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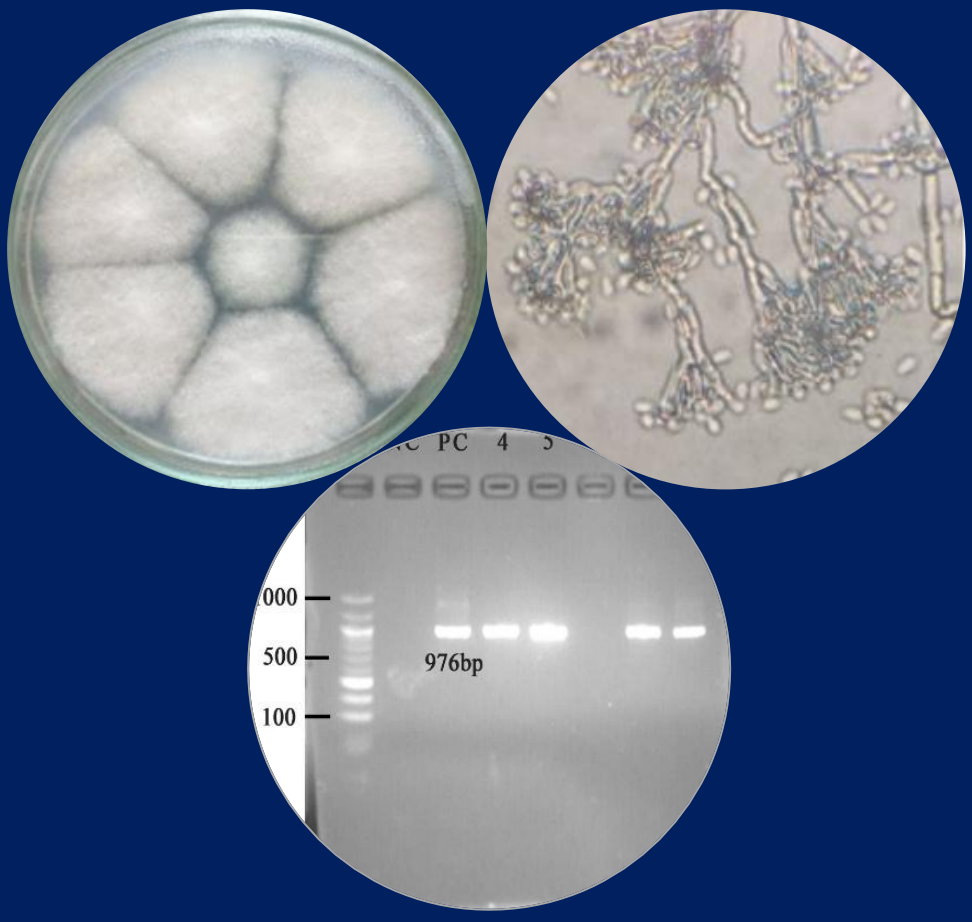

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Research Article

Antioxidant and Antidiabetic Potential of Extracts from Selected Species of *Smilax* L.

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Abstract

Smilax is a potential medicinal plant of the family Smilacaceae, distributed in Nepal's tropical, sub-tropical, and temperate zones. The present work focuses on preliminary phytochemical analysis, antioxidant properties, and antidiabetic activity of methanolic extracts from four different species of *Smilax*. Preliminary qualitative analysis had shown the presence of almost all tested secondary metabolites in methanolic extracts. Although, antioxidant activity was shown by all species of *Smilax*, the lowest IC_{50} value was found in methanolic extract of *S. ferox.* Percentage α-amylase inhibition activity was highest in the methanolic extract of *S. perfoliata* (79.13%) and lowest in *S. lanceifolia* (50.0%). Similarly, the highest percentage of α-Glucosidase inhibition activity was shown by the methanolic extract of *S. lanceifolia* (98.08%) and the lowest by *S. perfoliata* (29.71%). The present findings pave the way for a further comprehensive phytochemical investigation to validate their medicinal properties.

Keywords: α-amylase, α-glucosidase, antioxidant, flavonoids, phenol

Introduction

Smilax L., with about 255 known species (Xu & Chang, 2017), is the only genus of the family Smilacaceae. It is well-known in both temperate and tropical habitats. Species of *Smilax* are among the most abundant and easily recognized climbing plants in many ecosystems (Dong et al., 2021). They are distributed mostly in the forest areas of the Central and Eastern parts of China, Thailand, Vietnam, Myanmar, India and Nepal.

The species of *Smilax*, locally known as 'kukurdaino' are among the most important medicinal plants. In Nepal, there are 16 species of *Smilax* distributed mostly from tropical, to temperate zones, while a few species like *S. menispermoidea* DC and *S. minutiflora* A.DC are also found in the sub-alpine zone (Shrestha et al., 2022). These are among the multi-potential medicinal plants and are used by different tribes and communities of various parts of Nepal as well as other countries. The roots, leaves, and tender shoots are variably used in treating diseases like jaundice, skin problems, toothache, urinary complaints, muscular sprain, stomach pain, rheumatic arthritis, infertility, as a sexual stimulant, abnormal semen discharge, uterine diseases, dysentery, malaria, tuberculosis, wound healing, and also used as antibiotic, antifungal, antiseptic and blood purifier

(Sedai, 2010; Uprety et al., 2012; Kunwar et al., 2010). The plants are also used as vegetables in different countries. A single species has been used in various treatments for example *S. ovalifolia* root decoction is used in venereal disease, to increase appetite, and to cure different types of gastric disorders and sexual diseases (Shah, 2015). Besides, leaves and plants (Harba et al., 2009), shoots are also used as vegetables and unripe fruits are eaten (Acharya & Acharya, 2010).

Various authors have investigated the chemical constituents of *Smilax*. Preliminary phytochemical analysis of *S. zeylanica* revealed the presence of alkaloids, flavonoids, tannins, triterpenoids, and sterols in leaf and fruit extracts (Hossain et al., 2013). Additionally, several biological activities of *Smilax* extracts including anti-inflammatory (Hirota et al., 2016), antifungal (Belhouchet et al., 2008), anti-hyperuricemic (Chen et al., 2011), and antioxidant activities (Shah, 2015) have been also pointed out. Despite valuable medicinal properties, several species belonging to the genus *Smilax* are still unexplored phytochemically as well as for their bioactivity. The present study aimed to determine major phytochemicals in different species of *Smilax* and evaluate their biological activity to verify their ethnomedicinal potential and prospective pharmaceutical applications.

Materials and Methods

Collection and identification of plant materials

Plant leaves were collected from different parts of Kathmandu Valley during the flowering season. Identification was done with the help of comparison with sample species from the National Herbarium and Plant Laboratory (KATH), Godawari, Lalitpur.

Herbarium specimens were prepared and deposited in Tribhuvan University Central Herbarium (TUCH) of the Tribhuvan University for further reference. Details of collected sites and GPS coordinates are given below (Table 1, Figure 1).

Table 1: Collection sites of plant sample.

S.N.	Species	Collection site	Elevation (m)	GPS coordinates
1	*Smilax aspera* L	Lakuri bhanjyang, Lalitpur	1250	27° 61' 78" N, 85° 41' 43" E
2	*S. ferox* Wall. ex Kunth	Champadevi, Kathmandu	1610	27° 38' 71" N, 85° 15' 83" E
3	*S. lanceifolia* Roxb	Champadevi, Kathmandu	1650	27° 38' 71" N, 85° 15' 83" E
4	*S. perfoliata* Lour.	Champadevi, Kathmandu	1730	27° 38' 71" N, 85° 15' 83" E

Figure 1: Herbarium specimens of *Smilax* species; *S. aspera* (a), *S. ferox* (b), *S. lanceifolia* (c), *S. perfoliata* (d).

Preparation of plant extract and extract dilution

The collected plant materials were cleaned and powdered with the help of a grinder. Four-gram fine powder of each plant sample was taken separately and dissolved in 40 ml of 99% methanol. These samples were subjected to sonication for two hours and centrifuged in falcon tubes then the solvent was filtered and subjected to evaporation at reduced pressure in a rotatory evaporator. The condensed

extract thus obtained was transferred to a clean weighed glass petriplate and allowed to dry at room temperature. Then dried plant extract was removed from a petriplate with the help of a sterile blade and placed in small tubes. From the stock, 100 mg of crude plant extract was weighed accurately and dissolved in 1 ml methanol to make methanolic extracts and was used for the quantification of total phenolic content and total flavonoid content, and also for the evaluation of antioxidant and antidiabetic potential of plant extracts.

Qualitative phytochemical analysis

The methanolic extracts were used to screen for the presence of various secondary metabolites such as flavonoids, glycosides, steroids, terpenoids, alkaloids, tannins, and saponins by following the protocols suggested by Harborne & Baxter (1995) and Todkar et al. (2010).

Antioxidant activity via DPPH free radical scavenging assay

The antioxidant activity of the extract of four species of *Smilax* and standard (ascorbic acid) was evaluated based on the radical scavenging effect of the stable 1,1-diphenyl-2-picryhydrazyl (DPPH) free radical activity following the protocol of Singh et al. (2002). Different concentrations of plant extract and ascorbic acid (10-100 µg/ml) were prepared in methanol in the clean test tubes. A 0.5 ml sample of plant extract as well as ascorbic acid of each concentration was taken separately in clean test tubes. To this sample, 0.5 ml of the 0.2 mM DPPH solution was added. The tubes were shaken uniformly for proper mixing and incubated in the dark for half an hour. The control was prepared as above but without the plant extract or ascorbic acid, and methanol was taken as blank. Then absorbance was taken on a spectrophotometer at 517 nm. The radical scavenging activity was calculated using the following formula:

Percentage of DPPH radical scavenging activity = [(control abs. - sample abs.) / control abs.] * 100

A standard graph was plotted by the concentration of ascorbic acid on the X-axis and the percentage of DPPH scavenging activity on the Y-axis. Based on this standard graph IC_{50} was calculated using the linear equation of graph $Y = a* X + b$.

$$IC_{50} = (50 - b) / a$$

Where, X = concentration, Y = percentage of DPPH radical scavenging activity, a and b are the coefficient and constant of the linear equation. The IC_{50} value of the different species was compared. The species having the lowest IC_{50} was considered to have the best antioxidant properties.

Evaluation of antidiabetic activity

The antidiabetic activity was measured by α-amylase and α-glucosidase inhibition assays.

α-amylase inhibition assay

The α-amylase inhibition of extracts was assessed following the protocol of Ahmed et al. (2009) with modification. Firstly, the reaction medium was prepared by dissolving porcine pancreatic amylase (Sigma Aldrich, Germany) in 0.1 M potassium phosphate buffer (pH 6.8) to make a final concentration of 0.1 units/ml. Then 10 µl of pure methanol or methanolic solution of Acarbose (ARISTO Pharmaceutical Pvt. Ltd., India) or plant extract were mixed with 390 µl of reaction medium in a clean test tube. The respective negative control was also prepared by adding 200 µl of DNS reagent (Sigma Aldrich, Germany) to the reaction mixture. The tubes were incubated at 37 ˚C for 10 minutes. Then 200 µl of 1% soluble starch (Fisher Scientific, India) was added and the tubes were incubated for another 20 min. Then 200 µl of DNS reagent was added in all the tubes (positive control). The tubes were kept in a boiling water bath for 10 min and allowed to cool. Then 4 ml of distilled water was added in each tube and absorbance was taken at 540 nm in a spectrophotometer.

Percentage of α-amylase inhibition activity = (control abs. – sample abs./ control abs.) × 100

α-glucosidase inhibition assay

To test the α-glucosidase inhibition assay, the protocol of Si et al. (2010) was followed with slight modification. For this, the first reaction medium was prepared by dissolving Maltose (Sigma Aldrich, Germany) in 0.1 M Potassium phosphate buffer (pH 6.4) to a final concentration of 25 mM. Then 1 ml of the reaction mixture was taken in a clean test tube and 20 µl of pure methanol or methanolic solution of acarbose (1 mg/ml) or plant extract solution (1 mg/ml) was added to the previously taken reaction mixture solution. Respective negative controls were

also prepared for each methanol, acarbose, and plant extract. Now 250 µl of Na_2CO_3 was added in all test tubes of negative control. Then tubes were incubated for 5 minutes at 30°C. A 20 µl of α-glucosidase (Sigma Aldrich, Germany) was added in all test tubes including positive and negative control, and incubated for 45 minutes at 30 °C. Then 250 µl Na_2CO_3 was added in all tubes of positive control. At last, 710 µl of distilled water was added to make the volume of 2 ml and absorbance was measured at 405 nm in a UV spectrophotometer.

Percentage of α-glucosidase inhibition activity = (control abs. – sample abs./ control abs.) × 100

Data analysis

All the experiments were performed in triplicates for each sample and values were reported as mean ± S.D. The statistical analysis was done using Microsoft Excel 2013.

Results and Discussion

Qualitative phytochemical analysis

A summary of different tests performed on methanolic extracts from species of *Smilax* is tabulated in Table 2. Methanolic extracts showed the presence of almost all phytochemicals tested in different proportions except the absence of glycosides and the presence of saponin in the extracts of *S. aspera*. The preliminary phytochemical analysis provides a rough outline for the major secondary metabolites of the plant species. Different phytochemicals have been found to possess a wide range of activities, which may help protect against diseases. For example, alkaloids protect against chronic diseases, saponins protect against hypercholesterolemia and antibiotic properties, and steroids and triterpenoids show analgesic properties (Murali et al., 2011). Flavonoids and phenolic compounds in plants have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic, etc. (Dai & Mumper, 2010, Uthaman et al., 2024).

Several studies also showed the presence of alkaloids, phenol, flavonoids, tannins, steroids, and saponins in different species of *Smilax* (Dhanya Shree et al., 2018; Chandana et al., 2019; Paneru & Rajbhandari, 2020). Steroids, saponins, and flavonoids were thought to be the characteristic constituents of the genus *Smilax* (Ao et al., 2011). According to Saravanakumar et al. (2014), methanolic extract of *Smilax china* showed higher affinity for various phytochemicals than other solvents like chloroform, acetone, N- hexane, ethanol, and aqueous. As reported by Shah (2015) preliminary phytochemical analysis of the leaf extract showed the presence of varied phytochemicals like carbohydrates, protein, amino acid, saponin, alkaloid, steroid, terpenoid, phenol, glycoside and flavanoid in methanolic extract than aqueous, ethanolic, petroleum ether and chloroform extracts. In the present study, qualitative phytochemical analysis of methanolic extracts of different species of *Smilax* had shown varied phytochemicals like alkaloids, polyphenols, flavonoids, tannin, glycosides, terpenoids, and steroids suggested its potential against ailments.

Antioxidant activity

The antioxidant activity of species of *Smilax* was compared with standard ascorbic acid (Figure 2).

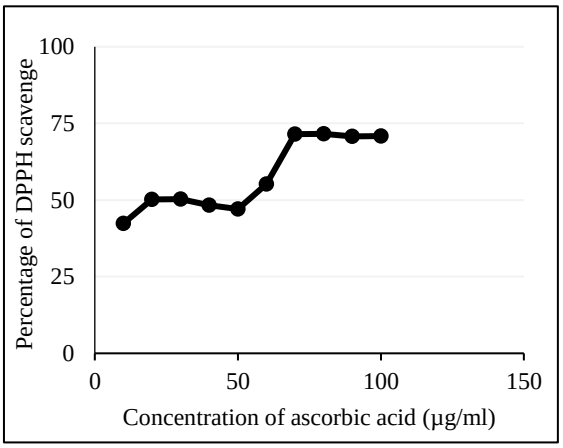

Figure 2: Standard curve of ascorbic acid.

The highest scavenging activity was seen in methanolic extracts of *Smilax ferox* and the lowest in *Smilax aspera* (Figure 3). The percentage radical scavenging activity of other species was found between these two extremes. Higher concentrations of plant extract showed an increasing percentage of radical scavenging activity in DPPH revealing concentration-dependent scavenging properties.

The antioxidant activity of different species of *Smilax* was compared with standard ascorbic acid. The highest IC_{50} value was obtained for the

methanol extract of *Smilax aspera* (188.29 µg/ml) and the lowest was found in *Smilax ferox* (48.05 µg/ml). As the lowest IC_{50} value shows strong antioxidant activity so graph shows *Smilax ferox* with strong antioxidant activity in comparison to other species of *Smilax* (Figure 4).

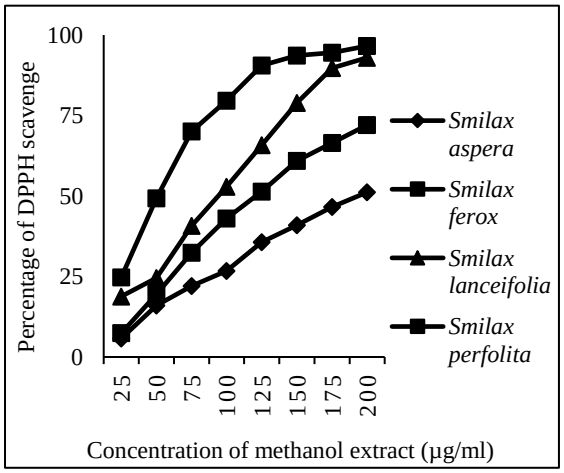

Figure 3: Percentage of radical scavenging activity by methanol extract of *Smilax* species.

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical-induced oxidative stress. There is an increasing interest in natural antioxidants, for example, polyphenols and flavonoids that are present mostly in medicinal plants, which might help to prevent oxidative damage (Antolovich et al., 2002). The DPPH is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants (Sanchez-Moreno, 2002). The methanol extract of *Smilax zeylenica* was found more effective than the aqueous extract (Murali et al., 2011). High amounts of phenol in the leaves resulted in the highest antioxidant capacities, as measured by DPPH, ABTS and FRAP assays in comparison to other samples of seed, stem and root (Acidri et al., 2020). The antioxidant activity depends on the extraction method and solvent used for extraction because the presence of various antioxidant compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent (Barchan et al., 2014). Barchan et al. (2014) found that polar solvents were important for obtaining fractions with high antioxidant activity. In the present study, the methanol extract of *Smilax ferox* showed strong antioxidant activity. Therefore, one of the possible mechanisms of methanol extract's strong antioxidant activity may be the presence of a good amount of phenolic and flavonoid contents as suggested by Uddin et al. (2015).

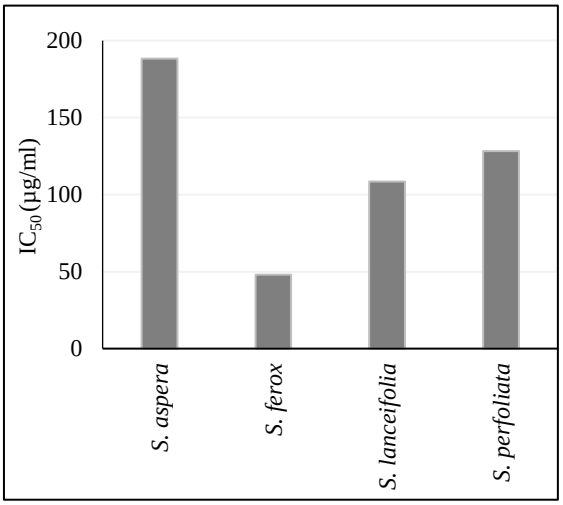

Figure 4: IC_{50} value of DPPH radical scavenging activity of methanol extracts of different *Smilax* species.

Antidiabetic activity

α-amylase inhibition activity

α-amylase inhibition activity of *Smilax* was found highest in the methanol extract of *S. perfoliata* (79.13%) and lowest in *S. lanceifolia* (50.0%) (Figure 5).

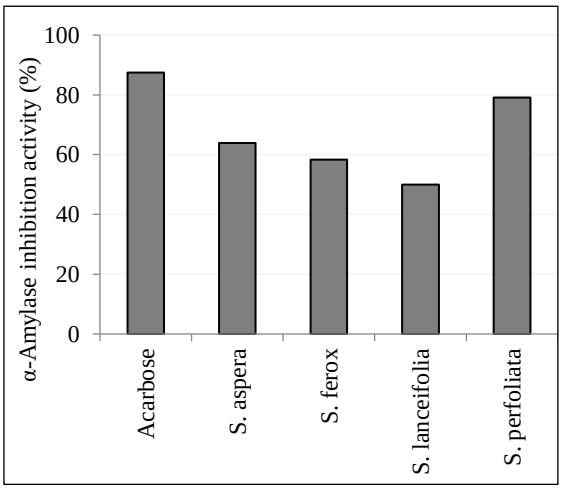

Figure 5: α-amylase inhibition activity of methanol extracts of *Smilax* species.

