n}	6.70±0.01^{a}	6.05±0.02^{b-e}	4.96±0.03^{g-J}
8	3.69±0.01^{r-x}	4.30±0.10^{k-q}	3.67±0.14^{r-y}	4.59±0.07^{i-n}	4.31±0.04^{k-q}	4.86±0.02^{h-k}	4.01±0.26^{n-u}	6.01±0.06^{b-e}	5.50±0.04^{d-g}	4.09±0.06^{m-t}
9	3.45±0.11^{u-ab}	3.46±0.06^{u-ab}	3.14±0.05^{x-ag}	2.81±0.04^{ac-al}	3.75±0.11^{q-w}	3.84±0.02^{p-v}	2.20±0.03^{am-ar}	4.31±0.05^{k-q}	4.44±0.06^{j-o}	2.12±0.04^{ao-as}
10	2.72±0.02^{ag-an}	2.49±0.19^{ai-ap}	2.83±0.02^{ac-ak}	2.38±0.01^{aj-aq}	2.95±0.05^{aa-aj}	3.49±0.09^{u-aa}	1.86±0.05^{aq-au}	2.75±0.08^{ae-am}	3.85±0.11^{o-v}	1.85±0.05^{aq-au}

Values are Means ± SD of three replicates and those within different letters indicate significance differences (P<0.05)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

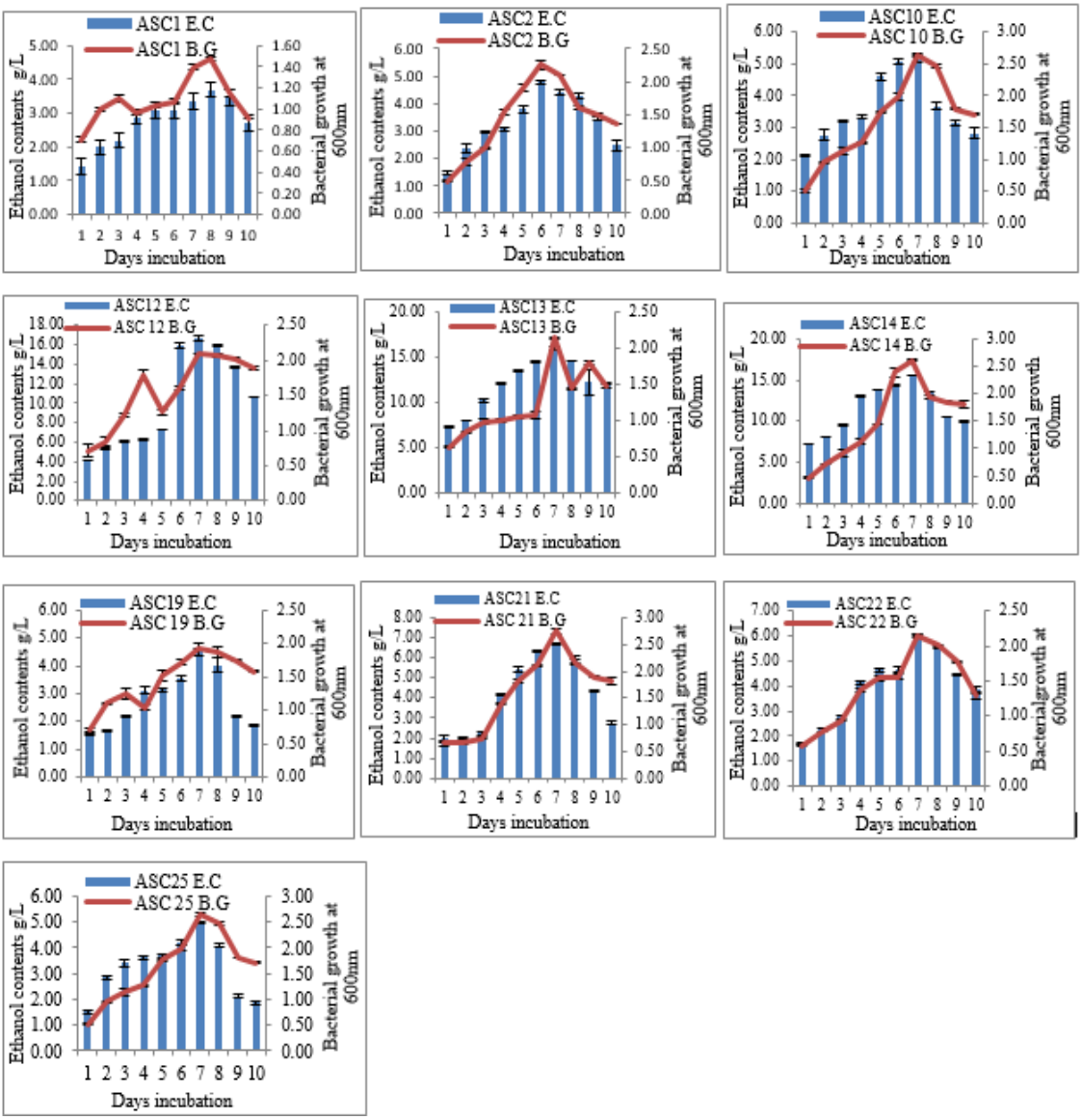
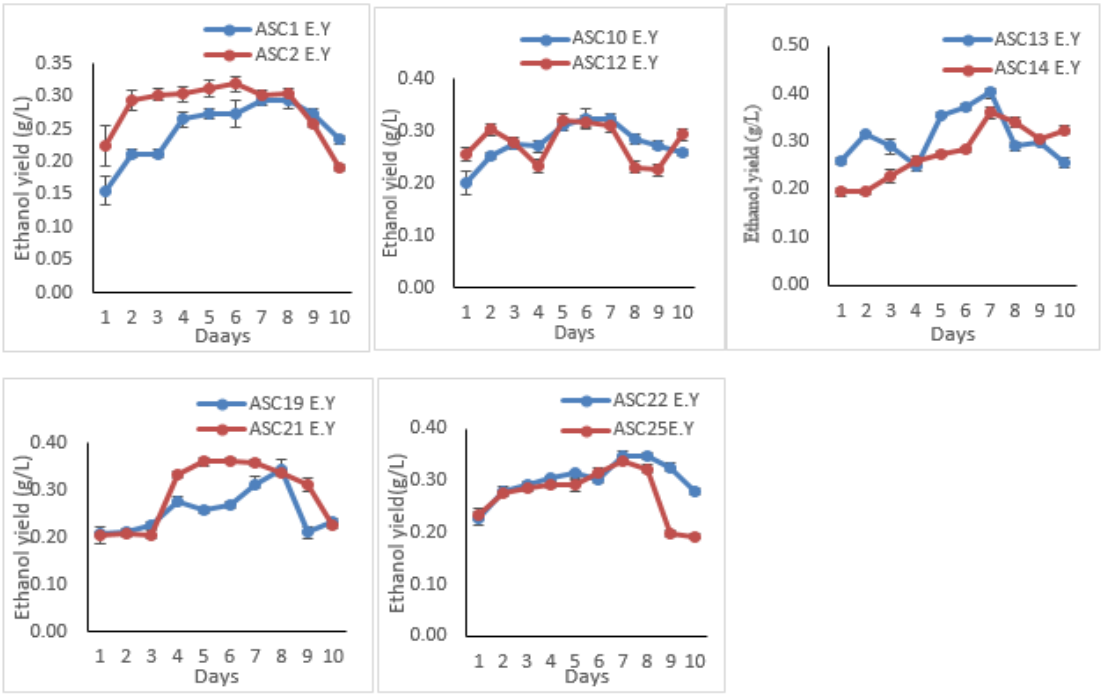


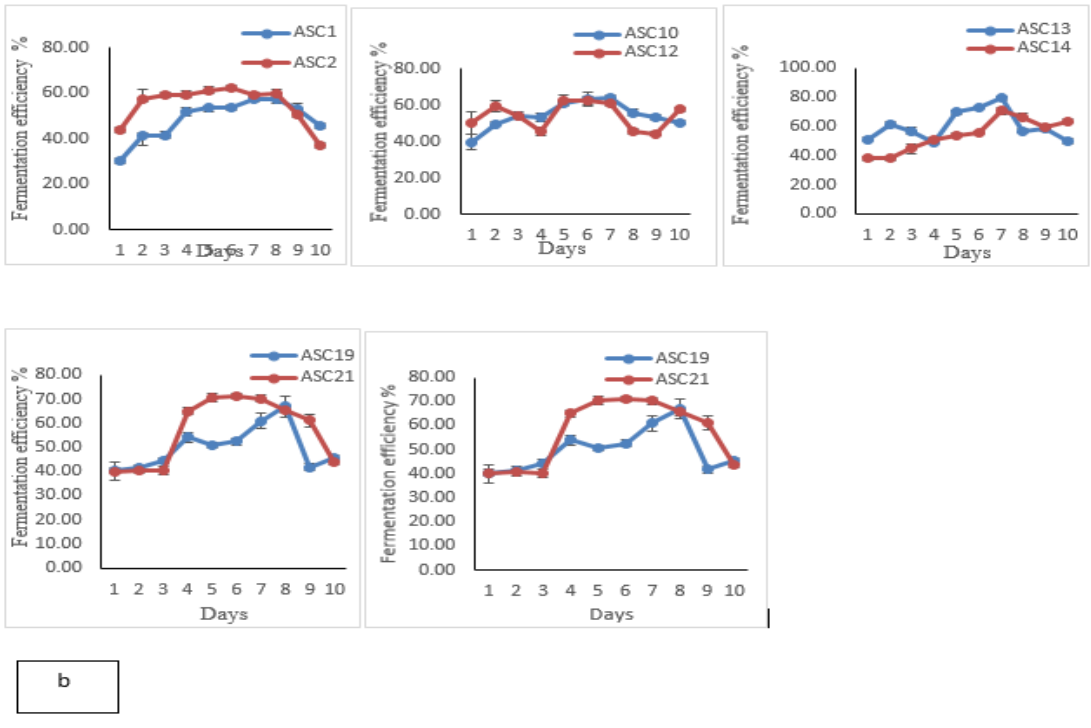
Figure 2. *Comparison of E.C (Ethanol contents) and B.G (bacterial growth) of isolates in fermentation medium supplemented with 2% cellulose by bacterial isolates. Bars denote Means ± standard error mean (SEM)*

Figure 3 showed the connection of ethanol contents and bacterial development in aging medium. In the fermentation medium, ethanologenesis (ethanol production) typically rises in tandem with bacterial growth. At days 7-8, the ethanol yield in 2% CMC-supplemented fermentation medium is shown in *Figure 3(a)*. The highest ethanol yield was observed in the bacterial isolates ASC12, ASC13, ASC21, and ASC22. In *Figure 3(b)* fermentation efficiency addressed. ASC21 showed greatest maturation effectiveness at day 8 and in general diminished at day 9-10. Based on the astounding ethanol yield four disengages ASC12, ASC13, ASC21 and ASC22 were chosen for subsequent molecular identification.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984



a



b

Figure 3. *Presentation of Ethanol yield (g/g) (a) and fermentation efficiency (%) (b) post ethanologenesis by different bacterial isolates*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Table 6. *Consumed Reducing Sugars (g/l) from Cellulose by different bacterial isolates*

Days	ASC1	ASC2	ASC10	ASC12	ASC13	ASC14	ASC19	ASC21	ASC22	ASC25
1	5.58±0.01^{am}	6.57±0.01^{ai-al}	7.23±0.02^{ah-ak}	4.15±0.03^{an}	7.24±0.04^{ah-ak}	7.20±0.02^{ah-ak}	7.42±0.03^{ah-ai}	7.29±0.02^{ah-aj}	7.90±0.04^{ag-ah}	6.41±0.07^{aj-am}
2	6.08±0.03^{al-am}	7.24±0.01^{ah-ak}	7.95±0.01^{ag-ah}	5.51±0.06^{am}	8.01±0.02^{ag-ah}	8.02±0.02^{ag-ah}	7.89±0.01^{ag-ah}	8.85±0.02^{af-ag}	8.02±0.02$^{ag\ ah}$	7.24±0.02^{ah-ak}
3	6.21±0.19^{al-am}	8.08±0.02^{ag-ah}	10.99±0.12^{x-aa}	6.04±0.10^{al-am}	10.18±0.13^{aa-ae}	9.51±0.01^{ac-af}	9.59±0.01^{ac-af}	11.49±0.04^{u-y}	9.38±0.01^{ae-af}	7.99±0.03^{ag-ah}
4	7.29±0.03^{ah-aj}	10.17±0.07^{aa-ae}	12.92±0.02^{o-s}	6.29±0.02^{ak-am}	12.05±0.05^{s-w}	12.96±0.03^{o-s}	10.50±0.01^{z-ac}	12.83±0.03^{p-t}	11.98±0.02^{s-w}	9.75±0.01^{ab-af}
5	8.05±0.05^{ag-ah}	12.13±0.01^{s-w}	13.82±0.03^{i-o}	7.29±0.01^{ah-aj}	13.38±0.10^{l-q}	13.76±0.04^{j-p}	11.36±0.02^{v-z}	14.33±0.06^{g-m}	13.75±0.02^{j-p}	10.41±0.02^{z-ad}
6	9.69±0.02^{ab-af}	14.20±0.06^{h-n}	15.39±0.07^{def}	15.93±0.27^{cd}	14.51±0.08^{f-k}	14.37±0.03^{g-k}	13.19±0.01^{o-r}	16.14±0.04^{cd}	15.30±0.05^{d-g}	12.44±0.03^{q-u}
7	11.19±0.10^{w-z}	12.92±0.02^{o-}	15.90±0.21^{cd}	16.66±0.24^{bc}	15.91±0.22^{cd}	15.53±0.29^{de}	14.80±0.01^{e-i}	17.97±0.02^{a}	17.42±0.22^{ab}	14.35±0.06^{g-l}
8	13.27±0.02^{n-q}	11.39±0.02^{v-z}	12.92±0.01^{o-s}	15.89±0.03^{cd}	14.61±0.03^{e-j}	13.53±0.08^{k-p}	11.74±0.01^{u-x}	15.17±0.02^{d-h}	13.37±0.01^{l-q}	12.94±0.01^{o-s}
9	12.10±0.02^{s-w}	9.57±0.03^{ac-af}	11.25±0.07^{w-z}	13.68±0.02^{j-p}	12.26±1.40^{r-v}	10.44±0.01^{z-ad}	9.73±0.01^{ab-af}	13.35±0.08^{m-q}	11.53±0.05^{u-y}	12.90±0.03^{o-s}
10	9.50±0.02^{ad-af}	8.02±0.03^{ag-ah}	9.59±0.04^{ac-af}	10.58±0.01^{y-ab}	11.90±0.03^{t-x}	9.95±0.03^{ab-ae}	8.03±0.01^{ag-ah}	12.28±0.01^{r-v}	9.70±0.01^{ab-af}	9.61±0.04^{ab-af}