α-glucosidase inhibition activity

The highest percentage of α-glucosidase inhibition was shown by the methanolic extract of *S. lanceifolia* (98.08 %) and the lowest by *S. perfoliata* (29.71%) (Figure 6).

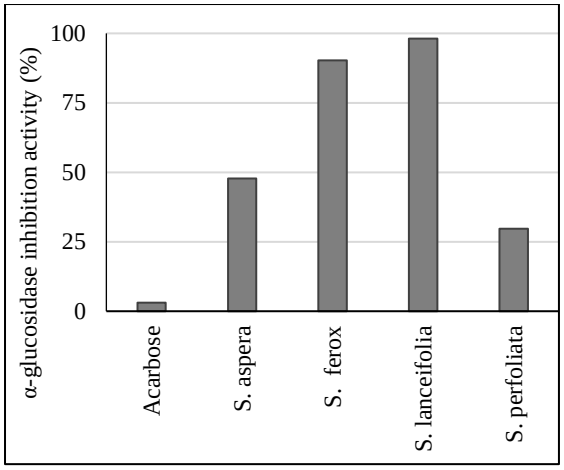

Figure 6: α-Glucosidase inhibition activity of methanol extracts of *Smilax* species.

Non-insulin-dependent diabetes mellitus or type-II diabetes mellitus is one of the most common and serious metabolic disorders with abnormally high blood glucose levels (hyper-glycaemia) due to defects in insulin secretion, action, or both (Rajesh & Perumal, 2014). Glucosidase and pancreatic amylase play a critical role in carbohydrate digestion and glycoprotein processing. So, the inhibitors of these enzymes might be used to treat diabetes (Perez-Najera *et al*., 2018). These inhibitors generally lower blood sugar levels by slowing or decreasing carbohydrate breakdown in the intestine (Nguyen et al., 2020). In *Smilax excelsa* different extract solvents had shown different inhibition activity. Among the solvents used, the methanol extract of leaves showed the highest (98.5%) α-amylase inhibition activity whereas α-glucosidase inhibition was the lowest (1.5%). Similarly, the ethanol extract of the stem also showed the highest (98.5%) α-amylase inhibition activity and α-glucosidase inhibition activity (58.9%) (Dehghan et al., 2016). According to Bhati et al. (2011), hydroalcoholic and aqueous extracts of *S. china* showed a significant reduction in blood glucose levels in comparison with the standard drug (gliclazide) in alloxan-induced diabetic rats. In the present research, *S. lanceifolia* and *S. ferox* had shown good α-glucosidase inhibition activity but α-amylase inhibition activity showed the least activity. The anti-diabetic effect of the extracts of *Smilax* species may be due to the presence of compounds like flavonoid, phenol, alkaloid, and other related compounds which could be beneficial to carbohydrate metabolism. This required further exploration and study to identify the detailed molecular mechanism of action.

Conclusion

The present study revealed variations in phytochemical constituents, antioxidant potential and antidiabetic activities among the methanolic extracts of different species of *Smilax*. For example, extracts of *S. ferox* possess the highest antioxidant potential than others. Similarly, extracts of *S. perfoliata* show the highest α-amylase inhibition activity comparable to the acarbose (control). Furthermore, the extracts of all tested species show higher α-glucosidase inhibition activity than acarbose. This is preliminary information that came out from in vitro studies. However, further in vivo scientific work will be recommended to ensure the medicinal properties of these plants.

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Research Article

Isolation and Purification of L-Asparaginase Producing Endophytic Fungi from *Ocimum tenuiflorum* L.

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Abstract

Endophytic fungi are essential sources of bioactive chemicals and enzymes. L-asparaginase is a key enzyme used in the treatment of acute lymphoblastic leukemia and lymphoblastic lymphoma. However, the usage of commercial L-asparaginase generated from prokaryotes is limited due to severe clinical hypersensitivity. Studies showed that L-asparaginase from eukaryotic sources can significantly reduce allergy reactions. Thus, nowadays, researchers are looking for eukaryotic sources to manufacture L-asparaginase. This work isolated and purified four endophytic fungi known as (Tulsi 'a', 'b', 'c', and 'd') from the leaf tissue of *Ocimum tenuiflorum* (Holy Basil). Morphological analysis revealed Tulsi 'b' corresponds to *Microsporum* sp., Tulsi 'c' corresponds to *Penicillium* sp., while Tulsi 'd' corresponds to *Trichoderma* sp. Tulsi 'a' did not produce spores so morphological identification of Tulsi 'a' was done. Screening for L-asparaginase biosynthesis on modified Czapek Dox (MCDox) medium showed varying activity: Tulsi 'b' and 'c' exhibited a faint pink hue (pH-dependent), but Tulsi 'a' and 'd' caused the medium to turn yellow. Submerged fermentation followed by nesslerization method was used to measure the concentration of ammonia formed. One unit of asparaginase catalyzes the formation of 1 μM of ammonia per minute. So, amount of ammonia in the sample gives enzyme activity. Tulsi 'a' demonstrated the maximum activity (31.24 U/ml) on day 5, while Tulsi 'd' reached its peak on day 16 (77.43 U/ml). Temporal variations in activity indicate phase-dependent enzyme synthesis. These findings highlighted the potential of endophytic fungi from *O. tenuiflorum* as a source of L-asparaginase could serve as an alternative to bacterial-derived enzymes in medical and industrial applications.

Keywords: Endophyte, L-asparaginase, Nesslerization, *Ocimum tenuiflorum*

Introduction

Endophytes are microorganisms that live in the internal tissues of living plants without harming them. They are found in all plants and are highly valuable since they are known to produce a variety of beneficial bioactive chemicals that play a role in phytopathogen defense mechanisms. Endophytic bioactive chemicals have been studied in recent years and found to have a wide range of antibacterial, anticancer, antioxidant, and anti-inflammatory properties (Chow & Ting, 2015).

L-Asparaginase (E.C. 3.5.1.1) is one of the enzymes being researched for its production from endophytes. It catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Yap et al., 2021). This enzyme is used to treat disorders such as acute lymphoblastic leukemia in conjunction with vincristine and a glucocorticoid (such as dezamethasone) (Vimal & Kumar, 2017). It is also used to treat autoimmune illnesses, canine and feline cancer, and has antibacterial properties (Vimal & Kumar, 2017). In the food business, it is used to make acrylamide-free food. Commercial L-asparaginase for clinical use is derived from the bacterium *E. chrysanthemi* and *E. coli* (Vimal & Kumar, 2017). However, clinical hypersensitivity in individuals receiving L-asparaginase has resulted in substantial drawbacks (Narta et al., 2007). Prokaryotic bacterial enzymes cause a modest to severe immunological response (Manasa & Nalini, 2014). Eukaryotic fungal L-asparaginase has been examined since it has fewer adverse effects than prokaryotic L-asparaginase (Kumar et al., 2016).

O. tenuiflorum known as Holy Basil in English and Tulsi in Nepali, is a member of the *Ocimum* genus in the Lamiaceae family of plants. It is a perennial plant which is about 30-60 cm tall. It is an erect and branched herb and have hair. The flower may be white or purple in a green or purplish stem. It is a traditional plant used for healing properties in Ayurveda. Different parts of plant like leaf, stem, root, flower and seed are used for the treatment of diseases such as bronchitis, arthritis, malaria diarrhea, dysentry, skin diseases, insect bites and many more (Pathak & Niraula, 2019). *O. tenuiflorum* contains a variety of therapeutic qualities, including antioxidant, anti-diabetic, anti-inflammatory, anti-cancer, anti-fertility, anti-helmintic, antibacterial, and cardio protective effects (Singh & Chaudhuri, 2018; Bhattarai et al., 2024). *O. tenuiflorum* contains phytochemicals including euginol, euginal, ursolic acid, carvacrol, linalool, limatrol, caryophyllene, methyl carvicol, rosmarinic acid, luteolin, ursollic acid, and limonene (Pattanayak et al., 2010).

Bacillus stratosphericus endophytic bacteria were isolated from *O. tenuiflorum* and showed considerable L-asparaginase production (Pola et al., 2018). Endophytic fungi identified from *O. tenuiflorum* include *Collectrichum* sp., *Pleosporales* sp., *Phomopsis* sp., *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., *Diaporthe* sp., *Rhizoctonia* sp., and *Chaetomium* sp. (Manikandan & Ramanathan, 2023). Different enzymes, such as protease, amylase, cellulase, and laccase have been reported from endophytic fungi isolated from *O.tenuiflorum*. One of the enzymes produced by *O. tenuiflorum* is L-asparaginase. *O. tenuiflorum*, a medicinal herb utilized in Ayurveda, possesses several endophytes, although study on its fungus population is limited. Thus, this study aimed to isolate endophytic fungi from *O. tenuiflorum* and evaluate their L-asparaginase production, with a focus on their potential as biocatalysts for medical and industrial applications.

Materials and Methods

Collection of plant sample and isolation of fungi

Healthy *O. tenuiflorum* leaves was collected from the local garden cultivation in Bagdol, Lalitpur district [27.6681° N; 85.3039° E]. Isolation of fungi was done by the method given by (Kumar et al., 2016). The plant sample was thoroughly washed with distilled water and then soaked in 4% sodium hypochlorite solution for one minute. Then it was surface sterilized with 90% ethanol.

After complete sterilization, the plant parts were cut into 2 mm x 2 mm pieces and seeded into PDA plate equidistantly at six points. The seeded petri plates were then incubated at 28 ± 2 °C for 1 to 2 weeks. The mycelial growth in the petri plate where the plant parts were seeded indicated the production of endophytic fungi.

Morphological characteristics of fungi

Morphological characteristics were observed macroscopically and microscopically. Macroscopic observation included the texture, colour of fungi at center and margin, colour of fungi in the reverse side, shape, presence or absence of exudates and sulcation, initial colour of colonies and colour of colonies after maturation. Similarly, microscopic observation was done under 4x, 10x, 40x and 100x magnification on the optical microscope (Alsohaili & Bani-Hasan, 2018).

Screening of L-asparaginase producing fungi

The screening of L-asparaginase producing fungi was done using the method given by (Chow and

Ting, 2015). The mycelial plug of the fungi was transferred to the modified Czapek Dox (MCDox) media [contains agar 20.0 g/l; glucose 2.0 g/l; L-asparagine 1.0 g/l; KH_2PO_4 1.52 g/l; KCl 0.052 g/l; $MgSO_4$.7H2O 0.052 g/l and trace amount of $CuNO_3.3H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$] at 6.8 pH and incubated at the temperature of 28 ± 2 °C. Phenol red indicator was used to indicate whether the given fungi produced L-asparaginase or not. For control, the MCDox agar medium did not contain L-asparagine but $NaNO_3$ as nitrogen source. Activity was indicated by pink (alkaline pH) or yellow (acidic metabolites) zones.

Enzyme activity assay

L-asparaginase converts L-asparagine into aspartic acid and ammonia. The amount of ammonia produced during the reaction is determined by Nesslerization method as it is highly specific for ammonia. The amount of ammonia produced during the reaction is equal to the enzyme activity of L-asparaginase enzyme (Zhao et al., 2019). In this study, the method given by (Hatamzadeh et al., 2020) is followed for the determination of L-asparaginase enzyme activity. The five days old mycelial plug was placed in the MCDox broth and incubated in a shaker at 150 rpm for five days at the temperature of 28 ± 2 °C. On the 5th day, 100 µl of broth, 100 µL of 0.1 M Tris-HCl (pH 7.2), 200 µl of 0.04 M L-asparagine and 100 µl of sterile distill water was incubated at 37 ± 2 °C. After 1 hour, 100 µl of TAA was added to the mixture to stop the enzymatic reaction.

From this, 100 µl of solution was taken and 750 µl of sterile distill water and 300 µl of Nessler's reagent was added. It was allowed to incubate at 28 ± 2 °C for 20 mins. The UV absorbance of the enzyme was measured at λ_{max} which was found to be 450 nm. The same procedure was done for the MCDox broth on 16th day. One unit of asparaginase is expressed as the amount of enzyme that catalyzes the formation of 1 µmol of ammonia per minute.

The concentration of ammonia in the broth was measured by using the formula adopted by Aisha et al. (2022).

$$\text{Concentration of ammonia in sample (µg/ml)} = \text{Abs. of sample at 450 nm / slope}$$

Similarly, enzyme activity (EA) was measured following Hatamzadeh et al. (2020).

$$EA = (\text{Conc. of ammonia} \times VTS) / VS \times T \times AA$$

Volume of Total Solution (VTS) = 0.6 ml
Volume of Sample (VS) = 0.1 ml
Time in Minutes (T) = 60 minutes
Amount of Asparagine (AA)= 0.2 ml

Results and Discussion

Isolation and morphology of Fungi

Four endophytic fungi (Tulsi 'a', Tulsi 'b', Tulsi 'c', and Tulsi 'd') were isolated from *O. tenuiflorum* leaves. Tulsi 'b', Tulsi 'c' and Tulsi 'd' exhibited sporulation, with morphological features resembling *Microsporum*, *Penicillium* and *Trichoderma* species respectively (Kidd et al., 2022; Tobeigei et al., 2023). Tulsi 'a' did not produce spores. All the isolated fungi were obtained from the leaf tissue of the sample plant as shown in Figure 1.

Screening of L-asparaginase producing fungi

During screening of L-asparaginase production, the isolated fungi Tulsi 'a' and Tulsi'd' did not produce pink colour but there was slight change in colour of the media to yellow in Tulsi 'c'. Tulsi 'b' showed slight pink colour in the culture media. The change in colour was due to the catalytic hydrolysis of L-asparagine into aspartic acid and ammonia.

Enzyme activity

The enzyme activities of the isolated fungi were studied for the 5th and 16th day. On the fifth day, the highest enzyme activity was observed for Tulsi 'a' which was 31.24 U/ml and the least was observed for Tulsi 'd' with 21.98 U/ml. Similarly, for the 16th day, the highest L-asparaginase activity was shown by Tulsi 'd' with 77.43 U/ml and the least was for Tulsi 'c' 21.07 U/ml. The activity for all the isolates were shown in Figure 2. This result suggested that the enzyme activity of the isolated fungi varied with time. Tulsi 'd' showed higher enzyme activity after some time than other isolates. In isolates Tulsi 'a', Tulsi 'b' and Tulsi 'd', enzyme activity increased from day 5 to day 16. Tulsi 'c' showed a decline in activity from 25.43 U/ml (day 5) to 21.07 U/ml (day 16).

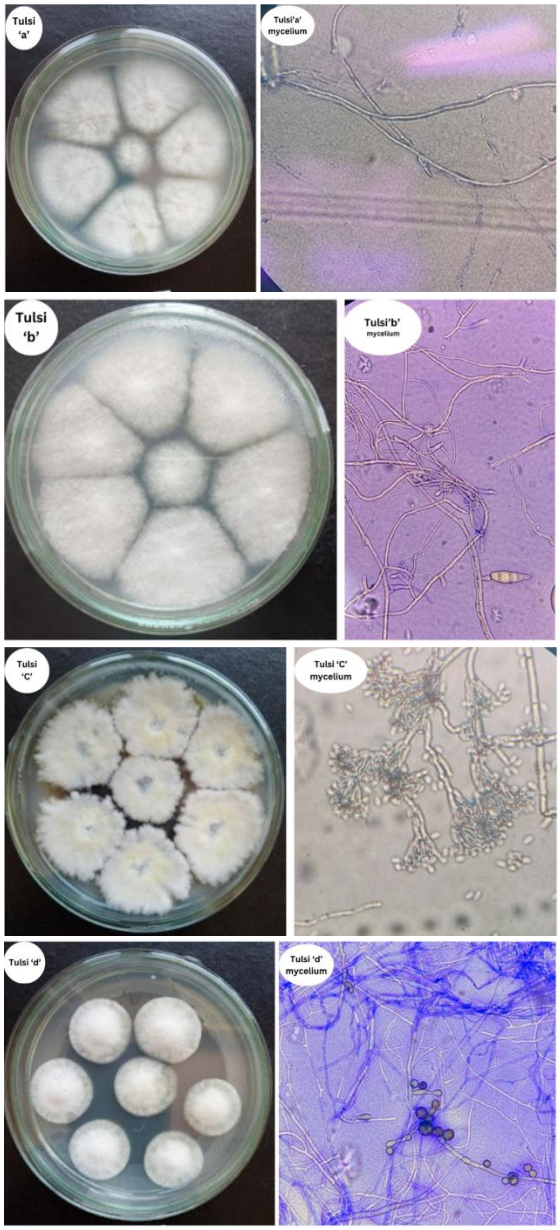

Figure 1: Morphological view of endophytic fungi isolated from *O. tenuiflorum* and microscopic observation of hyphae and sporulation in isolated fungi.

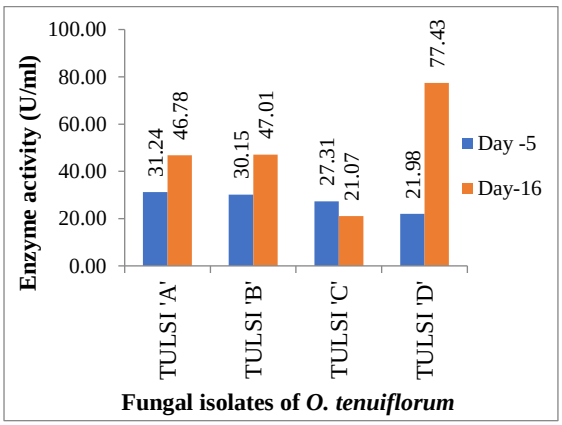

Figure 2: Enzyme Activity of isolated fungi of *O. tenuiflorum* on day 5 and day 16.

The study found that endophytic fungi from *O. tenuiflorum* can produce L-asparaginase, with activity levels changing between isolates and incubation periods. Tulsi 'a', Tulsi 'b', and Tulsi 'd' enzyme activity appears to be increasing over time, possibly because they are entering a secondary metabolic phase that enhances enzyme production. Tulsi 'c', on the other hand, has decreased activity over time, which can be attributed to nutrient depletion or autolysis.

The results of this investigation are highly aligned with previously reported literature (Cheruiyot et al., 2024), who isolated a *Penicillium* sp. with peak L-asparaginase activity on day 6, followed by a gradual decrease (Cheruiyot et al., 2024). Ahiravan & Gnanadoss (2022) had reported isolation of 10 endophytic fungi from *O. tenuiflorum*, among which five isolates showed positive L-asparaginase activity. The highest activity was shown by *Fusarium* sp. which was 9.36 ± 0.52 U/ml (Kathiravan & Gnanadoss, 2022). Notably, Tulsi 'd', tentatively identified as *Trichoderma* sp. based on conidial morphology, showed a significant increase in enzyme activity over time (21.98 U/ml on day 5 to 77.43 U/ml on day 16). To our knowledge, this is the first documentation of L-asparaginase production by *Trichoderma* sp., therefore augmenting the repertoire of fungal genera with biotechnological potential.

Comparably, Tulsi 'b,' displaying morphological traits like *Microsporum* sp., showed higher activity from 27.15 U/ml (day 5) to 45.67 U/ml (day 16) (Tobeigei et al., 2023). *Microsporum* is usually known as a dermatophyte, but its enzymatic properties are yet unknown, so this finding is unique. But, the molecular identification of these isolates, specifically *Trichoderma* and *Microsporum* is essential to verify their taxonomy and associate genetic characteristics with enzymatic activity.

The limits of phenotypic assays are shown by the difference between colorimetric screening (pink zone) and quantitative enzyme activity. For example, Tulsi 'a' lacked a pink zone but showed great activity (31.24 U/ml on day 5), most likely from melanin or another pigment interfering. The findings indicate the potential of endophytic fungi as a safer alternative to bacterial enzymes and a sustainable source of L-asparaginase. Future studies should look into the genetic underpinnings of enzyme synthesis and improve cultural settings including pH levels, carbon sources, temperature etc.

Conclusion

This study demonstrated that the amount of L-asparaginase generated varied even across endophytes isolated from the same plant. Furthermore, this work supports the hypothesis that endophytes derived from medicinal plants have therapeutic capabilities. Because endophytes have varied growth rates and enzyme activity, growing strategies must be tuned to get larger yields and increased L-asparaginase activity.