Values are Means ± SD of three replicates and those within different letters indicate significance differences (P<0.05)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

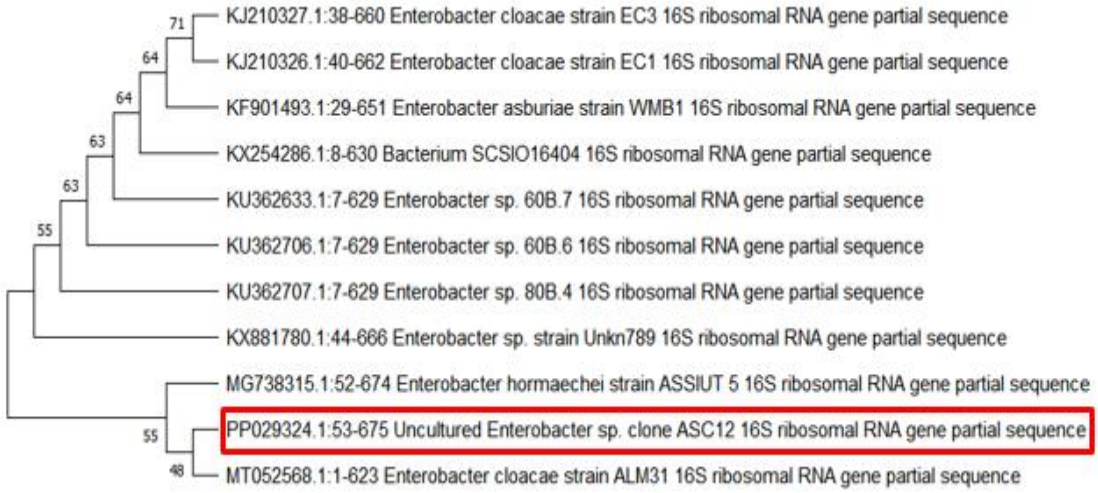


Figure 4. *The Neighbor-Joining approach of Enterobacter cloacae sp. ASC12 was utilized to construct the developmental history within bootstrap agreement tree that is gotten from 1000 imitates. Close to each branch is the extent of copy trees where the related taxa bunched together in the bootstrap test (1000 redundancies). The transformative distances are communicated in base replacements per site and were determined utilizing the Greatest Composite Probability method. There were 11 nucleotide groupings in this examination. For each succession pair, all muddled spots were disposed of (pairwise erasure choice). The last dataset contained 623 areas altogether. Evolutionary studies were conducted in MEGA X*

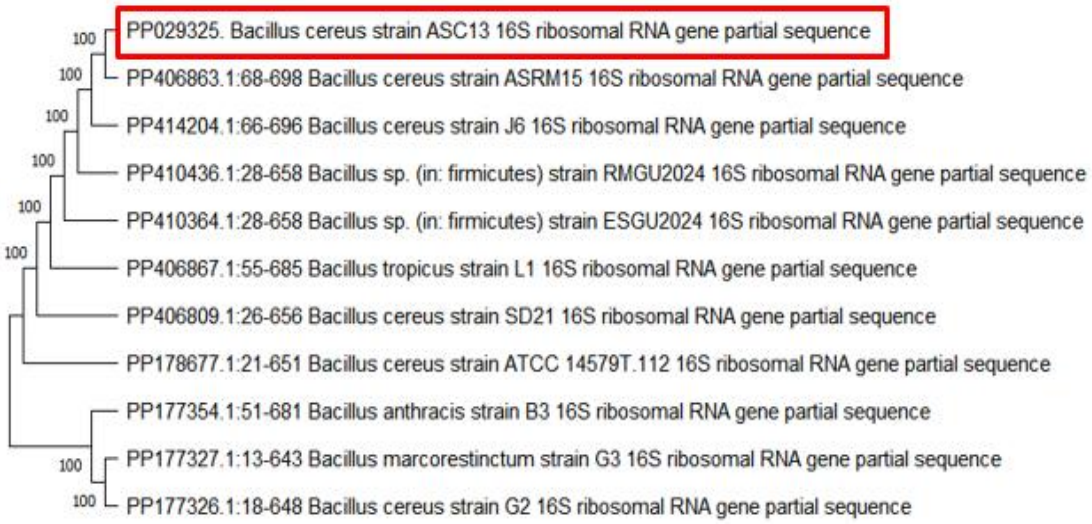


Figure 5. *The Neighbor-Joining approach of Bacillus paramycoides sp. ASC13 was utilized to construct the developmental history within bootstrap agreement that is obtained from 1000 imitates. Close to the branches are the level of copy trees where the related taxa grouped in the bootstrap test (1000 reiterations). The transformative distances are communicated in base replacements per site and were determined utilizing the Greatest Composite Probability method. There were eleven nucleotide successions in this examination. For each succession pair, all muddled spots were disposed of (pairwise erasure choice). The last dataset contained 631 areas altogether. In Uber X, developmental examinations were done*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

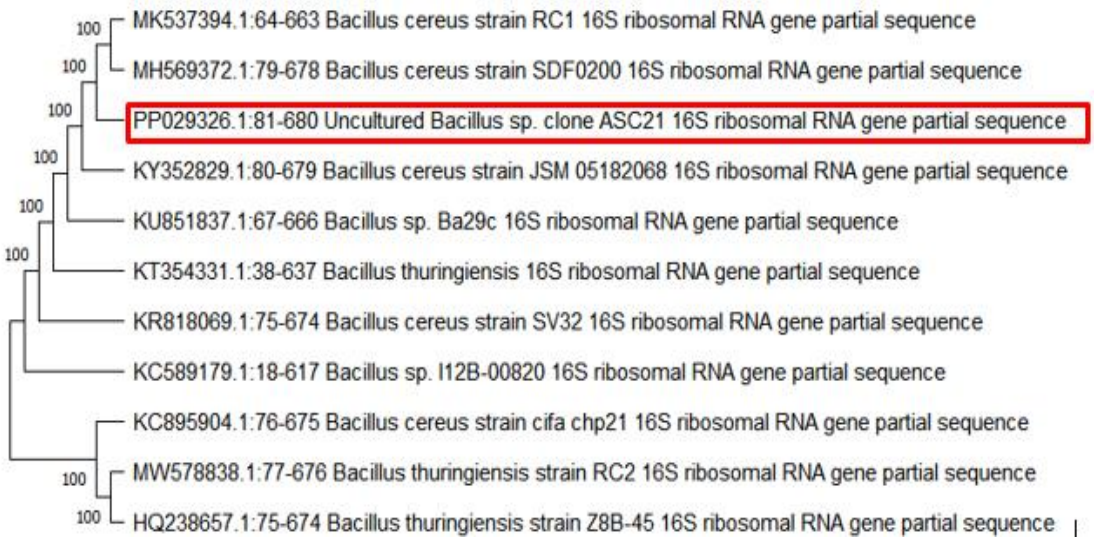


Figure 6. *The Neighbor-Joining approach of Bacillus cereus sp. ASC21 was utilized to construct the developmental history within1000 replicates, assumed to represent the taxonomic group's evolutionary history. Close to the branches are the level of copy trees where the related taxa grouped in the bootstrap test (1000 reiterations). The transformative distances are communicated in base replacements per site and were determined utilizing the Greatest Composite Probability method. There were eleven nucleotide groupings in this examination. For each succession pair, all muddled spots were disposed of (pairwise erasure choice). The last dataset contained 600 areas altogether. Evolutionary analyses were carried out in MEGA X*

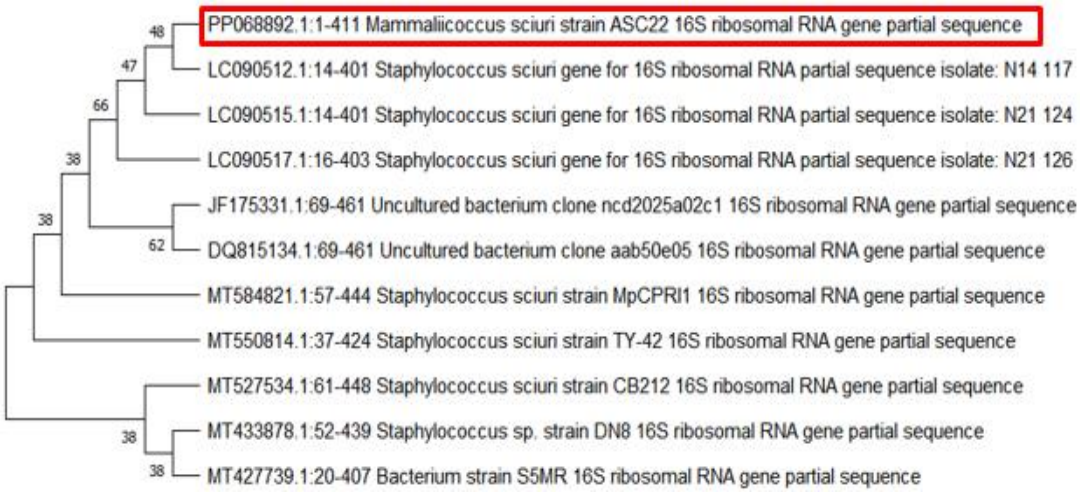


Figure 7. *The Neighbor-Joining approach of Staphylococcus sciuri sp. was utilized to construct the developmental history within the bootstrap agreement tree that is gotten from 1000 imitates. Close to the branches are the level of copy trees where the related taxa grouped in the bootstrap test (1000 reiterations). The transformative distances are communicated in base replacements per site and were determined utilizing the Greatest Composite Probability method. There were eleven nucleotide successions in this examination. For each succession pair, all muddled spots were disposed of (pairwise erasure choice). The last dataset contained 411 areas altogether. Evolutionary studies were conducted in MEGA X*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Discussion

The study involved finding bacteria in termites' gut that have the ability to break down cellulose and produce ethanol. The Sample was collected from different areas of Kasur, Pakistan on 13th July 2021. Termites consume wood and other cellulose-based materials. Symbiotic microbes in termites' gut help them break down cellulose, a component of wood. Because of this special capacity termites are able to recycle nutrients in forest ecosystems and perform significant ecological functions in the decomposition of dead wood. On the other hand, termites have the potential to seriously harm timber constructions and result in financial losses. Their social organization is well-known as they reside in colonies in many cases, each with distinct roles. Nymphs depending on their age and developmental stage, these juvenile termites perform a variety of activities inside the colony. Workers are often the most prevalent caste in the colony and have pale or white heads. Soldiers usually have black or reddish-colored heads and they often have larger mandibles or other protective characteristics (Wako, 2015). Termites were found causing damage to wood logs and extra attention was paid to the soldier termites throughout the collecting procedure. Soldiers are essential for classifying termite species because of their unique physical characteristics, especially with regard to head and body length. Effective termite control strategies depend on the ability to identify distinct species, since they may require different methods of prevention and treatment. Termite species may be precisely identified by researchers or pest management experts by focusing on the soldiers and using morphometrics to quantify their body and head length. This identification makes it possible to implement targeted treatments to lessen the harm of the insects that destroy wood (Roonwal and Chhotani, 1989; Scheffrahn et al., 2006; Engle et al., 2009; Saha et al., 2016; Amina et al., 2016; Mahapatro et al., 2018). Its mandibles range in color from red to brown and have an oval, pale brown head. Mandibles have a range of 0.8-0.9 mm and are smaller and less curled than the skull. The labrum is subtriangular and has a pointed tip (Bhanupr et al., 2023). Termites are a major concern in both agricultural and urban areas worldwide because they destroy a lot of plants, crops, structures, and woodwork and create a lot of financial loss. Termites coexist in a close mutualistic partnership with a range of endomicrobes that reside in the hindgut in order to break down and prepare cellulose for absorption. These endomicrobes primarily flagellates produce the enzyme cellulase, which enhances the assimilation of plant celluloses (Breznak, 2000).

While many bacteria are able to break down cellulose, only a small number are able to generate substantial amounts of free enzymes that are able to hydrolyze crystalline cellulose entirely. There are two types of bacterial cellulases: extracellular and cell bound. Several bacteria from the gut microbiota that were able to create extracellular cellulases were used in this study. Bacteria that break down cellulose are found in various environments, such as termite guts, scientists may learn more about the enzymes and metabolic processes that contribute to this process. This information may be used in a variety of contexts such as the generation of biofuel, waste management, and agriculture where sustainable methods depend on the effective breakdown of biomass (Shankar and Isaiarasu, 2011). They can degrade cellulose to glucose. It has been proposed that the intestinal microbes of these lignocellulose-degrading termites are necessary for the digestion of cellulose (Hu et al., 2014). The purpose of this study was to identify bacteria that could break down cellulosic biomass into glucose monomers and to explore the cellulolytic bacteria present in the gut symbionts of termite workers *Coptotermes heimi* (Wasmann). Durham tube testing is a technique used in microbiology to identify gas generation as a consequence of microbial metabolism. The gas produced is usually carbon

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

dioxide or another gas. This test is frequently used in conjunction with tests for the fermentation of carbohydrates. Durham tube testing is helpful in identifying gases produced by bacteria that are fermenting quickly but it might not be sensitive enough to identify gases produced by bacteria that are fermenting slowly or in low metabolic activity situations. In these situations, more sensitive gas detection techniques or longer incubation times may be required for accurate identifications (Scheffers, 1987). Tetrazolium chloride (TTC) and Congo red stain are often employed indicators in microbiology for a variety of applications, such as the identification of bacterial sugar hydrolysis. When certain metabolically active bacteria are present, the colorless molecule TTC is converted to the strongly colorful chemical formazan. When TTC is added to a growth medium with a sugar substrate, bacteria that can hydrolyze the sugar and digest the byproducts can decrease TTC, which causes formazan to develop and the medium's color to alter. This color shift usually varies from bright pink to dark maroon, depending on how much of a decrease there is. This shift in colors can be used to show if the tested bacteria are hydrolyzing sugar (Thom et al., 1993; Caviedes et al., 2002; Abate et al., 2004). Congo red staining is useful for detecting bacteria that can degrade cellulose. Bacteria break down cellulose by producing extracellular cellulolytic enzymes that hydrolyze the material into smaller pieces that the bacteria may use as a source of carbon (Lu et al., 2004). With the exception of four, every bacterial isolate created a different clearance zone surrounding its colony. The enzymes diffused into the medium as a result of the lysis or aging of the bacterial cell, interacting with the dye to lessen its color (Jalandoni-Buan et al., 2010). Congo red stain or Sulfonatedazo dye is unable to pass through bacterial plasma membranes. There were large zones of clearance and little dye retention as a result of the significant cellulolytic activity (Stolz, 2001; Fujimoto et al., 2011; Florencio et al., 2012). Techniques like Congo red staining, which shows the breakdown of cellulose by the creation of hollow zones surrounding bacterial colonies, may be used to assess the cellulolytic activity of bacterial isolates. This approach yields a quantitative measure of cellulolytic activity: the cellulolytic index, which is the diameter of the hollow zone divided by the diameter of the bacterial colony. In comparison to other isolates, isolate ASC21 exhibits a comparatively strong cellulolytic capacity, as seen by its cellulolytic index value of 3.0. The increased width of the hollow zone in comparison to the bacterial colony's diameter indicates that isolate ASC21 is particularly adept at breaking down cellulose.