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Research Article

Nutritional Composition of Wild Edible Mushrooms *Scleroderma cepa* and *Laccaria laccata*

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Abstract

Wild edible mushrooms are widely recognized for their high nutritional value and used as food. This study analyzed the macronutrient and mineral composition of *Laccaria laccata* and *Scleroderma cepa*, focusing on moisture, ash, protein, fiber, fat, carbohydrate, calcium, phosphorus and iron. The quantitative estimation of these components was conducted using standard analytical techniques: oven-dry method for moisture, Soxhlet extraction for fat, Kjeldahl digestion for protein, ignition method for ash, proximate analysis for carbohydrates, acid-base digestion for crude fiber, complexometric titration for calcium, molybdenum blue method for phosphorus, and colorimetric analysis for iron. The results revealed that carbohydrates were the most abundant macronutrient (54.93–60.42%), while fat was present in the low amount (0.38–0.55%). Among the minerals, phosphorus content ranged from 424.9 to 507.72 mg/100 g, calcium from 182.83 to 243.16 mg/100 g, and iron from 43.25 to 48.14 mg/100 g. These findings highlight the nutritional significance of these wild edible mushrooms reinforcing their potential as valuable dietary food supplements.

Keywords: *Laccaria laccata*, Macronutrient, Mushroom, Nutrient analysis, *Scleroderma cepa*

Introduction

Wild edible mushrooms have been consumed as food for centuries due to their rich nutritional value and they are also utilized as traditional medicine for health benefit. They are highly valued for their unique taste and texture. They serve as a significant source of essential nutrients, including proteins, carbohydrates, dietary fibers, vitamins, and minerals, while being low in calories and cholesterol (Demirbas, 2002; Mendil et al., 2004). Compared to many conventional food sources, mushrooms contain high-quality proteins and bioactive compounds, making them an excellent dietary choice for improving human health (Chang & Miles, 2004; Kakon et al., 2012).

The nutritional composition of wild edible mushrooms varies depending on species, habitat, and environmental conditions. Many species are rich in essential amino acids, unsaturated fatty acids and antioxidants, which contribute to their role in boosting immunity, reducing inflammation and preventing various chronic diseases (Kalač, 2009; Valverde et al., 2015). Additionally, wild edible mushrooms provide a significant number of micronutrients such as potassium, phosphorus, iron, zinc and selenium, which are crucial for metabolic

functions and overall well-beings (Mattila et al., 2000; Agahar-Murugkar & Subbulakshmi, 2005).

Despite their nutritional and medicinal significance, research on the composition and potential health benefits of wild mushrooms remains limited, particularly in regions with high fungal biodiversity. According to previous studies, mushrooms with low fat content are recommended as a good source for the people with cardiac problems (Pandey and Budhathoki, 2006; Upadhyaya et al., 2017; Shrestha et al., 2023). Therefore, this study aims to analyze the nutritional composition of wild edible mushrooms.

Materials and Methods

Collection of mushrooms

Two wild edible mushrooms species; *Scleroderma cepa* Pers. and *Laccaria laccata* (Figure 1) were collected from the Chaukot community forest in Kavreplanchowk district of central Nepal (27°37′N latitude and 85°33′E longitude, and elevation range of 1,500 to 1,700 meters). The forest is characterized by montane subtropical *Schima-Castanopsis* forest, dominated by *Pinus roxburghii*, *Schima wallichii*, *Myrica esculenta*, *Rhododendron arboreum*, and *Castanopsis tribuloides*.

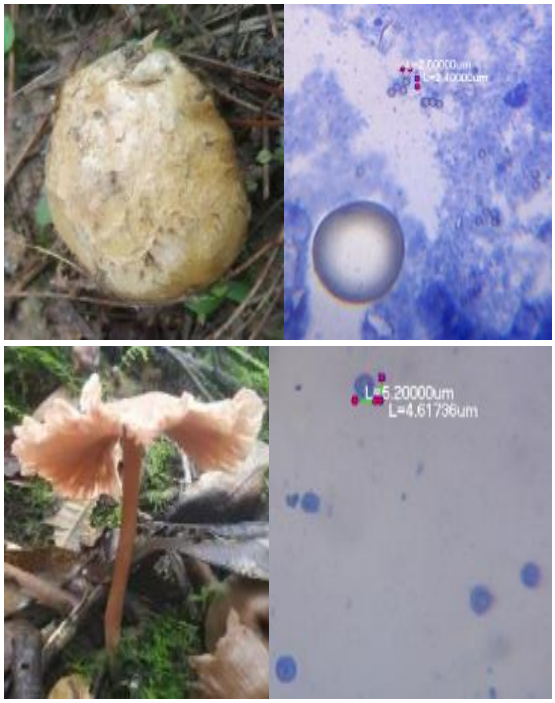

Figure 1: *Scleroderma cepa* with its spores (above) and *Laccaria laccata* with its spores (below).

Sample preparation

The mushrooms were thoroughly cleaned to remove any mud, dried on blotting paper, sliced without dividing the pileus and stipe, powdered to about 1 mm particle size, and stored at room temperature in polyethylene bottles until analysis (Mallikarjuna et al., 2013).

Determination of macronutrients

Nutrient contents in the wild edible mushroom species were determined in the National Agricultural Research Council (NARC) according to the Handbook of Analysis and Quality Control for Fruit and Vegetable Product (Ranganna, 2011).

Moisture content determination

The moisture content of the mushroom samples was determined using the hot air oven drying technique. In tarred oven dried crucible, 2 g air dried sample was placed and heated to 110 °C in hot air oven until its weight stayed constant. The dried sample allowed to cool in a desiccator until its final weight was determined. The percentage of moisture content was determined by using a formula given by (Raghuramulu *et al*., 2003).

Percentage of moisture content = (Fresh wt. – Dried wt.) / Fresh wt. x 100

Total ash content

A dried clean crucible was weighed. A dry sample of 1 g was measured. The samples were placed inside a muffle furnace at 525 ˚C and lit on a hot plate for 6 hours. After the entire process, the crucible with the ash was cooled in a desiccator and the sample's ash content and the crucible's final weight were determined. The following formula is used to get the percentage of ash content.

Percentage of total ash content = (Wt. of ash / Wt. of dried sample) x 100

Protein content determination

Kjeldal digestion technique was used to determine the total protein content. About 3 g of powdered was mixed with 10 g digesting mixture in the presence of 10 ml of Conc. H_2SO_4. It was heated till the solution turned transparent blue and white fumes started to form. Following digestion, the flask was allowed to

cool for 20 to 30 min at room temperature. Then, using a pipette, the digested sample was transferred into a volumetric flask, the volume was adjusted with distill water, and the flask was sealed.

The equipment was set up for distillation with cold water running through it constantly. A conical flask was filled with 5 ml of 2% boric acid, 4 drops of mixed indicator. Filling the burette with 0.01 N HCl. The steam trap liquid was then removed by opening the pinched clamp. Next, set the boric acid-filled conical flask underneath the condenser. After pipetting 5 ml of the digested material into the distilling flask, the funnel was washed with distilled water. When steam entered the distillation flask, 10 ml of 30% NaOH was added, stirring the sodium hydroxide and digestion mix. Thus, released ammonia escapes into the boric acid solution through the condenser along with steam, creating a solution that is bluish green.

Percentage of total Nitrogen = (14 x (V – V1) x 100 x S) / W x 1000

Percentage of total protein = Total Nitrogen (%) x 6.25

Where, 14 is molecular weight of Nitrogen, 6.25 is conversion factor, V = volume of standard acid used to neutralize the distillate, V1 = volume of standard acid used to neutralize the blank, S = Normality of standard acid, W = Weight of sample taken for digestion

Fat determination

The Soxhlet extraction method was used to assess the fat content of a sample of mushrooms. An oven-dried powdered sample weighing 10 g was kept in a thimble. Next, cotton is folded and inserted into the thimble so as to cover the sample. A dried round-bottom flask weight was noted. After that, the sample and thimble were put inside the Soxhlet apparatus, where they were extracted using petrolium spirit for four to five hours. In an evaporating dish covered in tar, the solvent was evaporated and then weighed. The following calculation was used to determine fat percentage (AOAC, 2005).

Percentage of fat = (M1 – M2)/E x 100

Where, M1= wt. of round bottom flask, M2 = wt. of round bottom flask with fat, E= wt. of sample

Carbohydrate determination

Total carbohydrate was calculated by using AOAC (Association of Official Analytical Chemists), 18th edition official method (Horwitz & Latimer, 2005).

Percentage of total carbohydrate = 100 – (Ash % + Fat % + Protein %)

Crude fiber determination

Acid base digestion method was used to quantify amount of crude fiber (AOAC, 2000). Firstly, 0.5- 1 g sample was taken in crucible and assembled in (fibrotron) crude fiber instrument connected with condenser. Then, digestion is carried out with 1.25% H_2SO_4 for 30 min. It was washed with warm H_2O to remove excess H_2SO_4. For digestion, 1.25 % of NaOH was used for 30 min followed by wash with warm water to remove excess NaOH. It was then wash with alcohol. The crucible was heated at 110 °C to constant weight and cooled in desiccator and weighted. The content of the crucible was ignited in muffle furnace for 20 min. Finally, it was cooled and weighted to get crude fiber quantity.

Percentage of Fiber = (Wt. of dried sample – Wt. of ash) / Wt. of dried sample x 100

Determination of minerals

Preparation of ash solution

The ash solution was prepared by adding 25 ml of 10% HCl in the ash obtained from ashing. The solution was filtered through Whatman filter paper no. 1 and volume was made up to 100 ml.

Phosphorus content

About 5 ml of ash solution obtained by dry ashing and 5 ml of molybdate reagent was mixed well. Aminonalphtholsulphonic acid solution of volume 2ml mixed and made the volume to 50 ml. Blank solution was prepared similarly using water in place of the sample. The sample solution was allowed to stand for 15 min and colour had been measured at 650 nm settings the blank at 100% transmission.

Phosphorus content (mg/100 g) = (mg of P in ash solution x vol. of ash solution x 100) / (ml. of ash solution x wt. of sample)

Standard curve of phosphorus

A 10 ml standard potassium phosphate solution was diluted by using 10 ml water. In a 50 ml of volumetric flask 40 ml of aliquot Pipetted out. Then 5ml of molybdate reagent was added and mixed. After that, 2 ml of aminonaphtholsulphonic acid reagent was added and mixed. The final volume was made 50ml and measured color as in sample. The plot concentration against absorbance was made.

Calcium content

An aliquot of 50 ml of the ash solution and 50 ml distilled water was pipetted into 250 ml beaker and 10 ml of saturated ammonium oxalate solution and 2 drops of methyl red indicator was added. To make solution slightly alkaline dil. ammonia was added dropwise until the color turns yellow and few drops of acetic acid was poured until it gets faint pink color to make the solution slightly acidic. Then, solution was heated to the boiling point and left overnight. The solution was filtered using Whatman filter paper no. 42 and precipitate was washed with distilled water. The precipitate was dissolved in dilute Sulphuric acid to make volume of 200ml. Thus, prepared 25 ml solution was pipetted in a conical flask and titrated with 0.01 N $KMnO_4$ until a persistent pink endpoint was achieved.

Calcium content (mg/100 g) = (Titer x N of KMnO4 x 20 x volume of ash x 100) / (volume of ash solution x wt. of sample)

Iron content

Iron content in mushroom samples was determined by a colorimetric method. A blank solution was prepared by using 0.5 ml of concentrated sulfuric acid (H_2SO_4), 15 ml of distilled water, 1 ml of potassium persulfate solution, and 2 ml of potassium thiocyanate solution. The solution of 10 ml of the mushroom sample extract, 5 ml of distilled water, 0.5 mL of concentrated H_2SO_4, 1 ml of potassium persulfate solution, and 2 ml of potassium thiocyanate solution was made in a separate container and allow the mixture to react for approximately 15 minutes to ensure complete color development. Similarly, a standard solution was prepared by combining 1 ml of a known iron standard solution, 14 ml of distilled water, 0.5 ml of concentrated H_2SO_4, 1 ml of potassium persulfate solution, and 2 ml of potassium thiocyanate solution and let it react for 15 minutes. After the reaction time, the absorbance of the sample, blank, and standard solutions were measured at 480 nm using a colorimeter.

Iron content (mg/100 g) = (Optical density of sample x 0.1 x total volume of ash solution x 100) / (Optical density of standard x 5 x wt. of sample)

Statistical analysis

Excel was utilized to assess the mean value of nutrients among species and conduct an independent sample T-test. Significance was recognized at the 5% significance level. To ensure that the results are accurate, the analysis was done three times. The experimental result was given as the mean ± standard error.

Results and Discussion

Nutrient analysis

Two species namely, *Laccaria laccata* and *Scleroderma cepa* were edible mushrooms whose nutrients analyzed. We performed analysis of six macronutrients (fiber, protein, fat, ash, carbohydrates and moisture) and three micronutrients (iron, phosphorous and calcium). All nutrient analysis was carried out on dry weight basis.

Macronutrient profile

Mushroom contained the highest percentage of carbohydrate (54.93-60.42) % followed by fiber (37.46-48.86) % and protein (22.95-24.14) % and lowest fat percentage (0.38-0.55) (Figure 2). Null hypothesis was rejected from the independent sample t-test and shows a difference between the *Scleroderma cepa* and *Laccaria laccata* with respect to the dependent variable moisture %, carbohydrate %, and fiber %. There was sufficient evidence to say that the result is statistically significant. However, the independent sample t-test with unequal variances results acceptance of null hypothesis in case of protein %, fat% and ash % of samples. The $p>0.05$ illustrates that there was not significant difference in protein, fat and ash of two species.

Depending on the kind of mushroom, nutrient level differed because of their ability to bioaccumulate the nutrients into their cells (Mshandete & cuff, 2007). Mushrooms that have been air-dried may have as little as 5–20% moisture whereas fresh mushrooms typically have 85–95% moisture (Crisan & Sands, 1978). In our research, mushrooms sample were air dried and we found out that the moisture content of *Scleroderma cepa* was lesser (6.1%) than that of *Laccaria laccata* (11.08%) resembles with the findings of Wu et al. (2023) which states that the dense basidiome structure of *Scleroderma cepa* contributes to lower moisture content compared to other fungi because the compact morphology limits water retention. Mushrooms having more moisture spoil quickly due to susceptibility to enzymatic and microbiological degradation (Bano, 1976; Djamila et al., 2020).

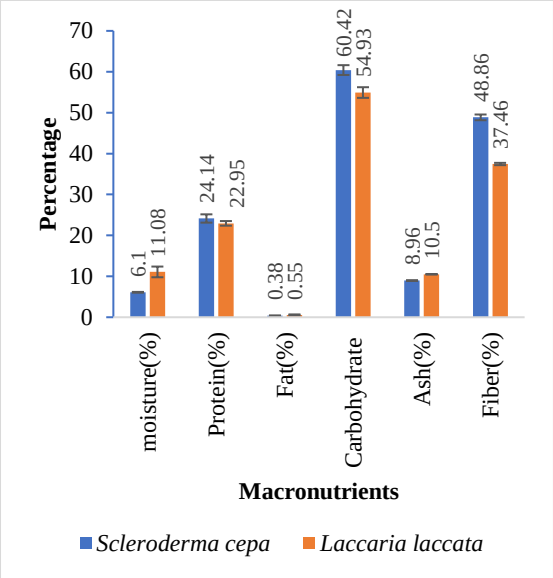

Figure 2: Macronutrient constituent in two species of mushrooms.

Crude protein content in edible mushrooms usually ranges from 19 to 40% (Kurtzman, 1978). According to our finding, *Laccaria* lacc*ata* had lower protein content (22.95%) than *Scleroderma cepa* (24.14%), as protein content fluctuates depending on the growing environment supported by the research of EC et al. (2011) stating that denser fungi like *Scleroderma cepa* tend to accumulate more protein due to their compact structures. Ash contents in two wild mushrooms range from 8.96% to 10.5% of the total weight of the mushroom. These results were similar to those published by (Singha et al., 2017; Shrestha et al., 2021) and lesser than as reported by those of (Panday & budhathoki, 2007; Egwim et al., 2011). Ash content varies in mushrooms may be due to substrate composition (Boadu et al., 2023). Mushrooms appear to be an excellent source of energy in the diet based on the measured content of carbohydrate. The results showed that *Laccaria laccata* had 54.93% carbohydrates in its total weight which is almost identical to the data from Shrestha et al. (2021). A substantial amount of carbohydrate is dietary fiber (Hamano, 1997). Mushrooms contain both soluble and insoluble dietary fiber (Park & Nile, 2014). Dietary fiber content in *Scleroderma cepa* was 48.86%. Fiber in both mushrooms differ in their content due to the fiber composition of edible mushrooms changes substantially depending on their morphological phases, such as the fruit body, mycelium, and sclerotium (Cheung, 2013). *Scleroderma cepa* and *Laccaria laccata* had fat contents of only 0.38% and 0.55%, respectively which indicate they may be an appropriate healthy diet for local people of Chaukot (Jequier & Bray, 2002).

Micronutrient profile

Phosphorus (424.9–507.72 mg/100 g) was the most prevalent micro element in all samples. It was followed by calcium (182.83-243.16 mg/100 g) and iron (43.25–48.14 mg/100 g) (Figure 3). Both of the micronutrients (phosphorus and calcium) were significantly higher ($p<0.05$) in *Laccaria laccata* compared to *Scleroderma cepa*. The iron content showed no significant differences ($p>0.05$) between two wild edible mushrooms.

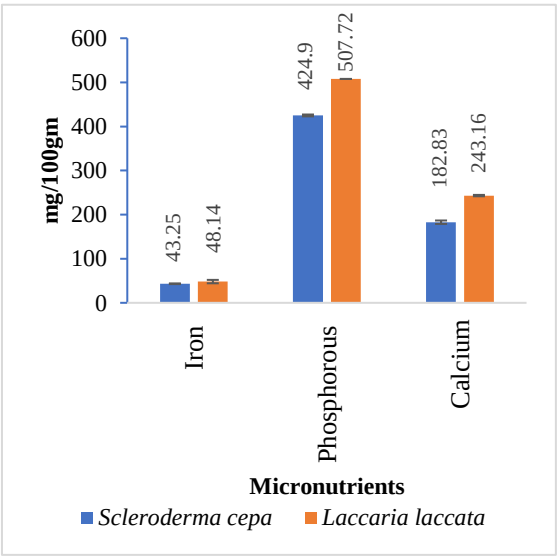

Figure 3: Micronutrient constituent in two species of mushrooms.

According to Duarte et al. (2006), concentration of micronutrients is influenced by the physiology of the species and by its ecological pattern. The study revealed high content of phosphorus as compared to iron and calcium which is in line with the result from Colak et al. (2009). It may be due to higher accumulation capacity of phosphorus by these mushroom as it was recorded in Zuo (2022) that ectomycorrhizal mushroom *Scleroderma* species improve plant growth and can replace the use of phosphate fertilizer in nursery. In comparison to *Scleroderma cepa*, *Laccaria laccata* had a greater calcium concentration. *Laccaria laccata* have a 395.5 mg/100g concentration (Egwim et al., 2011) which is higher in comparison to calcium content analysed in current investigation. The higher calcium in *Laccaria Laccata* in comparison with *Scleroderma cepa* is attributed by their effective nutrient uptake capability which is higher in comparison to calcium content analysed in current investigation. The higher calcium in *Laccaria laccata* in comparison with *Scleroderma cepa* is attributed by their effective nutrient uptake capability (Gu et al., 2019). In *Scleroderma cepa*, the iron concentration was approximately 43 mg/100g, while in *Laccaria laccata*, it was 48 mg/100g. Egwim et al. (2011) reported that the iron nutritional content was 177.69 mg/100g. Different varieties of mushrooms caused varying differences in iron concentration. These mushrooms have a mineral content range comparable to that of farmed species as noted by Crisan & Sands (1978).

Conclusion

The findings highlight that two wild edible mushrooms (*Scleroderma cepa* and *Laccaria laccata*) are an excellent source of proteins, fiber, carbohydrates, and essential minerals while being naturally low in fat, making them a highly nutritious and easily digestible food option. They contribute to improved dietary diversity and food security. Furthermore, the presence of both macro- and micronutrients in these mushrooms underscores their potential as a functional food with significant health benefits.

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Research Article

Antibacterial Activity of *Moringa Oleifera* Leaf Extract Against Clinically Significant Bacteria

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Abstract

The global rise of multidrug-resistant (MDR) bacteria calls for different alternative antimicrobial strategies. *Moringa oleifera*, which is found to be rich in secondary metabolites compounds, has shown some antibacterial potential. This study assesses the ethanol-extracted antibacterial activity of *M. oleifera* leaves against different clinically significant bacteria like *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis* and *Klebsiella pneumonia.* A laboratory-based experimental study was done using the disc diffusion method at two extract of different concentrations (500 mg/ml and 1000 mg/ml). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests were performed to observe bacteriostatic and bactericidal effects. The study identified the presence of alkaloids, flavonoids, tannins, saponins and terpenoids, highlighting the diverse phytochemical composition of the analyzed sample. The extract of different concentrations demonstrated the zone of inhibition for *S. aureus* (12 mm at both concentrations) and *P. aeruginosa* (17 mm at 500 mg/ml, 20 mm at 1000 mg/ml). *E. coli* only exhibited the inhibition (11 mm) at 1000 mg/ml. Likewise, *E. faecalis* and *K. pneumoniae* showed inhibition (16 mm and 12 mm) at 1000 mg/ml. However, MIC and MBC tests were found negative, where the regrowth of the bacteria was observed, which further suggested that the liquid culture has only limited efficacy. The selective secondary metabolites, particularly against *P. aeruginosa* advocates potential external applications or adjunctive use with antibiotics rather than the standalone. However, the lack of bactericidal activity in liquid culture shows the need for further research on higher concentrations, active compound purification, and synergistic effects with conventional antimicrobials.

Keywords: Antibacterial activities, *Moringa oleifera*, Multidrug-resistant bacteria, Secondary metabolites

Introduction

The rise of multidrug-resistant (MDR) bacteria has become a global health crisis due to the overuse and misuse of antibiotics. The World Health Organization (WHO) identifies antibiotic resistance as a major threat to public health, food security and development. Different pathogens such as

methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* are deeply linked to increased morbidity, mortality and healthcare costs. Despite the urgent need for antibiotics, the discovery has not kept pace with the emergence of resistant strains (Muteeb et al., 2023), entailing the exploration of alternative antimicrobial agents, particularly from natural sources (Newman & Cragg, 2020).

Moringa oleifera, known as the drumstick or miracle tree, has gained some attention for its different medicinal properties and nutritional benefits (Kashyap et al., 2022). Its leaves are found rich in essential nutrients and different bioactive compounds, such as flavonoids, phenolic acids, alkaloids, saponins, and tannins (Nepolean et al., 2009). These secondary metabolites show diverse pharmacological activities, including antimicrobial effects, through their mechanisms such as bacterial cell membrane disruption and inhibition of essential bacterial processes (Donadio et al., 2021). Previous studies have shown that *M. oleifera* extracts show antibacterial activity against both Gram-positive and Gram-negative pathogens, including *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* (Lar et al., 2011; Abalaka et al., 2012).

Despite its promising antimicrobial properties, the rate of research on *M. oleifera* where the use of ethanol as an extracting solvent remains low. Polar solvents are commonly used to extract polyphenols from plant matrices, with ethanol-based aqueous solutions being among the most effective options (Sultana et al., 2009). Particularly in screening its secondary metabolites and exploring potential synergies with conventional antibiotics. As a widely and locally available as well as easily cultivable plant, *M. oleifera* offers a sustainable and accessible approach to managing bacterial infections, especially in resource-limited settings where the burden of infectious disease is high. The WHO encourages integrating traditional medicine into healthcare systems, particularly in regions with limited access to conventional treatments.

The aim of this study is to evaluate the antimicrobial efficacy of *M. oleifera* leaf extract against clinically significant bacteria, assess its secondary metabolites components and also to explore its potential antibacterial activities. Given the increasing threat of antibiotic resistance, investigating *M. oleifera* as a potential antimicrobial agent is timely and may contribute to developing sustainable and effective healthcare solutions. The findings of this study could support evidence-based applications of *M. oleifera* in modern medicine, addressing the urgent need for alternative antimicrobial strategies.

Materials and Methods

Collection of plant materials

One kilogram of fresh *Moringa oleifera* leaves were collected from Narayani riverside, Bharatpur, Nepal (altitude: 252 m, latitude: 27.6765°N, longitude: 84.4357°E), ensuring they were free from disease, pests and contaminants. The leaves were washed with distilled water, shade-dried at room temperature (30°C) for 10 days, and finely ground into powder.

Extract preparation

About 50 g of powder was soaked in ethanol at a 1:10 (w/v) ratio. Soxhlet extraction was performed using a cellulose extraction thimble. The solvent was heated to its boiling point, evaporated, condensed, and repeatedly siphoned through the plant material until the solvent became colorless (Abubakar & Haque, 2020). The extract was concentrated using a water bath at 45°C, dried, weighed, and stored at 4°C in an airtight container.

Phytochemical screening

The presence of secondary metabolites was confirmed using standard qualitative tests conducted at the Microbiology Laboratory of Balkumari College, Narayangarh, Chitwan.

Alkaloids detection (Dragendorff's test & Wagner's test)

About 1 ml of extract was treated with a few drops of Dragendorff's reagent. The formation of a reddish-brown precipitate indicated the presence of alkaloids (Patel et al., 2014). For further confirmation, Wagner's reagent was added to another 1 ml of the extract. The appearance of a reddish-brown precipitate confirmed the presence of alkaloids (Santhi & Sengottuvel, 2016).

Flavonoids detection (Alkaline reagent test)

A few drops of sodium hydroxide (NaOH) were added to 1 ml of extract. A yellow coloration indicated flavonoids, which disappeared upon the addition of dilute hydrochloric acid (Sudha et al., 2021).

Phenolics and Tannins detection (Ferric chloride test and Lead acetate test)

A few drops of 5% ferric chloride solution were added to 1 ml of extract. The formation of a dark green or blue-black colour indicated phenolics and tannins. In the lead acetate test, 1 ml of extract was treated with 1% lead acetate solution. A yellow precipitate confirmed tannins.

Saponins detection (Foam test)

In the foam test, 1 ml of extract was vigorously shaken with 5 ml of distilled water. Persistent frothing for more than 10 minutes confirmed saponins (Santhi & Sengottuvel, 2016).

Terpenoids detection (Salkowski test)

For the Salkowski test, 1 ml of extract was mixed with 2 ml of chloroform then concentrated sulfuric acid was added on filtrated. A golden yellow colour confirmed terpenoids (Sudha et al., 2021).

Biochemical test of bacteria

The bacterial strains were isolated from clinical samples (urine and pus) at the Microbiology Laboratory of Narayani Samudayik Hospital, Bharatpur, Bagmati Province, Nepal. The pure cultures at hospital were transported to Balkumari College, where biochemical test was performed.

Antibacterial activity

Mueller-Hinton agar (MHA) medium was prepared, and bacterial suspensions were standardized to 0.5 McFarland (~10^8 CFU/mL). The obtaining of 0.5 McFarland suspension is controlled by the use of a spectrophotometer. The agar well diffusion method was used to measure the zone of inhibition (ZOI) against selected bacterial strains to determine the antimicrobial activity. The bacterial suspension was spread onto MHA plates. Wells (6 mm) were created, and the extract was added at two concentrations (500 mg/ml and 1000 mg/ml). The plates were incubated at 37°C for 24 hours, and the ZOI was measured.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

Stock extract solution (1000 mg/ml) was serially diluted (2-fold). Bacterial suspensions were adjusted to 0.5 McFarland and inoculated. MIC was determined as the lowest concentration inhibiting growth. MBC was determined by subculturing MIC-negative tubes. MBC/MIC ratio $\leq$ 4 indicated bactericidal activity, while >4 suggested bacteriostatic effects (Kowalska-Krochmal & Dudek-Wicher, 2021; Ishak et al., 2024). This ratio serves as a key parameter for determining whether an antibacterial agent is bactericidal or bacteriostatic.

Results and Discussion

Qualitative phytochemicals screening

The leaves were specifically chosen for this study due to several proven results from previous studies. *Moringa* leaves contain high concentrations of bioactive secondary metabolites such as phenols, flavonoids, tannins and alkaloids (Table 1), which contribute to their potent antibacterial properties (Azra et al., 2024; Jhones, 2022). Previous studies have extensively documented the antimicrobial effects of *Moringa* leaves, making them a well-established choice for further investigation (El-Sherbiny et al., 2024).

Qualitative phytochemical screening of *Moringa oleifera* leaf extract revealed various secondary metabolites. Alkaloids transitioned from green to radish brown, indicating their presence, which is associated with antimicrobial properties (Yan et al., 2021). Flavonoids conformed after became green to colourless, can be considered as their antioxidant significance (Dias et al., 2021). Tannins deepened in green colour, supporting their antimicrobial role. Foam formation confirmed saponins, which contribute to immune modulation and antimicrobial activity. Terpenoids changed from green to radish brown, confirming their therapeutic potential (Villanueva et al., 2023). These findings support the traditional medicinal use and pharmacological potential of *Moringa oleifera*. These findings

highlight the rich phytochemicals composition of *Moringa oleifera*, further supporting its potential as a natural source of bioactive compounds with therapeutic applications, and the results are supported by other studies too (Onyekwere, 2014). 2014).

Table 1: Qualitative tests of phytochemicals.

Phytochemical	Initial colour	Final colour	Remarks
Alkaloids	Green	Radish brown	Positive
Flavonoids	Green	Colourless	Positive
Tannins	Green	Dark green	Positive
Saponins	No foam	Foam formation	Positive
Terpenoids	Green	Radish brown	Positive

Biochemical test of bacteria

Biochemical tests characterized five bacterial strains. *S. aureus* and *E. faecalis* were Gram-positive cocci, while *P. aeruginosa*, *E. coli*, and *K. pneumoniae* were Gram-negative rods. Catalase positivity varied among strains. Coagulase positivity in *S. aureus* indicated its pathogenicity. Oxidase was positive only for *P. aeruginosa*. Indole and MR tests confirmed *E. coli*'s fermentative capabilities, while *K. pneumoniae* tested VP positive, signifying butanediol fermentation. Hemolysis patterns varied, highlighting strain-specific metabolic traits (Table 2).

Antibacterial activity

Moringa oleifera extract at 500 mg/ml exhibited antibacterial activity against *S. aureus* (12 mm ZOI) and *P. aeruginosa* (17 mm ZOI), Similar results are reported in previous studies (Khayra et al., 2020). No inhibition was observed against *E. coli*, *E. faecalis* and *K. pneumoniae*, suggesting resistance at this concentration (Figure 1). The absence of inhibition at 500 mg/ml could be due to the low concentration of active compounds in the extract may also be insufficient to exert an antibacterial effect. Furthermore, several crucial factors must be considered, including the selection of the medium, pH level, agar depth, moisture content and incubation conditions, along with maintaining accurate inoculum density. The size of the inhibition zone can be influenced by the tested substance's solubility, diffusion capacity and evaporation rate. Additionally, if water-insoluble substances precipitate on the disc, they can obstruct the diffusion of antimicrobial agents into the agar (Bubonja-Šonje et al., 2020).

At 1000 mg/ml, *Moringa oleifera* extract demonstrated broader antibacterial efficacy. *P. aeruginosa* had the highest ZOI (20 mm), similar ZOI was observed in previous studies (Abalaka et al., 2012) using chloroform as a extracting solvent. *E. coli* and *S. aureus* showed inhibition zone of 11 mm and 12 mm, consistent with previous report (Garba et al., 2021) highlighted an inhibitory effect at high concentrations of *M. oleifera* extract, supporting the concentration-dependent activity. *E. faecalis* and *K. pneumoniae* showed inhibition zone of 16 mm and 12 mm respectively indicating concentration-dependent activity (Garba et al., 2021). However, *S. aureus* showed no increase in ZOI compared to 500 mg/ml, suggesting its susceptibility plateaued. Average result reported in previous studies is consistence with our study (Pal et al., 1995), however, a significant increase with the concentration that could be due the variations in strain resistance (Garba et al., 2021). Our findings from the research do align with previous studies that highlighted the bioactive secondary metabolites in *M. oleifera*, which exhibit antimicrobial effects against a range of pathogenic bacteria (Kasolo et al., 2010; El-Sherbiny et al., 2024; Soto et al., 2025).

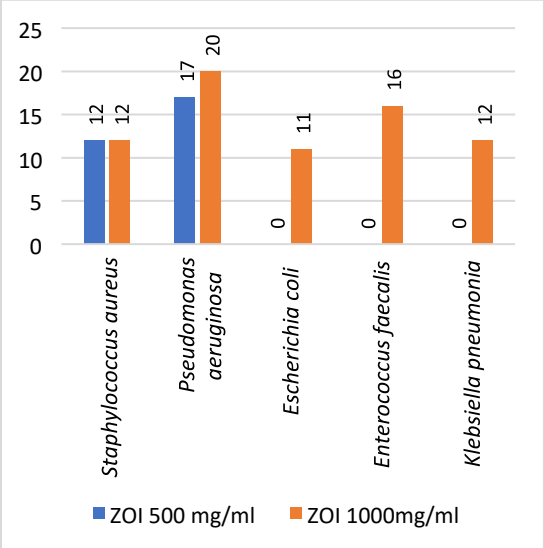

Figure 1: Comparative ZOI at 500 mg/ml and 1000 mg/ml concentrations.

Table 2: Biochemical test for bacteria.

Test	*S. aureus*	*P. aeruginosa*	*E. coli*	*E. faecalis*	*K. pneumonia*
Gram staining	+, cocci	-, rod	-, rod	+, cocci	-, rod
Catalase	+	+	+	-	+
Coagulase	+	-	-	-	-
Oxidase	-	+	-	-	-
Indole	-	-	+	-	-
MR	+	-	+	-	-
VP	+	-	-	+	+
Citrate	+	+	-	-	+
Urease	+	-	-	-	+
Hemolysis Beta	None	None	None	Alpha	None

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed on *Moringa oleifera* extract yielded negative results (Table 3). That could be due to the selection of appropriate methods is largely determined by the characteristics of the secondary metabolites, including its solubility and molecular weight. These factors affect how the compound interacts with the medium, influencing its diffusion, stability and overall effectiveness in experimental applications (Bubonja-Šonje et al., 2020). However, the minimum bactericidal concentration (MBC) revealed that there was the presence of more than 200 colonies. MBC/MIC ratio $\leq$ 4 indicating a bacteriostatic rather than a bactericidal effect at the tested concentrations. This finding suggests that *M. oleifera* may inhibit bacterial growth but may not completely eradicate the bacterial population at these specific concentrations. Compared to conventional antibiotics, the antibacterial efficacy of *M. oleifera* is modest, reinforcing its potential as an adjunct rather than a standalone antimicrobial agent. This supports previous findings that plant-based extracts can be effective against multidrug-resistant (MDR) bacteria (Al Alsheikh et al., 2020.; Košćak et al., 2023). However, it might require optimization in concentration or formulation for enhanced efficacy. This research suggests that further studies should focus on fractionating the extract to isolate specific bioactive compounds that are responsible for antibacterial action and then evaluate their synergistic effects with standard antibiotics.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of different concentrations in bacterial strains.

Bacterial Strains	MIC (mg/ml)	MBC (mg/ml)	MBC Result at 500 mg/ml	MBC Result at 1000 mg/ml
E. coli	>1000	>1000	>200 colonies (No effect)	>200 colonies (No effect)
E. faecalis	>1000	>1000	>200 colonies (No effect)	>200 colonies (No effect)
K. pneumoniae	>1000	>1000	>200 colonies (No effect)	>200 colonies (No effect)
S. aureus	>1000	>1000	>200 colonies (No effect)	>200 colonies (No effect)
P. aeruginosa	>1000	>1000	>200 colonies (No effect)	>200 colonies (No effect)

Additionally, the study was limited to ethanol extracts which suggests the exploring of different solvents which could provide a broader understanding of the extract's full antimicrobial potential (Arora & Onsare, 2014). Due to resource constraints, the experiment could not be conducted at multiple concentrations to determine the precise MIC and MBC values. Therefore, the exact MIC/MBC ratio remains uncertain, and further dilution testing is required for a more accurate analysis.

Conclusion

Moringa oleifera leaf extract exhibits promising antibacterial activity against certain pathogens, particularly *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but its efficacy is limited in liquid conditions, as indicated by the negative MIC and MBC results. Further research should explore higher extract concentrations, purification techniques, and possible synergistic effects with conventional antibiotics to enhance its potential as an alternative antimicrobial agent.

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Research Article

Physicochemical Assessment and Water Quality Index of Underground and River Water in Wet Season

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Abstract

Physicochemical and water quality index (WQI) are the commonly used parameters for evaluating water contamination. Water samples were collected from the Bagmati river (Shankhamul, B-1 and Balkhu, B-2) and well water in the summer season. The physiochemical parameters such as pH, turbidity, dissolved oxygen, oxidation-reduction potential (ORP) and conductivity were measured on the spot and chemical biological parameters such as alkalinity, hardness, chemical oxygen demand (COD) and total coliform colonies were determined in the laboratory. The results showed that well water is less polluted than river water. The contaminants in the river water increased after the confluence of tributaries in the Bagmati river. Although the dissolved oxygen content was low (6.87 ppm), the observed ORP value was higher in the well water (110.1 mV) than in the river water (33.4 mV). The dissolved oxygen content was 7.11 ppm however, the oxidation-reduction potential (ORP) value was negative after the confluence of the tributaries indicating anthropogenic influence. The result is supported by a very high concentration of colonies (20000) observed in the B-2 samples. WQI revealed that the river water quality falls in the 'C' grade, indicating poor water quality. It is concluded that the groundwater and river water are polluted hence treatment is essential before using for domestic purposes.

Keywords: Bagmati river, Oxidation-reduction potential, Underground water, Water pollution, WQI

Introduction

All living organisms need water at every moment for their survival and growth. Surface and underground water are the main sources of water for daily use. Currently, anthropogenic pollutants decidedly increase contaminants in the source of water (Patil et al., 2012). The consumption of contaminated water causes waterborne diseases. About 80% of diseases in human beings are caused by water (WHO, 1999; Leevanthi, 2016). The type and quantity of contaminants in natural water vary from place to place and season to season. The types of impurities/contaminants mostly depend on their sources such as sewage and industrial waste, natural

resources, the growth of bacteria, algae, viruses, and the atmosphere in the form of dissolved gases (Basavaraja et al., 2011; Leelavathi et al., 2016). The concentration of contaminants increased in the dry season but reduced in the wet season due to dilution by rain (Dahal & Joshi, 2023)

Kathmandu valley is one of the most populated cities in Nepal. The demand for freshwater has been tremendously increasing due to rapid population growth and unmanaged expansion city. Further, the Bagmati river is enormously important culturally and historically for the people in the Kathmandu valley (Milner et al., 2015). They are critical components of the hydrological cycle because they provide habitat and nourishment for organisms that rely on them. There is a lack of basic sanitation services and drinkable water for people living in the core city area (Warner, 2008). The abundantly available water sources are underground and river water for industrial, agricultural, irrigation and domestic uses. People are using underground water as one of the alternative sources to fulfil the overall demand in Kathmandu valley. The quality of underground and river water is mainly decided by the geological structure and season of the particular region however, unmanaged sewer connections are dramatically polluting sources of water (Leelavathi, 2016). The water sources of this area are being polluted at an alarming rate due to the unmanaged and uncontrolled disposal of sewage and drainage (Koju et al., 2014, Adhikari 2020). The people are disposing of sewer and solid waste directly and continuously in the rivers of Kathmandu (Mehta et al., 2017; Mishra et al., 2017). It was reported that the water quality of the Bagmati River is severely deteriorating in the present conditions due to the mixing of effluent and solid waste (Adhikari et al., 2021; Adhikari et al., 2024), which harms the river ecosystem and the health of communities relying on it. Hence determination of water quality is essential to determine the palatability of water for its use (Koju et al., 2014).

In this study, the different water quality parameters were measured at the sources and samples were collected and analyzed with physicochemical and bacteriological characteristics. The data was collected from two observation sites, before and after the confluences of five tributaries in the Bagmati river and one from a well near the Bagmati river.