The highest amounts of glucose and reducing sugar that *T. reesei* could generate under these circumstances were 4.6 g/L and 8.0 g/L at 60 hours, respectively. The maximum production of ethanol by *C. molischiana* was 3.0 g/L after 120 h (Bu, 2019**)**. The study tested different bacterial isolates for their ability to produce ethanol. The bacteria were grown on a medium supplemented with 2% CMC, which is an indicator of their ability to break down cellulose. The results showed that bacterial isolates ASC12, ASC13, ASC21, and ASC22 were able to produce the highest amount of ethanol (g/l) as 6.08±0.04, 6.42±0.05, 6.70±0.01, 6.05±0.02 by day 7 of the experiment.

Respective efficiency of percentage fermentation of ethanol yield productivity (g/g) 0.31, 0.40, 0.36 and 0.35, 60.73, 79.19, 70.25, and 68.15 were likewise determined for each isolate (ASC12, ASC13, ASC21, ASC22). These actions demonstrate how effectively the microscopic organisms had the option to change cellulose over completely to ethanol. Isolate ASC21 had the most elevated ethanol yield 0.40 g/g and percent fermentation productivity 79.19%, demonstrating that they were the best at delivering ethanol from cellulose. After being refined in an engineered medium utilizing β-glucan as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

the main carbon source, the resultant yeast cells could mature 45 g of β-glucan per liter straightforwardly, yielding 16.5 g of ethanol per liter in about 50 hours. The yield was 0.48 g/g, or 93.3% of the anticipated yield, estimated in grams of ethanol created per gram of sugar utilized. Cellulolytic catalysts delivering recombinant yeast cells are liable for this (Yasuya Fujita et al., 2002). Harun and Danquah (2011) concentrated on the enzymatic hydrolysis of microalgae (Chloroccum sp.) by utilizing cellulase obtained from Trichoderma reesei ATCC 26921. Wu et al. (2014) concentrated on the red green growth *Gracilaria sp.* which is utilized to create bioethanol. *Gracilaria sp.* was saccharified by progressive corrosive and protein hydrolysis in situ, bringing about a great hydrolysate that reliable ethanol creation during maturation. The last ethanol convergences of 4.72 g/l (0.48 g/g sugar consumed, 94% transformation proficiency) and ethanol efficiency of 4.93 g/l/d were accomplished. A great deal of research has been done on the performance of different ethanologenic microbes, with an emphasis on their ethanol yields from varied substrates. For example, refined cellulose and agricultural leftovers may be converted into ethanol with exceptional efficiency by the dual-functional yeast Clavispora NRRL Y-50464. While Saccharomyces cerevisiae and other modified strains generated just 24 g/L in the same time without additional enzymes, this strain reached a maximum ethanol titer of 47.74 g/L in 120 hours (Liu and Dien, 2022). In a review, Shokrkar et al. (2018) asserted the higher glucose yield (57%) at microalgal biomass centralization of 50 g/l, pH 5, and temperature of 50°C. Thus the outcomes approved the idea of the presence of cellulose degrading microbes in stomach related arrangement of termites. On medium improved with CMC, 25 bacterial confines were chosen from the examples. Gut of termites have enhanced microbiota in view of biochemical qualities and a phylogenetic examination of 16S rRNA quality pieces the detaches were recognized. Researchers previously produced bioethanol from Escherichia coli was hampered by plasmids, antibiotic resistance, and poor ethanol tolerance. In order to enhance this, they developed a novel strain that did not require plasmids or resistance genes since it had a chromosomally integrated ethanologenic pathway. For a month, the new strain's performance in CWP fermentation remained consistent. Through modification of fermentation parameters, such as inoculum dimensions and CWP content, the group detected toxicity and nutritional obstacles. They added ammonium sulfate and enhanced ethanol tolerance by adaptive evolution, which led to a considerable improvement in fermentation performance: 6.6% v/v ethanol, 1.2 g/L/h production rate, 82.5% yield, and a notable rise in cell survival (Pasotti et al., 2023).

The ongoing review's discoveries demonstrate that four cellulose degrading microorganisms were *Enterobacter cloacae* sp. ASC12, *Bacillus paramycoides* sp. ASC13, Bacillus cereus sp. ASC21 and Staphylococcus sciuri sp. ASC22. Four capable bacterial strains that chosen were described microscopically. The molecular identification of the four chosen effective bacterial strains as *Enterobacter cloacae* sp. ASC12, *Bacillus paramycoides* sp. ASC13, Bacillus cereus sp. ASC21, *Staphylococcus sciuri* sp. ASC22 is based on the most extreme cellulolytic list and ethanol yield. For molecular characterization bacterial 16S primers were used to amplify the 16S rRNA gene.

A number of studies conducted over the past ten years to distinguish cellulolytic bacteria from termites have shown that these bacteria can fully hydrolyze crystalline cellulose (Saha et al., 2006; Doi et al., 2008). The microscopic organisms develop more quickly than yeast and growth and consequently can be utilized broadly for cellulase creation and can be advanced effectively for specific social circumstances (Nakamura et al., 1988). The review was a venturing stone in laying out the enormous scope of process improvement of bioenergy creation. As the world's energy sources diminish at an

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

alarmingly rapid rate with each passing day, there is a pressing need to investigate alternatives that are more environmentally friendly, renewable, and efficient (Ahmad, 2021; Chaudhary, 2023). The transformation of cellulose into fermentable sugar through the utilization of cellulolytic bacteria is one of the most crucial steps in the production of biofuel (Kiran et al., 2024; Saleem et al., 2020). When this step is enhanced in an expense serious way, it might prompt the foundation of eco-feasible cycle improvement (Chaudhary et al., 2022). In this manner, the chosen bacterial separates could demonstrate be viable possibility for the business transformation of cellulosic biomass into bioethanol and could be utilized to separate plant waste to create fermentable sugars. Enormous scope bioremediation of cellulosic squanders can be achieved by the utilization of microorganism determined cellulases (Dixit et al., 2021). Furthermore, research suggests that a significant amount of the overall expenditures associated with producing cellulosic ethanol come from the cellulase. For instance, studies have demonstrated that generating cellulases locally can dramatically reduce the minimum ethanol selling price (MESP) by lowering the expenses associated with enzyme transfer (Ellilä et al., 2017). Although microbial enzymes in particular, cellulases have been used for millennia, their commercial application has just lately become popular. Numerous sectors, including agriculture, brewing, laundry, pulp and paper, and textiles, have found use for cellulases. Cellulases are used in the food industry for a variety of purposes, including hydrolyzing roasted coffee, decreasing roughage in dough, clarifying fruit liquids, and improving the extraction of essential oils and tea polyphenols. They can also enhance the food's flavor and scent. Cellulases have been underused in food processing despite these advantages (Ejaz et al., 2021). These chosen Isolates of bacteria be compelling business possibility for the change of cellulosic biomass into bioethanol and can likewise be utilized for the breakdown of plant waste to deliver fermentable sugars. The selected parameters in the current study will provide base line for the conversion of lignocellulosic biomass for further experimentation. In biomass the rate of substrate conversion, bacterial growth and ethenol production will be different as the substrate consisted of hemicellulose and lignin (Chaudhary et al., 2024).