Materials and Methods

Water samples and data were collected from a well, underground water (G-1) and river water from two different observation sites in the Bagmati river. The first river water sample is from the Shankhamul (B-1), just before the confluence of tributaries and 2nd sample is from the Balkhu (B-2) just after the confluence of five main tributaries of the Bagmati river during the wet (summer) season. Some physicochemical parameters, temperature, turbidity, pH, oxidation-reduction potential (ORP), dissolved oxygen (DO), conductivity, total dissolved solid (TDS) and salinity were observed and recorded in the observation sites by using a portable advanced multi-parameter analyzer (HANNA instruments, Hi-9829) (Adhikari et al., 2024). The water samples were collected in different bottles for chemical and microbiological analysis and transported to the laboratory using cooler boxes and analyzed as soon as possible using a standard method and samples were processed immediately for bacteriological analysis. The alkalinity, hardness, acidity, chloride ion, and dissolved CO_2 were determined using the titrimetric method (APHA, 2012; Rattan, 2012). Chemical oxygen demand (COD) was determined spectrophotometrically using hydrogen phthalate as standard (Bhatt et al., 2024)

The water quality index (WQI) was computed using the weighted arithmetic index of the water quality parameters. The value was calculated using eight variables. A quality rating was determined by using Equation 1.

$$Q_i = \left[\frac{(C_a - S_i)}{(C_s - S_i)}\right] x100 - - - (1)$$

Where, Q_i = quality rating for i^{th} parameters, C_a=concentration of i^{th} parameters in a water sample (mg/L), S_i = WHO or Nepal standard value and C_i = ideal value (0 for all parameters except DO=14.6 ppm and pH=7.0). The unit weight (W_i, Table 1) was calculated using Equations 2 and 3 (Akoteyon et al. 2011; Imneisi & Aydin, 2016).

$$W_i = \frac{k}{S_i} - - - (2)$$

$$k = \frac{1}{\sum_{i=1}^{n} 1/S_i} - - - (3)$$

Where, Wi = unit weight of i^{th} parameters, S_i = standard value for the i^{th} parameters n= no of parameters and k= relative constant.

From the quality rating (Qi) and unit weight (Wi), the index of i^{th} parameter I_i was calculated from Equation 4, then the WQI was calculated using Equation 5. Based on the WQI value water quality is classified into 5 categories (Table 1).

$$I_i = W_i Q_i \quad ---(4)$$

$$WQI = \sum_{i=1}^{n} \frac{I_i}{\sum W_i} \quad ---(5)$$

Table 1: Water quality classification based on the water quality index (WQI).

WQI value	Rating of water quality	Grading
0-25	Excellent water	A
26-50	Good	B
51-75	Poor	C
76-100	Very poor	D
Above 100	Unfit for drinking	E

Results and Discussion

The temperature of the river water was measured before noon and that of well water was recorded in the afternoon. The observed temperature was 24.99 and 25.29 and 28.47 °C in the B-1, B-2 and G-1 samples respectively (Figure 1a). The variation in water temperature was due to the diurnal variation of air temperature in the summer season. The turbidity was low about 130.2 FNU for well water, which was 2 to 3 times higher in the case of river water (Figure 1b).

The high turbidity of river water indicates the loading of suspended particles either soil particles from natural sources or organic matter from anthropogenic sources (Grobbelaar, 2009). pH is the negative logarithm of the hydrogen ion concentrations it ranges from 6.0 to 8.5 in surface and 6.5 to 8.5 in groundwater (Modoi et al., 2014). it is important in the disinfection, and metabolic activity of aquatic animals (Borse & Bahve, 2000), the formation of scaling, and corrosion prevention (Ahmad et al., 2020). The observed pH (7.25 to 7.67) of well and river water was slightly alkaline but within the range prescribed by WHO (WHO, 1999), the lowest pH (7.25) was observed in underground water (Figure 1c). Slightly alkaline pH indicates that river water contains carbonate and bicarbonates from the soil, limestone or waste discharge, and microbial decomposition of organic matter (Patil et al., 2012; Cuivillas et al., 2016). The conductivity measures the inorganic and organic ions in the water (Julian et al., 2018).

The inorganic ions having high mobility have a great influence but organic ions having less mobility have a low influence on conductivity (Gupta et al., 2009). The recorded conductivity of well water was highest (1084 µS/cm) which was lowest (322 µS/cm) for the B-1 sample and more than two times higher for the B-2 sample (745 µS/cm) (Figure 1d). The high conductivity of well water may be due to the presence of minerals from natural sources. The increase in conductivity in the B-2 sample indicated that the tributaries and local influence increased conducting species in the river. Dissolved oxygen is one of the most important parameters of the water ecosystem. The atmosphere and photosynthetic process are the main sources of dissolved oxygen. The concentration of dissolved oxygen depends on temperature, exposed surface area etc. (Yasin et al., 2015).

It is drastically reduced by the chemical or microbial decay process of organic materials, dead vegetation, and sewages (Bisht et al, 2013). Hence, dissolved oxygen (DO) is one of the important parameters which reveals the quality of water it makes water tasty. The observed DO (Figure 1e) value ranged from 6.87 to 7.11 ppm. The lack of aeration may be one of the causes of the low DO concentration of well water. Oxidation-reduction potential (ORP) is the capability of water to conduct a specific redox reaction. The redox reaction includes nitrification, denitrification, removal of phosphorus and organic matter, and production of biological malodor (Al-Samawi & Al-Hyssaini, 2016). Natural fresh water has a high and positive ORP whereas polluted water has a low and negative ORP. The observed ORP of well water was high (110.1 mV) but low (33.4 mV) in B-1 and negative (-0.4 mV) in B-2 (Figure 1f).

ORP higher than 100 mV is characterized by enough free oxygen, which is the most common condition of surface water. Denitrification occurs when the ORP values are between 50 to -50 mV by denitrifying bacteria. Based on the ORP value it is considered that reduction of nitrate occurs in the river water which releases nitrogen. The negative value of ORP and high turbidity in the B-2 indicate the presence of organic pollutants from domestic influents (Adhikari et al., 2024).

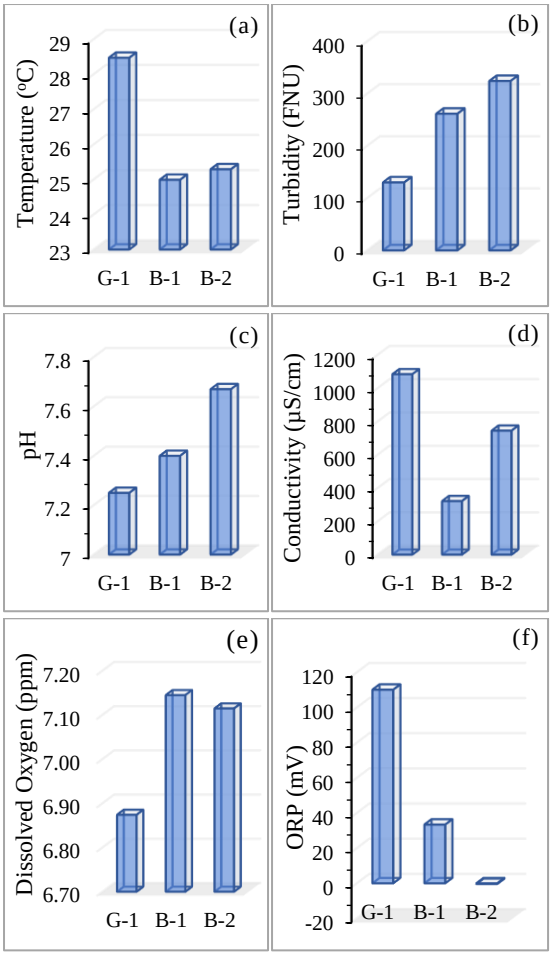

Figure 1: In-situ observed data of physicochemical parameters of the well and the Bagmati river water.

Hardness is the natural pollutants increased by soil and slow weathering of rocks containing calcium and magnesium. It develops scaling in pipes and water heaters and reduces the cleaning ability of water by decreasing lather formation with soap and increasing the boiling points (Ladipo et al., 2011). As expected, the total hardness of well water was the highest (91.39 ppm) and that of river water was lower by three folds (Figure 2a). It was reported that the hardness of nearly 150 ppm is generally perfect for its use. It is considered that water containing hardness less than 150 is soft and greater than 200 ppm is hard water. Soft water is corrosive and hard water develops scaling (WHO, 2009; Ahn et al., 2018). The hardness indicates the corrosive nature of water, especially the river water. The direct use of soft water is not harmful to humans however, it may cause corrosion in water pipes and tanks (WHO, 2009; Ahn et al., 2018). Khadka (1993) observed a total hardness of 80-300 ppm for the underground water in Kathmandu valley. Similarly, Adhikari (2020) observed 200 to 328 ppm hardness for underground water and 44 to 140 ppm for river water.

The alkalinity depends upon the dissolved minerals from soil, limestone, rock etc. (Milner et al., 2015). It measures the concentration of carbonate, bicarbonate, and hydroxyl ions. The alkalinity leads to the scale and sludge formation and corrosion in metals hence it is unpalatable. The observed alkalinity of well water was 170.89 ppm and 149.65 and 216.94 ppm, respectively of B-1 and B-2 samples (Figure 2b). The spatial variation of alkalinity of Bagmati river water in the winter season reported by Adhikari et al. (2024) showed that the alkalinity was 248.88, and 417.24 ppm respectively, before and after the confluence of the tributaries. The low concentration of alkalinity observed in this study may be due to the dilution of river water by rainfall in the wet season. The natural source of total hardness and alkalinity are minerals so their concentrations are usually nearly equal. The alkalinity of those samples was much higher than hardness which attributes that the alkalinity is due to the presence of contaminants rather than calcium and magnesium ions from soil and rock.

Acidity and dissolved CO_2 are related to each other. Dissolved CO_2 forms carbonic acid which makes water acidic. Carbonic acid ionizes to give bicarbonate and hydrogen ions making water acidic however, at a higher pH, bicarbonate takes hydrogen ions and water becomes alkaline. Respiration, photosynthesis, and decomposition influence the CO_2 levels. In well water, the CO_2 level is usually high because of decaying plants and the decomposition of organic substances. As expected, the acidity and dissolved carbon dioxide in well water were higher than in river water (Figure 2c & 2d). The high concentration of dissolved CO_2 and acidity reduced the pH of the well water.

Chloride is naturally present in wells and river water due to the leaching of soil and rocks in the form of calcium, magnesium, and sodium salt. Chloride ion concentration was 53.79, 39.1, 46.7 ppm in the G-1, B-1 and B-2, respectively (Figure 2e). The maximum limit of chloride in drinking water is 250 ppm commonly underground river water consists of less the 50 ppm (Hong et al., 2023). Chemical oxygen demand (COD) is the amount of oxygen needed to oxidize organic matter present in the water chemically. It indicates the level of pollution in water. The COD in well water is very low (0.03

ppm) which was more than two times higher in B-1 and three times in the B-2 samples (Figure 2f), indicating the presence of organic matter in the river water. The colony forming unit (CFU) indicates the pollution level of water. The clean unpolluted water consists of less the 100 CFU/mL whereas heavily polluted water consists of thousands of CFU/mL The CFU count of well water was 525 CFU/mL however it was 8300 and 20000 CFU/mL in B-1 and B-2 samples (Figure 2g). CFU counts and negative ORP value suggested that although other water quality parameters were within the WHO limits the river water consisted of excessive levels of bacteria, hence unfit to use for domestic uses especially downstream water. The computed water quality index (WQI) using 8 important water quality parameters (Figure 2f) shows that the well and Bagmati river water belongs to the C category. Although the water quality of B-1 and B-2 samples falls in the C category, the WQI value of B-2 (62.58) is higher than that (55.98) of B-1 (Figure 2h). As indicated by other parameters before the confluence of tributaries the Bagmati river water was comparatively less polluted. After the mixing of tributaries, the contaminants especially CFU counts increased extensively in the Bagmati river. The results attributed that the river water particularly downstream is hazardous and cannot be used for domestic purposes without treatment.

Conclusion

The observed pH of water samples was within the permissible range recommended by WHO. Most of the natural pollutants such as hardness, acidity, dissolved CO_2, chloride ion and conductivity were higher in the groundwater than in the river water. The anthropogenic pollutants such as alkalinity, COD, turbidity, and CFU counts were in the river water than in the underground water. The WQI suggested that the water quality of well and river water is poor and falls in the C category. Though, the WQI of Bagmati river water after the confluence of tributaries falls in the same category as other samples the CFU value was very high indicating the influence of sewer pollutants. It is concluded that the groundwater is less harmful than river water but cannot be used for domestic purposes without treatment. The Bagmati river water especially downstream is hazardous for human application.

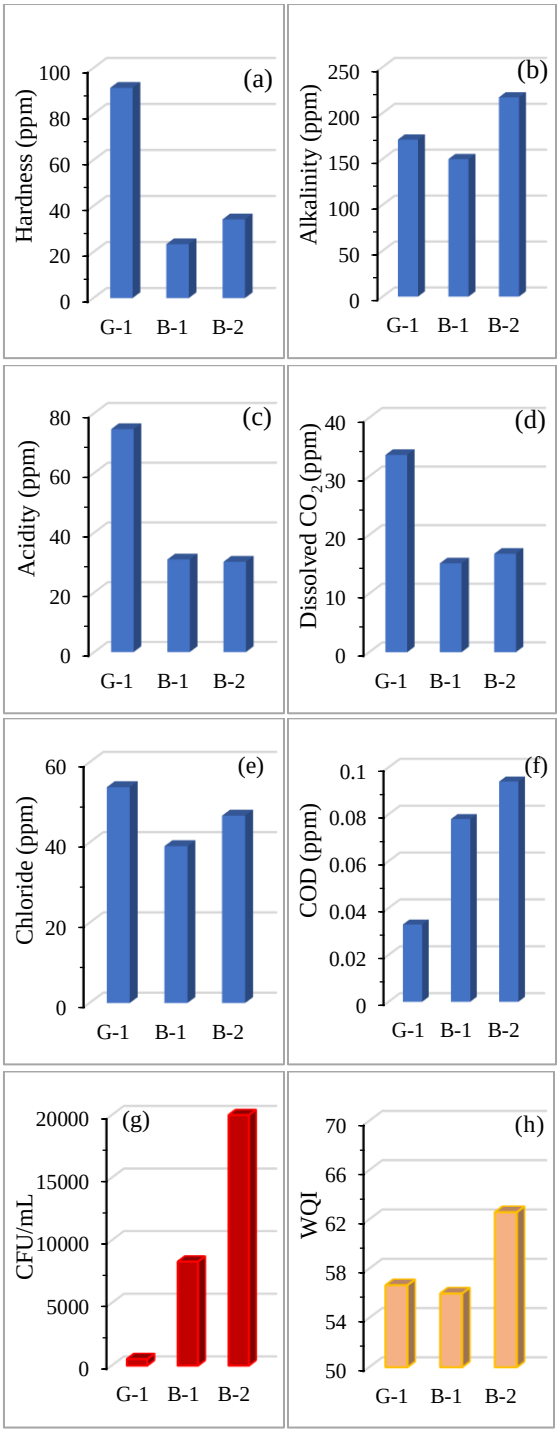

Figure 2: Physicochemical and biological parameters of the well and Bagmati river water.

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Research Article

Biogas Production from Cow-Dung at Low Temperature by Integration of Microbial Electrolysis System

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Abstract

Biogas, a sustainable energy source produced from anaerobic digestion of organic materials, holds significant potential in Nepal due to its abundant agricultural resources. This study investigates the production of biogas from cow dung integrating a microbial electrolysis cell (MEC) system to enhance performance of biogas production under low-temperature (15 ± 0.5°C). The biogas production process was assessed using a pilot-scale MEC system optimized at 2.5 V and 1:5 dilution. Performance metrics included biogas yield, methane content, and COD reduction. The MEC integration significantly improved biogas production, with a 20-fold increase at 2.5 V compared to the control reactor at 15°C. Scaling up to an 8.5 L pilot system demonstrated a fourfold increase in biogas production. Optimal conditions of 2.5 V and 1:5 dilution resulted in an 84.21% COD reduction at 15°C, increasing to 88.28% at the pilot scale. Molecular identification of microbial consortia isolated from biogas production medium were specifically *Bacillus cereus, Bacillus paramycoides, Bacillus licheniformis* and *Priestia flexa* which play crucial role in organic matter degradation and electron transfer in MEC operation at low temperature. Hence, integration of MEC technology substantially enhances biogas production and organic waste treatment efficiency, particularly at low temperature.

Keywords: Anaerobic digestion, Biogas, COD, Cow-Dung, MEC, Methane

Introduction

A viable source of renewable energy in Nepal is domestic biogas technology. Domestic biogas technology is widely used in Nepal's peri-urban and rural regions, and it may be scaled up to take the place of solid fuel cook stove (Lohani et al., 2022). In Nepal, wood is the most common fuel for cooking, followed by LPG, biogas, and other fuels including kerosene and electricity. Traditional indoor burning on inefficient indoor stoves or open fireplaces has caused numerous serious public health issues as well as environmental issues. Homes with inadequate ventilation expose occupants to a variety of air contaminants, including deadly carbon monoxide and particles smaller than 2.5 mm, which have been linked to cataracts, cardiovascular problems, respiratory illnesses and cancer (Gross et al., 2017).

Typically, biogas is composed of 50–70% methane, 30–40% carbondioxide, 5–10% hydrogen, 0-3% nitrogen, and trace amounts of hydrogen sulfide. Methane is the major energy carrier in biogas, which

has a greater heating value of around 38 $MJNm^{-3}$ and is produced by anaerobic decomposition of organic substrates (Gross et al., 2017). Anaerobic digestion (AD) helps to dramatically minimize the possibility of seepage of nitrates into groundwater, releasing nitrates and pathogens into surface water, and emitting aromas from storage lagoons. The digested organic substrates may be used as a beneficial fertilizer because of the increased nitrogen availability and decreased pathogen survival. According to widespread consensus, AD occurs in three steps that follow each other: the hydrolysis or solubilization phase, the acidogenesis or acid-producing phase, and the methanogenesis or methane-producing phase (Weiland, 2010). One of the crucial factors of AD is temperature because it has a great impact on microbial communities, thus varying the efficiency and stability of the digestion process. Methane-producing bacteria perform optimally at temperature ranging from 30-40°C or from 48-60°C (Baweja, 2018). Nepal, with diverse altitudes and climates across its regions, may experience impacts on biogas production in its cooler areas. Lowering temperature during winter season decreases the biogas production because low temperature result in reduced activity and growth rate of methanogens. To minimize the effect of low temperature, microbial electrolysis cell (MEC) can be integrated with AD to enhance the biogas production (Liu et al., 2016). An external voltage is applied between the electrodes to surpass the thermodynamic barrier. Integrating a MEC-AD system enhance performance through several key benefits: boosting methane production, stabilizing processes more effectively, achieving thorough removal of organic matter, efficiently producing methane from hydrogen ions and volatile fatty acids (Carrillo-Peña et al., 2024). This research attempts to enhance production of biogas using electro-methanogenesis technology.

Materials and Methods

Sample collection

Sample of fresh cow-dung was collected from a cow-farm in Kirtipur, Kathmandu.

Physiochemical analysis of sample

For pH analysis, sample was mixed with distilled water, 1:10 dilution and measured pH (HI2002-02 Edge® pH Meter, HANNA) (Estefan et al., 2013). The Association of Official Analytical Chemists' (AOAC, 2000) guidelines were followed in determining the total solid (TS) and moisture content for which sample was weighed and heated at 105 °C overnight. The dried sample was kept in muffle furnace at 550 °C for 2 h to determine ash content and volatile solid (VS) content. For determination of chemical oxygen demand (COD), sample was digested using digestion solution and catalyst solution and 1000 mg/L potassium acid phthalate was used as standard solution as described by Pisal, (2003). The reducing sugar of sample was determined spectrophotometrically using DNSA method (Miller et al., 1961). The ammonical-nitrogen was determined using Nesslers reagent and ammonium chloride (1000 mg/l) as standard solution. For determination of phosphorus and trace elements, sample was digested using H_2SO_4-Salicylic acid–H_2O_2 assay method. The phosphorus was determined by spectrophotometer using potassium dihydrogen phosphate as standard solution while trace elements were determined using atomic absorption spectroscopy (Pisal, 2003).

Biogas production in microbial electrochemical cell (MEC)

For biogas production, inoculum was prepared by using indigenous microorganisms present in cow-dung. About 100 mg of cow-dung was inoculated into 100 ml of anaerobic nutrient broth medium and subjected to anaerobic conditions for 7 days at 28 °C to enrich microbes present in cow-dung indigenously. After two to three subcultures, the culture was utilized as the inoculum in MEC system with or without voltage supply. A pair of carbon felt of dimension 10 cm × 3 cm × 1.5 cm were inserted into the 2 l three neck round bottom flask to make a biological reactor referred as microbial electrochemical cell (MEC) anaerobic reactor (Liu et al., 2016). The voltage supplied was fixed at 1.5 V, 2 V, 2.5 V and 3 V. The experimental control was constructed in common reactor similar to MEC-anaerobic reactor without application of voltage. Before digestion, anaerobic conditions were maintained by eliminating oxygen by the bubbling of nitrogen gas for 10 minutes. The gas volume was measured using measuring cylinder by downward displacement of water and 1 M KOH solution. The experiments were carried out at 15 °C and 28 °C. The sample was diluted at 1:2, 1:5 and 1:10 ratios. The reactors were operated in batch mode for 15

days. Samples after 15 days of digestion were taken and processed for determination of COD, reducing sugar and pH.

Experimental design for scale up process

Large scale digestion was carried out in a carboxy jar of 10 l size. Treated carbon felt electrodes having dimension of 24 cm × 4 cm × 1.5 cm were kept at 6 cm distance. The sample was diluted at 1:5 ratio, and digester was kept at 15°C with 2.4 V supply for 15 days. Measurement of biogas was done by downward displacement of KOH. For analysis of composition, gas was collected in a plastic bag and analyzed by infrared syngas analyzer. The presence of possible different gases like methane, CO_2, N_2, H_2 etc. was analyzed using gas analyzer. Samples after 15 days of digestion were taken from the digester and processed for determination of COD, reducing sugar and pH.

Molecular identification of microorganism

Isolation of microorganism

About 0.1 ml sample after digestion was spread on the agar plate of DSMZ 825 Methanobacterium II media (MMII). The plates were incubated at 37 °C for 24 h in an anaerobic jar. After incubation, the different colonies of bacteria were selected and isolation of pure culture was performed. Liquid culture of bacteria was prepared by inoculating pure colony of bacteria into MMII broth culture tube. The culture tubes were sealed, kept in anaerobic jar and incubated at 37 °C for 24 h (Lozano et al., 2009).

Molecular characterization of microorganism

The genomic DNA of isolated microorganism was extracted using SDS-based technique from the broth culture of isolates (Sambrook & Russell, 2001). The amplification of genomic DNA (gDNA) was carried out by using 16S rRNA primers. The forward primer, U1971-C070-27F-01, 5'-AGAGTTTGATYMTGGCTCAG-3', and reverse primer, U1971-C070-515R-02, 5'-TTACCGCGGCKGCTGGCAC-3' were used. The PCR conditions were initial denaturation (94 °C for 3 min), final denaturation (94 °C for 30 sec), annealing (64 °C for 40 sec), extension (72 °C for 1 min) and final extension (72 °C for 5 min). After completion of 28 cycles of PCR, gDNA was run in 2% gel-electrophoresis at 60 V for 60 minutes and was visualized under gel documentation (Hathway et al., 2021). The amplicons were subjected to sequence analysis. The acquired 16S rRNA gene sequences were input into the MUSCLE algorithm for multiple sequence alignment and phylogeny tree was constructed using Neighbor-joining (NJ) method with bootstrap value 1000 using MEGA v.11 software.

Data analysis

Statistical analysis of data and visualization of generated data was done using GraphPad Prism 8.0.2 (GraphPad Software, 2024), JASP v 0.17.3 (JASP Team, 2024), and Microsoft office excel (Microsoft Corporation, 2018). The associations between two categorical values were evaluated using chi-square test. Student's t-test was used for both paired and independent sample for determination of level of significance. Correlations between two datasets were accessed by Pearson correlation coefficient test and the level of significance was set at p value <0.01.

Results and Discussion

Physiochemical characteristics of cow-dung substrate

The physical and chemical characteristics of cow-dung were shown in Table 1. The pH of sample was found to be 7 and moisture content was 85±0.01%. Total solid (TS) and volatile solid (VS) were found 14.705±0.445 % and 78.905±0.389 % respectively. Our analysis showed the similarity with the study conducted by Gautam et al. (2009); Rawat et al. (2019); and Singh et al. (2021) in which TS and VS were found to be within range of 15-24% and 80-88 % respectively. The total chemical oxygen demand (COD) of cow-dung was found to be 81.91±1.15 mg/g. The reducing sugar content of cow dung was found to be 105.29±4.07 mg/g, indicating a substantial concentration of easily degradable carbohydrates. The amount of ammonia (0.0054±0.0001 mg/g) and phosphorous (0.015±0.001 mg/g), however, in manure is greatly affected by moisture, temperature, pH of manure as well as different microbiological activities (Bleizgys & Naujokienė, 2023). On analysis of trace elements and heavy metals, i.e.; iron (0.22 mg/g), zinc (0.0053 mg/g) and manganese (0.27 mg/g) were found. However, copper and nickel were below the

detection limit of the atomic absorption spectroscopy (AAS).

Table 1: Physicochemical parameters of cow-dung.

Characteristics of sample	Concentration
Total solid	14.705 ± 0.44 %
Volatile solid	78.905 ± 0.38 %
Ash content	21.095 ± 0.38 %
Moisture Content	85.295 ± 0.445 %
Total reducing sugar	105.29 ± 4.07 mg/g
Ammoniacal nitrogen	0.0054 ± 0.0001 mg/g
Chemical oxygen demand	81.91 ± 1.15 mg/g
Phosphorus	0.015 ± 0.001 mg/g
Iron	0.22 mg/g
Copper	Below detection limit
Nickel	Below detection limit
Zinc	0.0053 mg/g
Manganese	0.27 mg/g

Optimization of biogas production at low temperature

Biogas production at low temperature is a crucial topic as production rate deeply falls as temperature decreases. This might be due to reduction in microbial growth and activity at lower temperature. To enhance the biogas production at low temperature, microbial electrolysis cell can be used which provides additional energy to the microbial consortium, particularly the electro-active bacteria, which can facilitate the degradation of organic substrates into the biogas more efficiently under low temperature conditions.

Voltage optimization

To maximize the biogas production, different voltages i.e., 1.5 V, 2 V, 2.5 V & 3 V were applied, in which highest biogas production was observed from the MEC with 2.5 V, which was found to be 464 ± 8.49 ml after 15 days. The MEC with 1.5 V, 2 V & 3 V also showed biogas production of 108.5 ± 12.02 ml, 251 ± 15.56 ml and 346 ± 5.66 ml respectively while control reactor showed least biogas production i.e., 20 ± 5.66 ml in 15 days (Figure 1). The MEC with 2.5 V produced biogas more than 20 times higher than control. The volume of gas produced in MEC reactor upon application of different voltage was found to be statistically significant, which further suggested that when voltage was altered, the amount of gas produced in reactor was also altered significantly. In a related study, Liu et al. (2016) reported that when the cathodic potential was 0.90 V, the methane production was 5.3–6.6 times higher than that of the control group at 10 ºC. The applied voltage drives process of electromethanogenesis, as methanogenic microbes utilize electrons and protons producing methane which is typically coupled with the reduction of CO_2, where the applied voltage reduces CO_2 to methane at the cathode. This reduction works efficiently at certain voltage threshold (Blasco-Gómez et al., 2017). On top of that methanogens are sensitive to the applied voltage. Different methanogens might have optimal voltage ranges for their activity (Siegert et al., 2015). The increased gas generation at low temperatures may be caused by the MEC system's improved microbial metabolism in a number of ways, including increased activity of key enzymes. However, research on the primary methane generation pathway, the structure of the functioning microbial population, and the activity of a crucial enzyme in MES was rarely conducted at low temperatures (Wang et al., 2022).

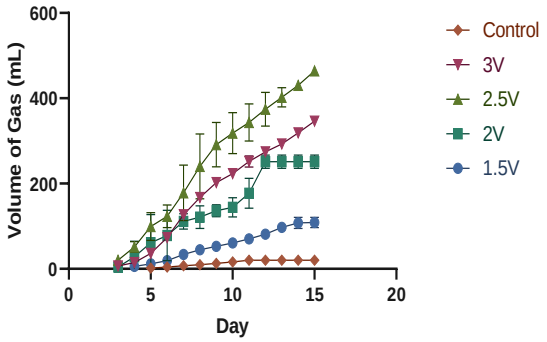

Figure 1: Biogas production from MEC with application of different voltages at 15 ºC and 1:10 dilution.

Dilution optimization

Three dilutions (1:10, 1:5 and 1:2) were used at optimized voltage of 2.5 V at 15 °C in which the MEC of 1:5 dilution showed the highest biogas production of 607 ± 24.04 ml whereas 1:10 and 1:2 dilution produced 464 ± 8.49 ml and 228 ± 16.97 ml biogas respectively. The control system without voltage supply with dilution of 1:10, 1:5 and 1:2 produced 20 ± 5.66 ml, 44.5 ± 3.54 ml and 72.5 ± 12.02 ml respectively after 15 days (Figure 2). The volume of gas produced in MEC reactor upon application of different dilution was statistically

significant (p<0.0001), which further suggested that alteration in dilution of substrate results in the alteration of biogas production significantly also indicated by strong positive Pearson's correlation coefficients across dilutions. Lower dilutions is likely led to substrate inhibition or mass transfer limitations, reducing gas production, whereas higher dilutions might have provided insufficient substrate for optimal microbial activity, as observed in previous studies by Siegert et al. (2015) and Zhang et al. (2019).

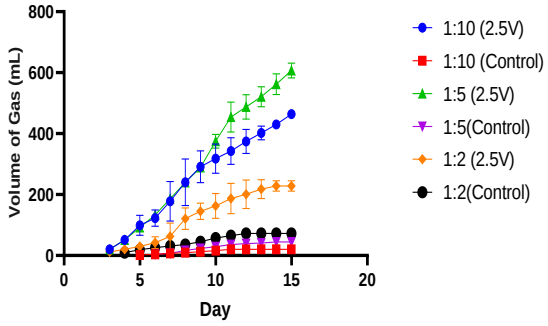

Figure 2: Biogas production by 1:10, 1:5 and 1:2 dilution at 15 °C and 2.5 V.

Scaling up biogas production

The biogas production was scaled up to pilot scale of working volume 10 L at 15 °C, in which MEC produced biogas of 3333 ± 29.69 ml and control produced 779.5 ± 20.51 ml after 15 days (Figure 3). The MEC produced 4 times more biogas as compared to control. This significant increase in biogas production is likely due to the applied voltage (2.5 V as optimized in lab scale) in the MEC, which is found to enhance the electrochemical reactions and microbial activity, leading to more efficient substrate utilization and higher biogas yields (Villano et al., 2010). The pilot scale also has higher and improved mass transfer and substrate availability, which further supports microbial activity and this leads in increase in overall gas production (Blasco-Gómez et al., 2017; Siegert et al., 2015).

Comparison of biogas collection using KOH and water displacement method

The total collected biogas volume was observed to be higher in MEC with water displacement which was found to be 741.5 ± 13.44 ml as compared with KOH displacement i.e., 607 ± 24.04 ml, indicating that 18% of total biogas thus produced was CO_2 in MEC reactor at 15 °C, 2.5 V and 1:5 dilution (Figure 4). The biogas produced in control reactor with water and KOH displacement was found to be 95.5 ± 2.12 ml and 44.5 ± 3.54 ml respectively, suggesting presence of 53% CO_2 in total biogas composition. The statistically significant difference shows that the choice of displacement solution has a significant impact on the biogas collection. According to Ahn & Logan (2010) and Mourad et al. (2022), KOH resulted a more accurate reflection of methane content but overall lower total biogas volume was collected comparing to that of water displacement method.

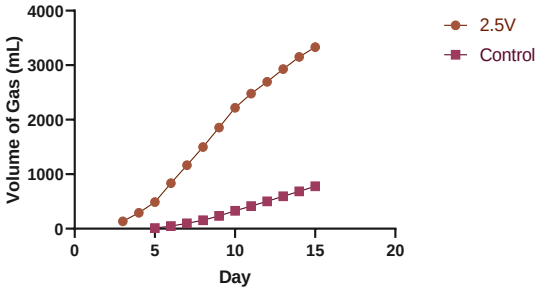

Figure 3: Biogas production in pilot scale of working volume 8.5 l at 15°C, 1:5 dilution and 2.5 V.

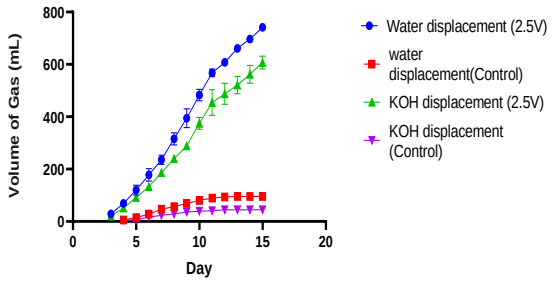

Figure 4: Comparison of biogas collected by water and KOH displacement at 15°C, 2.5V and 1:5 dilution.

Reduction in pH, COD and reducing sugar after digestion

The COD reduction results (Figure 5a) indicated that MEC system significantly enhanced treatment efficiency compared to control, with the most effective conditions found to be at 2.5 V and a 1:5 dilution. At this optimized voltage condition, the 81.59% COD reduction at 15 °C was observed in MEC, which increased to 88.28% when applied to pilot scale. This COD reduction is more than double that of control setup, which signifies MEC's performance in organic matter degradation. The

COD reduction data showed statistical significance with different voltages and dilutions, with *p*-values nearly equal to zero, confirming that 2.5 V and a 1:5 dilution were optimal for maximizing treatment efficiency as stated by Li et al. (2021); Logan et al. (2006); Mandal & Das (2018). There was significant reduction in soluble reducing sugar using MEC under various conditions, with the highest reduction (56.03%) observed at 2.5 V and 15 °C (Figure 5b). In a similar study, Feng et al. (2010) identified 2.5 V as optimal for enhancing organic matter degradation. According to Prathiba et al. (2022), microbial activity and electron transfer efficiency are also influenced by substrate concentration.

A strong correlation was seen between voltage and gas production (r = 0.878), as well as COD reduction (r = 0.854), suggesting that optimized voltage enhance metabolic activity of microorganisms and efficiency of degradation of organic substrate. Ding et al. (2016); Marmanis et al. (2022) further validates our findings. Dilution levels also significantly affected MEC performance, with correlations between dilution and gas production (r = 0.851) and COD reduction (r = 0.972). The pH levels remained relatively stable during the anaerobic digestion process, with only minimal fluctuations observed across various conditions as shown in Figure 5c. The pH values consistently remained between 6.7 and 7.0, indicating that the changes were statistically insignificant for voltage optimization as well as for dilution optimization.

Analysis of biogas composition

The analysis of biogas composition showed that the MEC system produced a significantly higher methane content than that of control reactor with lower CO_2 level in MEC compared to control reactor as shown in Table 2. The statistically significant differences in methane and CO_2 composition in those reactors (p = 0.02) indicated that MEC also helps in enhancing methane production and reducing CO_2 content. Thus, we can suggest that the MEC system improves the overall efficiency and quality of biogas production by increasing the methane to CO_2 ratio. Wellinger (2013) and Li et al. (2019) suggests that the raw biogas is normally composed of methane (50–75%), carbon dioxide (25–50%), and smaller amounts of nitrogen and other trace elements which also depends on the feedstock used. The higher production of methane in MEC reactor may be attributed to the favorable condition for efficient electron transfer processes that favor methanogenesis over other competing microbial processes (Wang et al., 2021; Zhao et al., 2021).

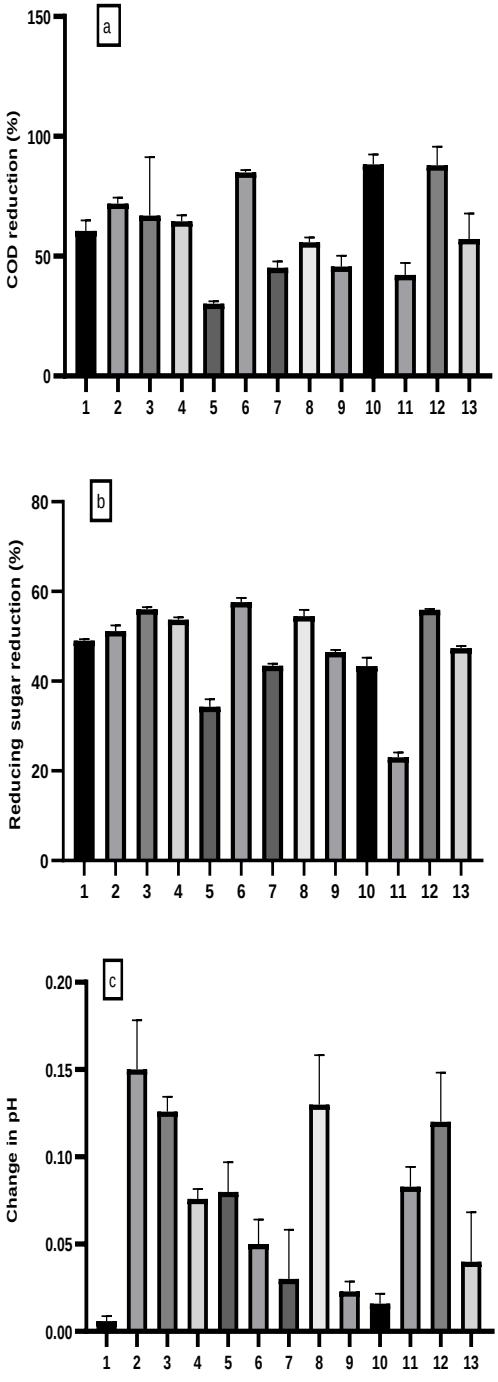

Figure 5: Reduction in a) Chemical oxygen demand b) Reducing sugar and c) Change in pH after 15 days of digestion under different conditions (1. MEC 1.5V (1:10), 2. MEC 2V (1:10), 3. MEC 2.5V (1:10), 4. MEC 3V (1:10), 5. Control (1:10), 6. MEC 2.5V (1:5), 7. Control (1:5), 8. MEC 2.5V (1:2), 9. Control (1:2), 10. MEC 2.5V (1:5, 8.5L), 11. Control (1:5, 8.5L), 12. MEC 2.5V (1:5,28°C) and 13. Control (1:5, 28°C)).

Table 2: Biogas composition in MEC and control setup and their p-value.

Gas	MEC	Control	Percentage of difference	*p*-value
CH_4	82.45±1.58	72.66±1.58	11.86	0.02
CO_2	11.35±1.25	20.68±1.59	45.15	0.02
H_2	4.67±0.64	4.41±0.64	5.67	0.71
Other	1.55±0.30	2.25±0.66	31.18	0.30

Isolation and molecular identification of microorganisms

From the sample after digestion in MEC, four different bacteria (MK1, MK2, MK3, and MK4) were isolated. The genomic DNA of four isolates were extracted and amplified by PCR using 16S rRNA primer. The amplified DNA was run on gel electrophoresis and visualized under gel documentation in which size of the amplified DNA of the four isolates was found to be 516 bp, which falls just above 500 bp on the ladder as shown in Figure 6.

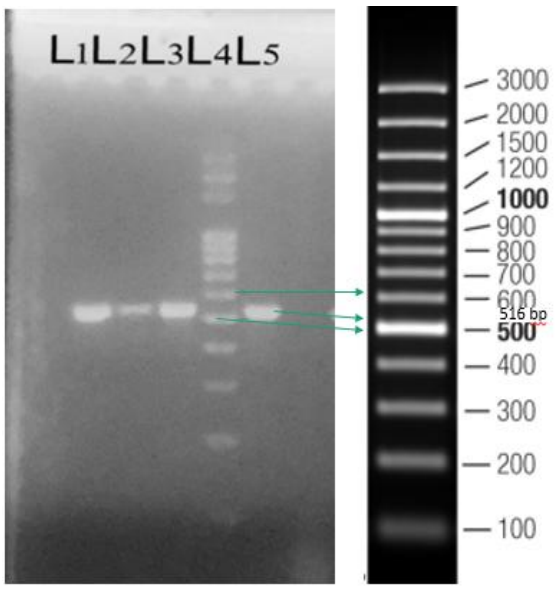

Figure 6: Gel electrophoresis of PCR products; L1, L2, L3 and L5 represents amplified DNA of isolate MK1, MK2, MK3 and MK4 respectively and L4 indicates Generuler 100 bp ladder (0.1 µg/µl).