Conclusions

The present study gave robust affirmation of an unprecedented method for identifying and evaluating the power of cellulolytic enzymes from the bacteria associated with the termite *Coptotermes heimi*'s gut. *Bacillus cereus sp*. ASC21, which was isolated from the gut of *C. heimi*, had spectacular characteristics with a cellulolytic index of 3.0 and a CMCase of 1.83 ± 0.01 umol/ml/min. *Bacillus cereus sp*. ASC21 attained a remarkable ethanol yield of 6.70 ± 0.01 g/L, an ethanol yield of 0.40 g/g, and a fermentation efficiency of 79.19%. Higher termite guts have been shown to be a viable and natural source for the screening of new cellulolytic enzymes and microorganisms. The selected cellulolytic bacteria can be used for composting, enzyme purification, and biofuel generation since they can biotransform cellulose into glucose. It is suggested that in order to better enhance these bacteria's performance, future studies should investigate the genetic and metabolic processes involved in their cellulolytic activity. Furthermore, examining the synergistic effects of co-culturing distinct bacterial strains enhances ethanol yields and cellulose breakdown rates, opening the door to more effective bioconversion procedures in bioenergy applications.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984

Conflicts of Interest. No authors have disclosed any conflicts of interest.

Data Availability Statement. All the data produced or generated during the study has been given in the manuscript.

Acknowledgments. Authors are thankful to the Researchers Supporting Project number (RSP2025R335), King Saud University, Riyadh, Saudi Arabia.

REFERENCES

[1] Abate, G., Aseffa, A., Selassie, A., Goshu, S., Fekade, B., Wolde Meskal, D., Miörner, H. (2004): Direct colorimetric assay for rapid detection of rifampin-resistant Mycobacterium tuberculosis. – Journal of Clinical Microbiology 42(2): 871-873.

[2] Abu-Gharbia, M. A., El-Sawy, N. M., Nasr, A. M., Zedan, L. A. (2018): Isolation, optimization and characterization of cellulases and hemicellulases from *Bacillus cereus* LAZ 518 isolated from cow dung using corn cobs as lignocellulosic waste. – Journal of Pharmaceutical and Applied Chemistry 4(2): 1-13.

[3] Ahmad, M. (1955): A new termite from E. Pakistan (Isoptera: Termitidae). – Biologia 25-27(1).

[4] Ahmad, Q., Manzoor, M., Chaudhary, A., Yang, S. T., Shim, H., Qazi, J. I. (2021): Bench-scale fermentation for second generation ethanol and hydrogen production by *Clostridium thermocellum* DSMZ 1313 from sugarcane bagasse. – Environmental Progress & Sustainable Energy 40(2): e13516.

[5] Akhtar, S., Wig, N. N., Varma, V. K., Pcrshad, D., Verma, S. K. (1975): A phenomenological analysis of symptoms in obsessive-compulsive neurosis. – The British Journal of Psychiatry 127(4): 342-348.

[6] Akhtar, M. S. (1983): Wood destroying termites (Isoptera) of Pakistan: Key to the most important species, their distribution and pattern of attack. – Materials and Organisms, pp. 277-291.

[7] Amina, B., Miloud, A., Samir, L., Abdelylah, B., Solano, J. P. (2016): Heat transfer enhancement in a parabolic trough solar receiver using longitudinal fins and nanofluids. – Journal of Thermal Science 25: 410-417.

[8] Arumugam, R., Manikandan, M. (2011): Fermentation of pretreated hydrolyzates of banana and mango fruit wastes for ethanol production. – Asian J. Exp. Biol. Sci 2(2): 246-256.

[9] Azhar, S., Aihetasham, A., Chaudhary, A., Hussain, Z., Abdul Rehman, R., Abbas, G., Qamer, S. (2024): Cellulolytic and Ethanologenic Evaluation of Heterotermes indicola's Gut-Associated Bacterial Isolates. – ACS omega 9(10): 12084-12100.

[10] Aziz, T., Shah, Z., Sarwar, A. Ullah N, Khan AA,Sameeh MY, Cui H, Lin L. (2023). Production of bioethanol from pretreated rice straw, an integrated and mediated upstream fermentation process. Biomass Conv. Bioref. 2023, 1-11.

[11] Bai, M. Y., Fan, M., Oh, E., Wang, Z. Y. (2012): A triple helix-loop-helix/basic helix-loop-helix cascade controls cell elongation downstream of multiple hormonal and environmental signaling pathways in Arabidopsis. – The Plant Cell 24(12): 4917-4929.

[12] Benson, H. J., Benson, H., Brown, A. E., Brown, A. (1984): Benson's microbiological applications: Laboratory manual in general microbiology. – 9th ed. McGraw-Hill Higher Education.

[13] Bhanupriya, P., Ramachandran, B. (2023): Imposition recognition in vehicular networks using hybrid offloading techniques with improved radial bias neural network. – Soft Computing 27(20): 15425-15441.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