The sequences of isolates MK1, MK2, MK3 and MK4 showed the evolutionary relationship with *Bacillus cereus*, *Bacillus paramycoides*, *Priestia flexa* and *Bacillus licheniformis* respectively based on 16S rRNA sequences (Figure 7). Li et al. (2023); Senés-Guerrero et al. (2019); Siddharth et al. (2024); Sun et al. (2023) reported that *Bacillus* species were frequently isolated from anaerobic environments, including biogas reactors and wastewater treatment plants. The close relationship of isolate MK3 with *Priestia flexa*, a relatively lesser-known member of the Bacillaceae family, which can degrade mucin and is better acclimatization in the human GI environment (Deswal et al., 2023) suggests that this strain may also play a specialized role in the anaerobic digestion process, potentially contributing to specific biochemical pathways that support the microbial community's stability and efficiency. *Bacillus* species, commonly enriched in cow dung due to its nutrient-rich environment (Behera & Ray, 2021; Bhatt & Maheshwari, 2019), play a crucial role during operation of MEC at low temperature. These microbes are essential to carry out decomposition of complex organic compounds into a much simpler forms necessary for sustaining microbial processes within the MEC, even at low temperatures i.e. 15 °C (Bazina et al., 2023). These species produces cellulases and proteases which are necessary for degradation of organic matter, crucial at low-temperature environments where microbial metabolic rates generally decrease (Li et al., 2019). *Bacillus* species have also been observed to facilitate transfer of extracellular electron, an essential process in MECs, by forming biofilms on the anode that enhance electron transfer from the oxidation of organic compounds at an anode to cathode (Chen et al., 2019; Yu et al., 2023). The ability of extracellular electron transfer is particularly significant at low temperatures, where the efficiency of electron transfer ability of other various species often diminishes, but *Bacillus* species can sustain this process by adopting different changes in membrane structure at low temperature (Beranová et al., 2010; Deng et al., 2023).

Conclusion

The study demonstrates the significant potential of microbial electrolysis cells (MEC) in enhancing biogas production from cow dung under low-temperature conditions (15 ± 0.5°C). The optimized voltage of 2.5 V led to a substantial increase in biogas production. Dilution optimization revealed that 1:5 dilution produced the highest biogas yield. Additionally, scaling up to a pilot reactor (10 l) confirmed the efficacy of MEC. The MEC also showed significant reduction of COD and reducing sugars. The MEC system enhanced biogas quality, increasing methane and reducing CO_2 content. The

microbial community identified four major strains of bacteria showing close evolutionary relationship with *Bacillus cereus*, *Bacillus paramycoides*, *Bacillus licheniformis*, and *Priestia flexa*, which play crucial roles in organic matter degradation and electron transfer, vital for MEC operation at low temperatures. These findings effectively illustrated the potential of MEC technology to enhance both the quantity and quality of biogas production from cow dung.

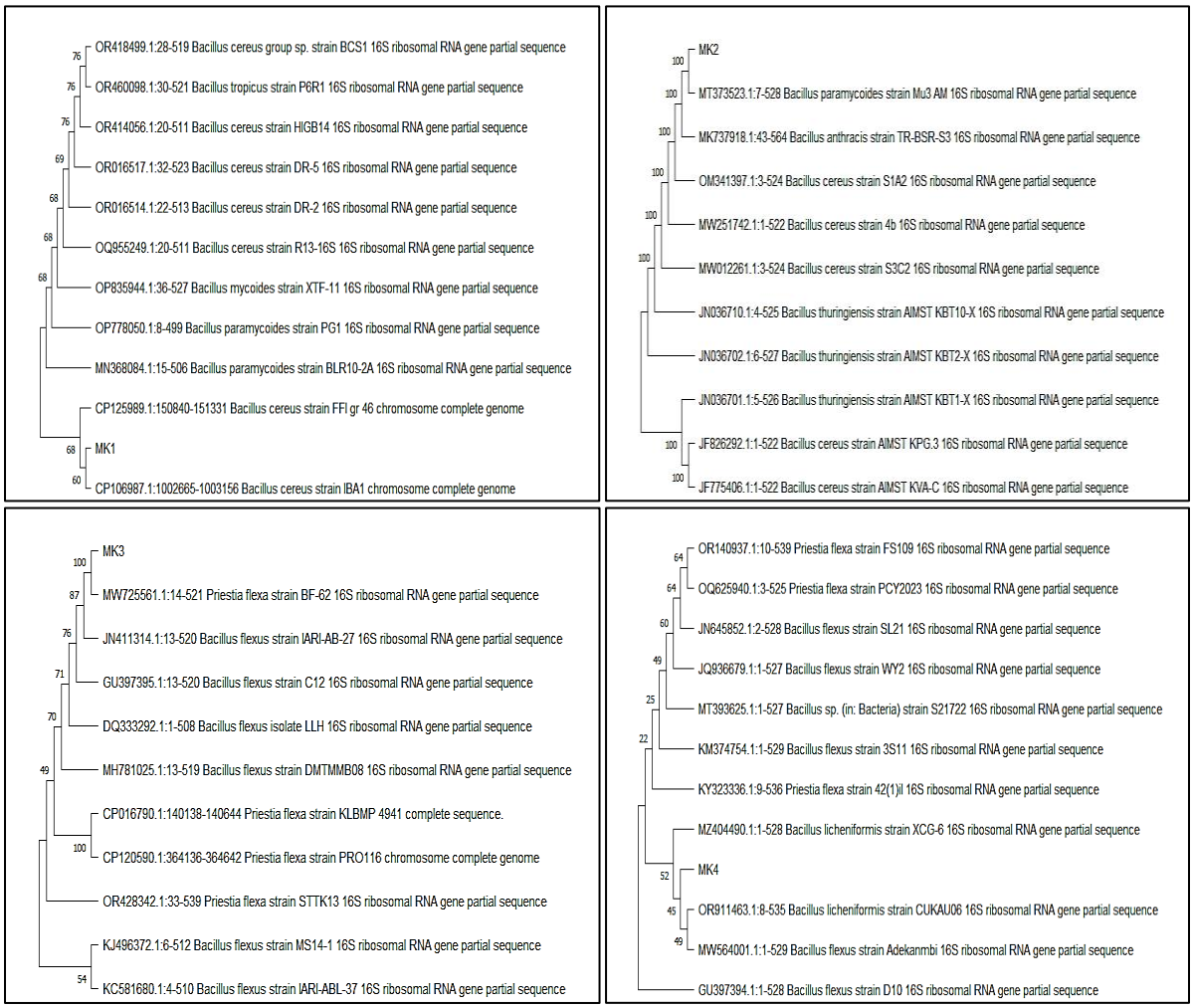

Figure 7: Phylogenetic tree of isolates MK1, MK2, MK3 and MK4.

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Research Article

Molecular detection of blaOXA-23, csuE and ompA genes from carbapenem-resistant and biofilm producing *Acinetobacter baumannii* isolated from clinical samples

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Abstract

Acinetobacter baumannii has become a critical hospital pathogen due to its biofilm formation and multidrug resistance, particularly against carbapenems. This study aimed to detect blaOXA-23, ompA and csuE genes in carbapenem-resistant, biofilm-producing *A. baumannii* in clinical isolates. Among 521 clinical samples, 39 MDR and carbapenem-resistant isolates were selected. Antibiotic susceptibility was tested via Kirby-Bauer disc diffusion with carbapenemase production confirmed by the combined-modified carbapenem inactivation method. Biofilm production was assessed by congo red agar and modified microtiter plate assay. PCR was used to detect blaOXA-23, ompA and csuE genes. All isolates were resistant to ceftazidime and ampicillin but sensitive to colistin. Of the 39 isolates, 29 produced biofilms (10 strong, 7 moderate, 12 weak and 10 non-biofilm producers). The prevalence of blaOXA-23, ompA and csuE genes was 61.53%, 41.02%, and 46.15%, respectively with a significant correlation between antibiotic resistance, biofilm formation and gene presence. The high prevalence of MDR *A. baumannii* with biofilm-associated genes and carbapenem resistance in hospitals highlights the need for stringent control measures and regular monitoring.

Keywords: *Acinetobacter baumannii*, Biofilm formation, blaOXA-23, csuE, MDR, ompA

Introduction

Antibiotic resistance has become a global issue, severely limiting the treatment of common infectious diseases (Nemec et al., 2016). Once considered a low-level pathogen, *Acinetobacter baumannii* has emerged as a significant cause of nosocomial infections, particularly septicemia and pneumonia in immunocompromised ICU patients. Its persistence in hospital environments and ability

to form biofilms make it a growing concern worldwide (Almasaudi, 2018, Gedefie et al., 2021).

Acinetobacter baumannii is known for its biofilm-forming ability and resistance to multiple antibiotics, which complicates treatment (Zeighami et al., 2019; Roy et al., 2022). Carbapenem-resistant *A. baumannii* (CRAB) is associated with various carbapenemase classes and international clones, making infection management challenging (Doi et al., 2009; Peleg et al., 2007). Carbapenem resistance mechanisms include carbapenemase production, reduced membrane permeability, altered penicillin-binding proteins, and efflux pump overexpression (Joshi et al., 2017). Among these, blaOXA-23 is the most prevalent gene, especially in Asian countries (Poirel et al., 2010; Peleg et al., 2008; Turton et al., 2006).

The World Health Organization (WHO) has labelled *A. baumannii* a "Priority Organism" due to its rapid spread of antibiotic resistance through biofilm formation (Gedefie et al., 2021). Biofilm production involves complex mechanisms such as collagen addition, pili expression, iron acquisition and quorum sensing (Gaddy & Actis, 2009; Tomaras et al., 2003). Virulence factors linked to biofilm formation include ompA, csuE, Bap, PNAG, and others (Ghasemi et al., 2018; Thummeepak et al., 2016).

The ompA gene contributes to cell adhesion, biofilm formation, and immune response modulation (Asif et al., 2018; Cassin & Tseng, 2019). The csuE gene, part of the csu operon, is essential for pilus formation and biofilm development (Cincarova et al., 2016; Tomaras et al., 2008). Studies in Taiwan and Nepal have reported high prevalence rates of csuE and ompA genes, with significant correlations between antibiotic resistance, biofilm formation and associated genes (Yang et al., 2019; Shrestha et al., 2015).

In Nepal, *A. baumannii* isolates have shown high carbapenem resistance rates, with blaOXA-23 being the most common gene detected. Studies have also reported biofilm-producing *A. baumannii* from various clinical settings, including 53.97% at B.P. Koirala Institute of Health Sciences and 14% at Shree Birendra Hospital (Baniya et al., 2019; Dumaru et al., 2019; Yadav et al., 2017). This study aimed to investigate the antimicrobial susceptibility, carbapenemase production, and biofilm-related genotypes of carbapenem-resistant *A. baumannii* isolated from clinical samples in Nepal.

Materials and Methods

Sample collection and processing

This descriptive cross-sectional study was conducted from August 2022 to February 2023 at the Annapurna Neurological Institute and Allied Sciences, Kathmandu, in collaboration with Golden Gate International College, Kathmandu. Samples were collected from both inpatients and outpatients of all age groups with suspected bacterial infections. Ethical approval was obtained from the Institutional Review Committee (Reg no: 011/2022) at Annapurna Neurological Institute and Allied Sciences. Written informed consent was obtained from all participants.

A total of 521 clinical samples (blood, urine, sputum, catheter tips, CSF, tracheal aspirates and CVP tips) were analyzed. *A. baumannii* isolates were identified through standard microbiological techniques, including gram staining, biochemical tests (catalase, oxidase, citrate, urease and TSI), and cultural characteristics (Cheesbrough, 2006).

Antibiotic susceptibility testing (AST)

AST was performed by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar according to CLSI guidelines (2022). Antibiotic discs used included ampicillin, amikacin, gentamycin, ceftazidime, cotrimoxazole, levofloxacin, meropenem, imipenem, piperacillin-tazobactam and colistin.

Biofilm and carbapenemase detection

Biofilm production was assessed using the congo red agar (CRA) method and modified microtiter plate assay (Stepanovic et al., 2007). Carbapenemase production was evaluated using the combined-modified carbapenem inactivation method (mCIM and eCIM) following CLSI protocols (Vander et al., 2015).

Table 1: Specific primers used in the study for the amplification of target genes.

Target gene	Primer	Sequence	Size / annealing temperature
*bla*OXA-23	*bla*OXA-23^{F}	5' GATCGGATTGGAGAACCAGA 3'	501 bp / 52℃
	*bla*OXA-23^{R}	5' ATTTCTGACCGCATTTCCAT 3'	
OmpA	*ompA*$^{-F}$	5' CTGGTGTTGGTGCTTTCTGG 3'	352 bp / 49℃
	ompA^{-R}	5' GTGTGACCTTCGATACGTGC 3'	
CsuE	*csuE*$^{-F}$	5' ATGCATGTTCTCTGGACTGATGTTGAC 3'	976 bp / 60℃
	csuE^{-R}	5' CGACTTGTACCGTGACCGTATCTTGATAAG 3'	

Molecular analysis

DNA extraction was performed by the phenol:chloroform method. The PCR reaction mixture (15 µl) was prepared as follows; for the blank, 7.5 µl PCR water, 6.5 µl master mix and 0.5 µl each of forward and reverse primers, for the positive control, 4.5 µl PCR water, 3 µl positive control, 6.5 µl master mix and 0.5 µl each of forward and reverse primers, and for the sample, 4.5 µl PCR water, 3 µl genomic DNA, 6.5 µl master mix and 0.5 µl each of forward and reverse primers were added. PCR amplification was performed using genomic DNA under thermal cycling conditions as described by Li et al. (2014) for blaOXA-23, Ghasemi et al. (2018) for ompA and Seifi et al. (2016) for csuE (Table 1). The amplified products were characterized by 2% agarose gel electrophoresis in tris-acetate-EDTA with 0.6 µl ethidium bromide as a tracking dye. After solidification, 2 µl of 100 bp DNA ladder, 3 µl positive control, 3 µl negative control and 3 µl PCR products were loaded into the wells (Figure 1-3).

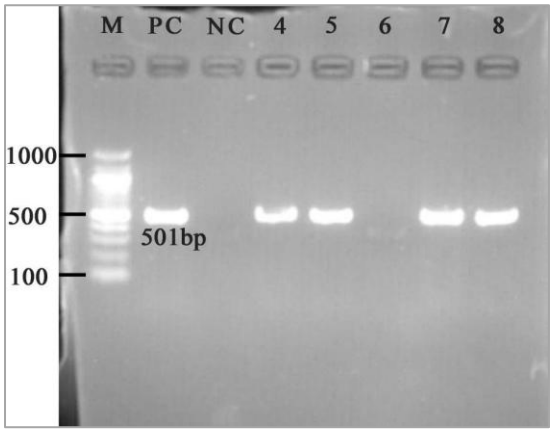

Figure 1: Gel Electrophoresis; Lane M: 100 bp ladder, lane PC: positive control, lane NC; negative control, lane 4, 5, 7 and 8 positive samples for blaOXa-23 gene and lane 6 negative samples with band appearing at 501 bp.

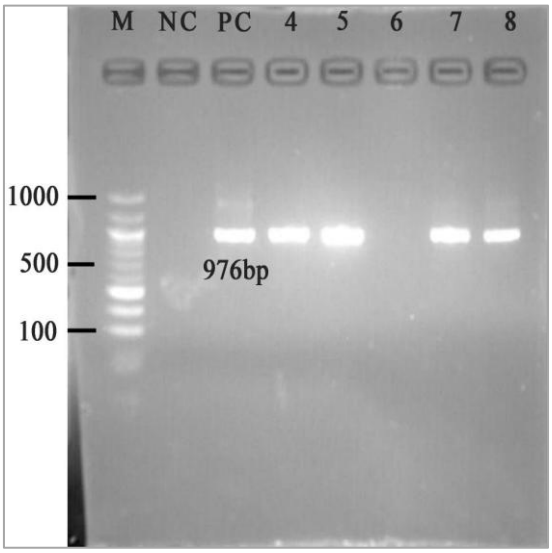

Figure 2: Gel Electrophoresis; Lane M: 100 bp ladder, lane NC: negative control, lane PC; positive control, lane 4, 5, 7 and 8 positive samples for csuE gene and lane 6 negative samples with band appearing at 976 bp.

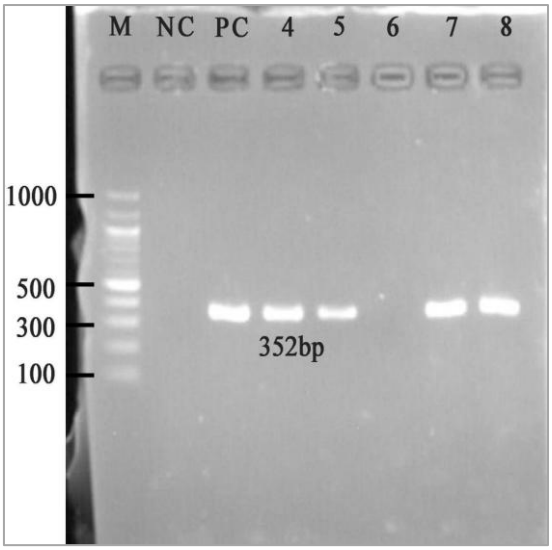

Figure 3: Gel Electrophoresis; Lane 1: 100 bp ladder, lane 2: negative control, lane 3; positive control, lane 4, 5, 7 and 8 positive samples for ompA gene and lane 6 negative samples with band appearing at 352 bp.

Statistical analysis

Data were analyzed using R software. Chi-square and regression analysis were applied to determine associations between variables with significance set at $p \leq 0.05$.

Results and Discussion

Among 521 clinical samples, 154 (29.55%) showed growth, and *A. baumannii* accounting for 25% (39/154) of isolates. Out of 39 isolates of *A. baumannii*, the majority of *A. baumannii* was isolated from sputum specimen 48.71% (19/39) followed by CSF 15.38% (6/39), urine 10.25% (4/39), pus 7.69% (3/39), CVP tips, tracheal aspirates, wound swab 5.12% (2/39), and throat swab 2.56% (1/39) while no *A. baumannii* isolates were isolated from blood, synovial fluid, tissue and pleural fluid specimens. No isolates were detected from blood, synovial fluid, tissue or pleural fluid.

Infections were more prevalent in male patients (58.97%) than female patients (41.02%). The highest prevalence was in patients aged 16–32 years (25.64%). The majority of *A. baumannii* isolates were from ICU patients (51.28%), followed by the post-operative ward (25.64%) and general ward (23.07%).

All isolates were resistant to ampicillin and ceftazidime (100%), with high resistance also observed against imipenem (97.43%), levofloxacin (94.87%), meropenem (92.30%) and gentamycin (92.30%). All isolates were sensitive to colistin. Phenotypic tests showed 36/39 (92.30%) isolates as carbapenemase producers by AST and 25/39 (64.10%) by mCIM. A significant association was observed between mCIM and blaOXA-23 gene occurrence ($p < 0.05$). Using the modified microtiter plate assay, 29 isolates (74.36%) were biofilm producers: 10 strong, 7 moderate and 12 weak. The congo red agar method identified 25 strong biofilm producers (64.10%).

Among 25 carbapenemase producers, 7 were strong, 5 were moderate, 8 were weak, and 5 were non-biofilm producers. A significant correlation was found between biofilm formation and carbapenem resistance ($p = 0.0069$) (Table 2). Prevalence of genes: blaOXA-23 (61.53%), ompA (41.02%) and csuE (46.15%) have shown significant associations between the occurrence of these genes, carbapenem resistance and biofilm formation ($p < 0.05$) (Table 3).

Table 2: Association between carbapenem resistance and biofilm production.

Biofilm production	**Carbapenemase producer**	**Carbapenemase non-producer**	**Total**	**p-value**
	Number (%)	Number (%)	Number (%)	
Strong	7 (17.94)	3 (7.69)	10 (25.64)	
Moderate	5 (12.82)	2 (5.12)	7 (17.94)	
Weak	8 (20.51)	4 (10.25)	12 (30.76)	0.0069
Non-producer	5 (12.82)	5 (12.82)	10 (25.64)	
Total	**25 (64.09)**	**14 (35.88)**	**39 (100)**	

Table 3: Comparison of combined-modified carbapenem inactivation method with bla_{OXA-23}.