[14] Boontanom, P., Chantarasiri, A. (2021): Diversity and cellulolytic activity of culturable bacteria isolated from the gut of higher termites (Odontotermes sp.) in eastern Thailand. – Biodiversitas-Journal of Biological Diversity 22(8).
[15] Breznak, J. A. (2000): Ecology of prokaryotic microbes in the guts of wood-and litter-feeding termites. – In: Abe, T., Bignell, D. E., Higashi, M. (eds.) Termites: Evolution, Sociality, Symbioses, Ecology. Springer, Dordrecht, pp. 209-231.
[16] Bu, Y., Alkotaini, B., Salunke, B. K., Deshmukh, A. R., Saha, P., Kim, B. S. (2019): Direct ethanol production from cellulose by consortium of *Trichoderma reesei* and *Candida molischiana*. – Green Processing and Synthesis 8(1): 416-420.
[17] Caviedes, L., Delgado, J., Gilman, R. H. (2002): Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of Mycobacterium tuberculosis. – Journal of Clinical Microbiology 40(5): 1873-1874.
[18] Chapman, E. N., Kaatz, A., Carnes, M. (2013): Physicians and implicit bias: how doctors may unwittingly perpetuate health care disparities. – Journal of General Internal Medicine 28: 1504-1510.
[19] Chatterjee, S., Sharma, S., Prasad, R. K., Datta, S., Dubey, D., Meghvansi, M. K., Veer, V. (2015): Cellulase enzyme based biodegradation of cellulosic materials: an overview. – South Asia J. Exp. Biol. 5: 271-282.
[20] Chaudhary, A., Aihetasham, A., Younas, S., Basheer, N., Hussain, N., Naz, S., Aziz, T., & Albekairi, T. H. (2024). Statistical optimization for comparative hydrolysis and fermentation for hemicellulosic ethanolgenesis. Italian Journal of Food Science, 36(2), 231-245.
[21] Chaudhary, A., Hussain, A., Ahmad, Q. U. A., Manzoor, M., Tahira, S. A., Karita, S. (2022): Statistical optimization of alkaline treatment of pomegranate peel waste for bioethanol production. – Biomass Conv. Boiref.
[22] Shehzadi, A., Chaudhary, A., Aihetasham, A., Hussain, N., Naz, S., Aziz, T., & Alasmari, A. F. (2024). Determination of hydrolyzing and ethanolic potential of cellulolytic bacteria isolated from fruit waste. Italian Journal of Food Science, 36(1), 127-141.
[23] Chaudhry, I. M., Ahmad, M. (1972): Termites of Pakistan, identity, distribution and ecological relationships. – Final Technical Report, pp. 13-19. Pakistan Forest Institute, Peshawar.
[24] Demissie, M. S., Legesse, N. H., Tesema, A. A. (2024): Isolation and characterization of cellulase producing bacteria from forest, cow dung, Dashen brewery and agro-industrial waste. – Plos One 19(4): 0301607.
[25] Dixit, M., Gupta, G. K., Usmani, Z., Sharma, M., Shukla, P. (2021): Enhanced bioremediation of pulp effluents through improved enzymatic treatment strategies: A greener approach. – Renewable and Sustainable Energy Reviews 152: 111664.
[26] Doi, R. H. (2008): Cellulases of mesophilic microorganisms: cellulosome and noncellulosome producers. – Ann. N.Y. Acad. Sci 1125: 267-279.
[27] Ejaz, U., Sohail, M., Ghanemi, A. (2021): Cellulases: from bioactivity to a variety of industrial applications. – Biomimetics 6(3): 44.
[28] Ellilä, S., Fonseca, L., Uchima, C., Cota, J., Goldman, G. H., Saloheimo, M., Siika-Aho, M. (2017): Development of a low-cost cellulase production process using *Trichoderma reesei* for Brazilian biorefineries. – Biotechnology for Biofuels 10: 1-17.
[29] Engel, M. S., Grimaldi, D. A., Krishna, K. (2009): Termites (Isoptera): Their phylogeny, classification and rise to ecological dominance. – American Museum Novitates 3650: 127.
[30] Engel, P., Moran, N. A. (2013): The gut microbiota of insects–diversity in structure and function. – FEMS Microbiology Reviews 37(5): 699-735.
[31] Florencio, C., Couri, S, Farinas, C. S. (2012): Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by Trichoderma strains. – Enzyme Res 2012: 793708.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

[32] Fujimoto, N., Kosaka, T., Nakao, T., Yamada, M. (2011): Bearing a high cellulose-degrading activity, which was isolated as a heat-resistant and micro-aerophilic microorganism from bovine rumen. – Open Biotechnol J. 5(1).
[33] Gay, J., Cole, M. (1967): The new mathematics and an old culture: A study of learning among the Kpelle of Liberia. – Holt, Rinehart and Winston.
[34] Hu, P., Wang, D., Cassidy, M. J., Stanier, S. A. (2014): Predicting the resistance profile of a spudcan penetrating sand overlying clay. – Canadian Geotechnical Journal 51(10).
[35] Ire, F. S., Ezebuiro, V., Ogugbue, C. J. (2016): Production of bioethanol by bacterial co-culture from agro-waste-impacted soil through simultaneous saccharification and co-fermentation of steam-exploded bagasse. – Bioresources and Bioprocessing 3: 1-12.
[36] Irfan, M., Safdar, A., Syed, Q., Nadeem, M. (2012): Isolation and screening of cellulolytic bacteria from soil and optimization of cellulose production and activity. – Turk Journal of Biochemistry 37: 287-293.
[37] Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., Weinstock, G. M. (2019): Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. – Nature Communications 10(1): 5029.
[38] Kiran, N., Yunus, F.-U.-N., Nadeem, M., Manzoor, F., Nelofar, R., Sarwar, A., Ullah, N., Aziz, T., S. Alamri, A., Alsanie, W. F., Alhomrani, M. (2024). Potato peel as substrate for Single Cell Protein in animal feed by submerged fermentation. Italian Journal of Food Science, 36(4), 57-63.
[39] Lee, S. M., Koo, Y. M. (2001): Pilot-scale production of cellulase using *Trichoderma Reesei* rut C-30 fed-batch mode. – Journal of Microbiology and Biotechnology 11(2): 229-233.
[40] Li, J., Pósfai, M., Hobbs, P. V., Buseck, P. R. (2003): Individual aerosol particles from biomass burning in southern Africa: 2, Compositions and aging of inorganic particles. – Journal of Geophysical Research, Atmospheres 108(D13).
[41] Liu, Z. L., Dien, B. S. (2022): Cellulosic ethanol production using a dual functional novel yeast. – International Journal of Microbiology 2022(1): 7853935.
[42] Lu, P. D., Harding, H. P., Ron, D. (2004): Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. – The Journal of Cell Biology 167(1): 27-33.
[43] Mahapatro, G. K., Kumar, S., Kumar, M. (2018): A new record of termite *Amitermes belli* (Desneux) from Himachal Pradesh. – Indian Journal of Entomology 80(2): 457-459.
[44] Miller, G. L., Blum, R., Glennon, W. E., Burton, A. L. (1960): Measurement of acarboxymethylcellulase activity. – Analytical Biochemistry 1(2): 127-132.
[45] Mtui, G. Y. S. (2009): Recent advances in pretreatment of lignocellulosic wastes and production of value added products. – African Journal of Biotechnology 8(8): 1398-1415.
[46] Nakamura, K., Kitamura, K. (1988): Cellulases of *Cellulomonas uda*. – Methods in Enzymology 160: 211-216.
[47] Pan, X., Shang-Guan, P., Li, S., Zhang, C., Lou, J., Guo, L, Lu, Y. (2025). The influence of carbon dioxide on fermentation products, microbial community, and functional gene in food waste fermentation with uncontrol pH. Environmental Research, 267, 120645. doi:
[48] Pasotti, L., De Marchi, D., Casanova, M., Frusteri Chiacchiera, A., Cusella De Angelis, M. G., Calvio, C., Magni, P. (2023): Design of a stable ethanologenic bacterial strain without heterologous plasmids and antibiotic resistance genes for efficient ethanol production from concentrated dairy waste. – Biotechnology for Biofuels and Bioproducts 16(1): 57.
[49] Prillinger, H., Messner, R., König, H., Bauer, R., Lopandic, K., Molnar, O., Sigler, L. (1996): Yeasts associated with termites: a phenotypic and genotypic characterization and use of coevolution for dating evolutionary radiations in asco-and basidiomycetes. – Systematic and Applied Microbiology 19(2): 265-283.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

[50] Promon, S. K., Kamal, W., Rahman, S. S., Hossain, M. M., Choudhury, N. (2018): Bioethanol production using vegetable peels medium and the effective role of cellulolytic bacterial (*Bacillus subtilis*) pre-treatment. – F1000Research 7: 271.
[51] Ramin, M. (2008): Isolation, Identification and In-vitro Fermentation Activity of Cellulolytic Bacteria from the Gut of Termites. – Doctoral dissertation, Universiti Putra Malaysia.
[52] Roonwal, M. L., Chatterjee, P. N., Thapa, R. S. (1959): Results of laboratory and field experiments on protection of bamboos in storage against ghoon beetles, Dinoderus spp. (Coleoptera: Bostryohidae). – Indian forest bulletin no. 216. (New series) Entomology.
[53] Roonwal, M. L., Chhotani, O. B. (1962): Termite fauna of Assam region, Eastren India. – Proct. Nalt. Inst. Sci. India, New Dehli 72: 281-406.
[54] Roonwal, M. L. (1970): Termites of the Oriental region. – In: Krishna, K., Weesner, F. M. (eds.) Biology of Termites. Academic Press, New York 2: 315-391.
[55] Roonwal, M. L., Chhotani, O. B. (1989): Fauna of India-Isoptera (Termites). – Part 1 and 2; Zoological survey of India.
[56] Russell, B. C., Torralba, A., Murphy, K. P., Freeman, W. T. (2008): LabelMe: a database and web-based tool for image annotation. – International Journal of Computer Vision 77: 157-173.
[57] Saha, S., Roy, R. N., Sen, S. K., Ray, A. K. (2006): Characterization of cellulase-producing bacteria from the digestive tract of tilapia, Oreochromis mossambica (Peters) and grass carp, Ctenopharyngodon idella (Valenciennes). – Aquaculture Research 37(4): 380-388.
[58] Saha, N., Mazumdar, P. C., Basak, J., Raha, A., Majumder, A., Chandra, K. (2016): Subterranian termite genus Odontotermes (Blattaria: Isoptera: Termitidae) from Chhattisgarh, India with its annotated checklist and revised key. – Journal of Threatened Taxa 8(3): 8602-8610.
[59] Saleem, A., Hussain, A., Chaudhary, A., Ahmad, Q. U. A., Iqtedar, M., Javid, A., Akram, A. M. (2020): Acid hydrolysis optimization of pomegranate peels waste using response surface methodology for ethanol production. – Biomass Conversion and Biorefinery 12: 1513-1524.
[60] Sari, S. A. (2016): The development of mind mapping media in flood material using ADDIE. – Journal of Education and Learning (Edu Learn) 10(1): 53-62.
[61] Schachter, J. B., Li, Q., Boyer, J. L., Nicholas, R. A., Harden, T. K. (1996): Second messenger cascade specificity and pharmacological selectivity of the human P2Y1-purinoceptor. – British Journal of Pharmacology 118(1): 167-173.
[62] Scheffers, W. A. (1987): Alcoholic fermentation. – Stud Mycol 30: 321-332.
[63] Scheffrahn, R. H., Křeček, J., Chase, J. A., Maharajh, B., Mangold, J. R. (2006): Taxonomy, biogeography, and notes on termites (Isoptera: Kalotermitidae, Rhinotermitidae, Termitidae) of the Bahamas and Turks and Caicos Islands. – Annals of the Entomological Society of America 99(3): 463-486.
[64] Shah, T.A., Majeed, T., Rahman, SU, Ihsan T, Aziz T, Alharbi M, , Alasmari AF, Alshammari A. (2023). Synergistic treatment of crude enzymes from Bacillus sp. strains to boost anaerobic fermentation of rice straw. Biomass Conv. Bioref. 15, 465–475 (2025)
[65] Shankar, T., Isaiarasu, L. (2011): Cellulase production by *Bacillus pumilus* EWBCM1 under varying cultural conditions. – Middle-East Journal of Scientific Research 8(1): 40-45.
[66] Stolz, A. (2001): Basic and applied aspects in the microbial degradation of azo dyes. – Appl Microbiol Biotechnol 56: 69-80.
[67] Sun, J. Z., Scharf, M. E. (2010): Exploring and integrating cellulolytic systems of insects to advance biofuel technology. – Insect Science 17(3): 163-165.
[68] Thom, S. M., Horobin, R. W., Seidler, E., Barer, M. R. (1993): Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. – Journal of Applied Microbiology 74(4): 433-443.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984
2025, ALÖKI Kft., Budapest, Hungary

[69] Tokuda, H., Hayamizu, K., Ishii, K., Susan, M. A. B. H., Watanabe, M. (2004): Physicochemical properties and structures of room temperature ionic liquids. 1. Variation of anionic species. – The Journal of Physical Chemistry B 108(42): 16593-16600.
[70] Tokuda, H., Hayamizu, K., Ishii, K., Susan, M. A. B. H., Watanabe, M. (2005): Physicochemical properties and structures of room temperature ionic liquids. 2. Variation of alkyl chain length in imidazolium cation. – The Journal of Physical Chemistry B 109(13): 6103-6110.
[71] Vandenbossche, V., Brault, J., Vilarem, G., Hernández-Meléndez, O., Vivaldo-Lima, E., Hernández-Luna, M., Rigal, L. (2014): A new lignocellulosic biomass deconstruction process combining thermo-mechano chemical action and bio-catalytic enzymatic hydrolysis in a twin-screw extruder. – Industrial Crops and Products 55: 258-266.
[72] Wako, S. E. (2015): Behaviour and ecological impacts of termites: fecundity investigations in mounds. – Ekológia (Bratislava) 34(1): 72-81.
[73] Wenzel, M. (2002): The impact of outcome orientation and justice concerns on tax compliance: the role of taxpayers' identity. – Journal of Applied Psychology 87(4): 629.
[74] Wu, Z., Wang, S., Zhao, J., Chen, L., Meng, H. (2014): Synergistic effect on thermal behavior during co-pyrolysis of lignocellulosic biomass model components blend with bituminous coal. – Bioresource Technology 169: 220-228.
[75] Xie, R., Dong, C., Wang, S., Danso, B., Dar, M. A., Pandit, R. S., Sun, J. (2023): Host-specific diversity of culturable bacteria in the gut systems of fungus-growing termites and their potential functions towards lignocellulose bioconversion. – Insects 14(4): 403.
[76] Yarrow, D. (1998): Methods for the isolation, maintenance and identification of yeasts. – In: The yeasts, pp. 77-100. Elsevier.
[77] Yu, K., Chai, B., Zhuo, T., Tang, Q., Gao, X., Wang, J. Chen, B. (2023). Hydrostatic pressure drives microbe-mediated biodegradation of microplastics in surface sediments of deep reservoirs: Novel findings from hydrostatic pressure simulation experiments. Water Research, 242, 120185.
[78] Zambare, V. P. (2011): Optimization of Amylase Production from *Bacillus* Sp. Using Statisticsbased Experimental Design. – Emirates Journal of Food and Agriculture 23(1): 37-47.
[79] Zhang, Z., Li, J., Zhao, X., Wang, J., Wong, G. K-S., Yu, J. (2006): KaKs_Calculator: calculating Ka and Ks through model selection and model averaging. – Genomics, Proteomics & Bioinformatics 4(4): 259-263.
[80] Zhang, J., Lü, F., Shao, L., He, P. (2014a): The use of biochar-amended composting to improve the humification and degradation of sewage sludge. – Bioresource Technology 168: 252-258.
[81] Zhang, J., Sun, M., Liu, X., Han, Y. (2014b): Catalytic oxidative conversion of cellulosic biomass to formic acid and acetic acid with exceptionally high yields. – Catalysis Today 233: 77-82.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2302_19571984