Combined-modified carbapenem inactivation method	***blaOXA*-23**		**Total**	**p-value**
	Positive	Negative		
Positive	24	1	25	
Negative	0	14	14	0.0253
Total	**24**	**15**	**39**	

Table 4: Association between biofilm formation and biofilm related genes.

Biofilm Formation	**Isolates / Biofilm formation (%)**	**Biofilm-related genes isolates / Genes (%)**	
		ompA	*csuE*
Non-biofilm	10 (25.64)	0.00	2 (5.12)
Weak biofilm	12 (30.76)	5 (12.82)	5 (12.82)
Moderate biofilm	7 (17.94)	3 (7.69)	2 (5.12)
Strong biofilm	10 (25.64)	8 (20.51)	9 (23.07)
Total	**39 (100)**	**16 (41.02)**	**18 (46.15)**
p-value		0.004	0.008

The presence of both ompA and csuE genes is more common in strong biofilm producers, suggesting a potential link between these genes and the ability to form stronger biofilms. The statistically significant p-values indicate a significant association between biofilm formation and the presence of both genes ($p < 0.05$) (Table 4).

This study highlights the high prevalence of multidrug-resistant (MDR) *A. baumannii* and its association with biofilm formation and carbapenem resistance. The presence of blaOXA-23, ompA and csuE genes among isolates indicates a strong correlation between genetic factors, antimicrobial resistance and biofilm production.

The dominance of *A. baumannii* in respiratory samples (48.71%) aligns with previous studies associating it with ventilator-associated pneumonia (Yadav et al., 2020; Shrestha et al., 2015). The high prevalence in ICU patients (51.28%) further supports its role as a major nosocomial pathogen. Antibiotic susceptibility testing revealed complete resistance to ampicillin and ceftazidime, with high resistance to carbapenems (imipenem and meropenem), consistent with previous reports from Nepal and other countries (Joshi et al., 2017; Ghimire et al., 2021). The 100% susceptibility to colistin highlights its effectiveness as a last-resort treatment for *A. baumannii* infections.

Phenotypic tests identified 92.30% of isolates as carbapenemase producers, and 64.10% were confirmed via mCIM. This discrepancy indicates that mCIM may not detect all carbapenemase-producing strains. The blaOXA-23 gene was present in 61.53% of isolates, consistent with its role as a predominant carbapenem resistance gene in Asian countries (Poirel et al., 2010). Biofilm formation was detected in 74.36% of isolates by the modified microtiter plate assay. Strong biofilm producers were mostly associated with carbapenem resistance, confirming the protective role of biofilms against antibiotics (Ghasemi et al., 2018). The congo red agar method was effective in detecting strong biofilm producers but less sensitive in distinguishing moderate and weak biofilm producers.

The ompA and csuE genes were detected in 41.02% and 46.15% of isolates, respectively. These genes are essential for biofilm formation and play a significant role in enhancing antibiotic resistance. The substantial correlation between biofilm formation, carbapenem resistance, and these genes suggests that targeting biofilm-related genes may be an effective therapeutic strategy. The findings support the urgent need for improved infection control measures and regular surveillance of MDR *A. baumannii*. Effective monitoring of carbapenem resistance genes and biofilm-related genes could help in designing better strategies to combat this pathogen.

Conclusion

This study demonstrates a high prevalence of MDR *A. baumannii* isolates associated with carbapenem resistance and biofilm formation. The majority of the isolates harboured the blaOXA-23 gene, confirming its role as a key determinant of carbapenem resistance in Nepal. Significant associations were found between carbapenem resistance, biofilm formation, and the presence of ompA and csuE genes. The findings emphasize the need for enhanced infection control measures, periodic surveillance of resistance genes, and strategic use of effective antibiotics like colistin to

manage infections. Targeting biofilm-related genes could be a promising approach to combatting *A. baumannii* infections.

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Review Article

Microbial Metabolic Engineering for Biopolymers Production

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Abstract

This review delves into microbial engineering strategies for biopolymer production, which is crucial for tackling contemporary environmental challenges. It surveys microbial species capable of synthesizing biopolymers and examines genetic engineering techniques to enhance biopolymer yields. For the genetic manipulation approach, mainly the over-expression of the genes responsible for the production, the downregulation/knock out of competing branches, or the abundant supply of cofactors needed for the reaction are mostly tested, among others. This study provides insights into a few applications of metabolic engineering endeavors towards the production of some biopolymers as for poly-γ-glutamic acid (PGA), polyhydroxyalkanoates (PHAs), starch-based materials, and polylactic acid (PLA). This review highlights the importance of genetic manipulation in optimizing microbial strains and fermentation processes for sustainable biopolymer production.

Keywords: Biopolymers, genetic manipulation, microbial engineering

Introduction

The increasing focus on natural biodegradable bio-based polymers in the scientific and industrial realms suggests an urgent imperative for sustainable development. With the environmental toll of petroleum-derived polymers becoming increasingly evident, there is a growing shift toward renewable alternatives such as proteins, polysaccharides, and lipids. This transition addresses pressing environmental concerns and meets the rising demand for eco-conscious materials. Biotechnology plays a pivotal role in this transformation, offering innovative solutions like genetic manipulation of metabolic pathways, heterologous gene expression, and alternative expression systems. This review explores key applications of genetic engineering in the development of biodegradable materials, highlighting its potential to drive sustainable innovation.

For decades, bacteria have emerged as an up-and-coming alternative for synthesizing polymers due to their metabolic versatility and efficiency. Their ability to produce biopolymers such as polysaccharides, polyamides, polyesters, and polyhydroxyalkanoates (PHAs) has been extensively explored as highlighted. This review also highlights the potential of different fungal genus such as *Aspergillus*, *Trichoderma*,

Penicillium, *Absida* etc. to produce various polysacchrides. Furthermore, besides bacteria and fungi, the role of algal species in producing different types of biopolymers such as polylactic acid, polyhydroxyalkanoates and polyhydroxybutyrates is also noted by the authors (Akinsemolu & Onyeaka, 2023). Other polymers, such as polyesters, polyamides, and polysaccharides, are frequently produced using microbes, including wild-type strains, mutated variants, or genetically modified organisms (GMOs). To elaborate, wild-type microbes are natural, unmodified strains found in the environment. Mutations can be introduced by inducing random changes in their DNA, often through exposure to mutagens like UV light or chemicals, in order to improve specific traits. In contrast, GMOs are engineered through precise genetic modification, where particular genes are inserted, deleted, or altered to achieve desired functions. These microbial approaches offer versatile and sustainable pathways for producing biopolymers. Xanthan gum, the first commercial polysaccharide—a polymer composed of long chains of carbohydrate (sugar) molecules—was initially produced using bacteria (Kumar et al., 2018). Through genetic manipulation, researchers produced a highly white-colored xanthan gum while reducing ethanol usage, thereby lowering downstream processing costs. This improvement was achieved by introducing the *Vitreoscilla* globin gene (vgb) into *Xanthomonas campestris* to enhance its metabolic capabilities (Dai et al., 2019). This review explores microbial-based polymer production and genetic engineering techniques aimed at enhancing synthesis, with a primary focus on key polymers such as PGA (poly-ϒ- glutamic acid), PHAs, starch, and PLA (polylactic acid).

Improvement of PGA yield

One of the most widely recognized polypeptides, frequently discussed for its biopolymer potential, is poly-γ-glutamic acid (PGA). This versatile biopolymer and its derivatives have numerous applications, including uses in the food (Tanimoto, 2010), and medical industries as a flocculant in water treatment (Campos et al., 2016), a chelating agent in wastewater treatment (Mark et al., 2006), an ingredient in cosmetics (Serra et al., 2024), and a carrier molecule for drug and gene delivery (Schlechter et al., 1989). PGA is a polymerized product of L- and/or D-glutamic acid by means of ϒ -amide linkage. A common genus used to produce PGA is *Bacillus* and its strains, such as *B. licheniformis* and *B. subtilis* (Xu et al., 2005). These are well reported to be a PGA producer by microbial fermentation. Genetic engineering techniques were employed in *Bacillus* species to increase the PGA yield (Li et al., 2022). Despite the potential of *Bacillus* sp. for PGA production, the presence of hydrolytic enzymes such as *pgdS* (formerly known as *YwtD*), *ggt* and *CWLO* poses a challenge as these enzymes can degrade the synthesized PGA (Yao et al., 2009; Scoffone et al., 2013; Mitsui et al., 2011) (Figure 1). To address this, some of the engineering strategies have been employed to increase the PGA yield, including the plasmid based γ-PGA expression, deletion of genes *pgdS* and *ggt*, and expression of glutamate racemase (Scoffone et al., 2013; Ashiuchi et al., 2006). Expression of the glutamate racemase (*glr*) gene positively impacts PGA yield, likely due to increased uptake of L-glutamic acid, a precursor of PGA. The *glr* gene encodes an enzyme responsible for converting L-glutamate to its D-isomer and vice versa, thereby enhancing the availability of substrates for PGA synthesis (Jiang et al., 2011).

Counterintuitively, overexpression of the PGA degradation gene *pgdS*, increased γ-PGA yield by 54%. Higher *pgdS* expression positively influences the glutamate transporter (glutamate being a precursor of PGA) and increases the expression level of the PGA synthetase complex. Together, these factors likely contributed to the increased product yield (Tian et al., 2014).

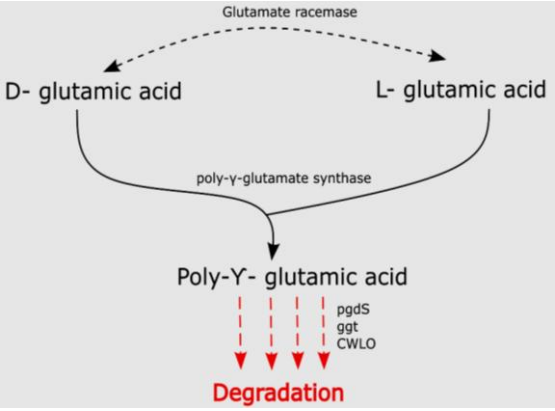

Figure 1: Microbial synthesis of poly-ϒ- glutamic acid (PGA). D- glutamic acid can be converted to L- form and vice versa using glutamate racemase. Polyglutamate synthetase complex can produce poly-ϒ- glutamic acid from its monomer. The degradation of PGA is mediated by the enzymatic activity of genes *pgdS*, *ggt* and *CWLO*.

Production of PHAs

Polyhydroxyalkanoates (PHAs), as naturally occurring biopolymers, have attracted considerable attention in various industries due to their biodegradable nature, biocompatibility, and physicochemical properties resembling those of petroleum-based counterparts (Sharma et al., 2021). Wang and their colleagues provide a thorough review of polyhydroxyalkanoates (PHAs) production, covering substrate roles, fermentation methods, and genetic engineering advances. Some notable examples listed include *Escherichia coli (E. coli)* modifications for ethanol-based poly(3-hydroxybutyrate) production and optimization of PHAs synthesis through propionate utilization (Wang et al., 2023). Propionate assimilation is crucial for generating propionyl-CoA, a precursor for medium-chain-length PHAs with odd-chain monomers (Zhuang & Qi, 2019). Microbes, particularly bacteria, are widely regarded as the most efficient systems for producing PHAs due to their natural ability to synthesize and accumulate these biopolymers. However, the high production cost remains a major barrier to scaling up PHA production (Tsang et al., 2019). Since substrate cost is the main expense, it can account for up to 50% of the total production cost (Pérez et al., 2020). To enhance the economic feasibility of PHAs commercialization, the utilization of low-cost carbon sources, as opposed to pure reducing sugar substrates, has been explored. For instance, studies have demonstrated that PHAs production can exceed 80% of the cell dry weight when waste rapeseed oil and propanol are used as substrates (Obruca et al., 2010). Among the various types of PHAs, polyhydroxybutyrate (PHB), which is composed of 3-hydroxybutyrate (3HB) monomer units, is one of the most extensively studied. PHB is produced by a wide range of microbial species (Markl et al., 2018).

There are numerous genetic modifications approaches tried to boost up the level of PHAs. The deletion of glucose dehydrogenase gene in *Pseudomonas putida* increased the titer of PHAs by 60%. The deletion of glucose dehydrogenase, an enzyme that directs glucose to gluconate, likely redirected more carbon toward PHA biosynthesis, resulting in increased production (Poblete-Castro et al., 2013). Similarly, a study from Salvachúa and the coworkers found that the knockout of several genes (*phaZ* and, β- oxidation related genes *fadBA1* and *fadBA2*) combined with overexpression of PHAs synthesizing genes (*phaC*, *phaG*, and *alkK*) was able to increase the yield of PHAs by using *P. putida.* Here, Salvachúa and the colleagues report a significant increase in PHAs yield by using solubilized lignin (derived from corn stover) as a primary carbon source for PHAs production. Genes *phaG, alkK,* and *phaC* catalyze forward reactions to produce 3-hydroxy acyl ACP and direct them towards mcl (medium chain length) – PHAs, while PhaZ reversely catalyzes this conversion (Ren et al., 2009; De Eugenio et al., 2010). Similarly, β-oxidation genes were deleted in this study to decrease 3-hydroxyacyl-CoA flux towards fatty acid β-oxidation and to increase the flux of 3-hydroxyacyl-CoA towards mcl- PHAs (Tsuge et al., 2000). Altogether, this genetic manipulation (Figure 2) done in *Pseudomonas putida* was able to give the highest titer and resulted in about a 200% increase in the yield of mcl polyhydroxyalkanoates (Salvachúa et al., 2020). This underscores the importance of a detailed understanding of metabolic pathways, where strategic interventions—such as overexpressing genes in the forward direction, downregulating competing branches, and minimizing reverse reactions—can significantly enhance the titer of a desired product, provided that the metabolic network is carefully balanced and optimized. In the context of other microbes, it has been shown with the over expression of *phbC,* P3HB yield was increased 1.4 times in *Ralstonia eutropha* (Barati et al., 2021). *phbC* is the gene encoding the main enzyme PHB synthase for PHB biosynthesis in *Ralstonia eutropha.* Another study carried out on Purple non-sulfur bacteria *Rhodobacter sphaeroides,* strain modified with the overexpressed PHA genes and deleted *phaZ* exhibited 1.7–3.9 folds higher production than the parent strain (Kobayashi & Kondo, 2019).

P3HB4HB is a biodegradable polymer within the PHAs family having good mechanical properties and thereby possess wide range of potential applications including tissue engineering (Wee et al., 2017, Luo et al., 2007, Crétois et al., 2015, Li et al., 2015). Acetyl CoA is converted to P3HB4HB in 3 steps. PhaA converts two acetyl CoA to acetoacetyl CoA. This acetoacetyl CoA is then converted to 3- hydroxy- butyryl CoA using PhaB. 3- hydroxy- butyryl CoA finally gets converted to P3HB4HB by PhaC. Chen and the colleagues

engineered *E. coli* to enhance P3HB4HB production by introducing *phaA*, *phaB*, and *phaC* from *Ralstonia eutropha* while deleting sad and *gabD* to increase succinate semialdehyde levels. Additionally, *sucD*, *4hbD*, and *orfZ* from *Clostridium kluyveri* were expressed to synthesize the key precursor, 4-hydroxybutyryl-CoA, leading to a titer of 0.85 g/l. Further optimization by expressing *ackA* and *pta* boosted acetyl-CoA levels, increasing P3HB4HB production to 1.71 g/l—nearly a 100% improvement. This study demonstrates how metabolic engineering can enhance biosynthetic pathways in *E. coli* for higher yields (Chen et al., 2018).

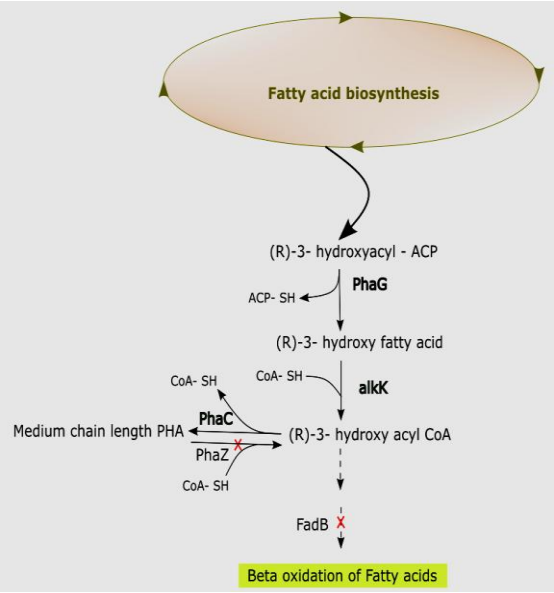

Figure 2: PHA metabolic pathway existing in *Pseudomonas putida*. Dotted lines and multiple arrows indicate multistep reactions. Red cross mark symbol in the arrow indicates deletion of the respective genes (*phaZ* and *fadB*), and bold letters shown for genes *phaG*, *alkK*, and *phaC* are the over expressed genes for high yield of PHA as done by Salvachúa et al., 2020.

The genetic modification of *Halomonas bluephagenesis* to enhance the xylose metabolic pathway, enabling the strain to utilize xylose as a substrate and achieve a poly-3-hydroxybutyrate (PHB) titer of 0.39 g/l (Tan et al., 2022). Further engineering of the strain, including optimization of the ribulose-5-P pathway and phosphoketolase pathway, resulted in a final PHB titer of 5.37 g/l. This highlights the potential of engineering diverse metabolic networks in microbial hosts to achieve high PHB yields. *Halomonas bluephagenesis*, a halophilic PHA-producing bacterium, has also been optimized in shake flask systems, where the expression of the *phaCAB* operon led to a PHAs content of up to 94% (Ren et al., 2018). Similarly, in *Pseudomonas putida*, genetic modifications such as the deletion of the *phaZ* gene, combined with DO-stat fed-batch fermentation, enabled the strain to utilize crude glycerol as a substrate and achieve a 48% increase in PHAs titer (Borrero-de Acuña et al., 2021).

Beyond the manipulation of metabolic pathways, studies have highlighted the importance of engineering cofactors, regulatory elements, promoter systems, and cell morphology to enhance PHAs synthesis (Wang et al., 2023). As an additional strategy, increasing the supply of cofactors required for PHAs synthesis has been explored in various studies. For instance, PHAs synthesis in most microbial strains relies on NADPH-dependent acetoacetyl-CoA reductase, making NADPH availability a critical factor for efficient production. To increase the supply of this cofactor, overexpression of the NAD salvage pathway genes *pncB* and *nadE* was employed, enhancing the NAD(P)H pool through improved NAD^+ biosynthesis. In *Cupriavidus necator*, overexpression of *pncB* and *nadE* not only increased PHB production efficiency but also improved tolerance to lignocellulose-derived inhibitors (Lee et al., 2022). Similarly, another study done with transhydrogenase gene overexpressed was shown to increase the NADPH pool for P3HB production. Transhydrogenase facilitates the conversion of NADH to NADPH, thereby boosting the NADPH pool, an essential cofactor for P3HB biosynthesis (Jung et al., 2019; Tadi et al., 2021).

Indeed, factors beyond pathway gene expression, such as cell structure, size, and intracellular space modifications, also influence cell morphology and thereby impact PHAs production, as discussed by Wang and the co- authors (Wang et al., 2023). Another study (Zhao et al., 2019) investigated *minC* and *minD*, known as FtsZ inhibitors (Bi & Lutkenhaus, 1993), and found that their deletion resulted in elongated cells with improved PHA titers. FtsZ is a bacterial protein that has a crucial role in coordinating cell wall and membrane growth, hence plays a key role during bacterial cell division Furthermore, in this engineered elongated strain background, it was shown that the individual overexpression of morphology related genes *mreB* and *ftsZ* made an increased titer of mcl- PHAs. However, when these genes *mreB* and *ftsZ* were simultaneously overexpressed, it resulted in the cells

with diverse shapes. In this scenario, the production went down, possibly due to adverse effects on cell division (Zhao et al., 2019). In another investigation, *E. coli* expressing PHB synthase underwent engineering, resulting in a notable increase in polyhydroxybutyrate (PHB) production, with the PHB titer rising from 5.72 g/l to 9.29 g/l. This enhancement was achieved by modifying the size of *E. coli* cells through engineering strategies, which included disrupting the *mreB* gene followed by its overexpression. The larger cell size was identified as a contributing factor to the higher accumulation of PHB granules (Jiang et al., 2015). Beside *mreB*, *sulA* was identified as another target to improve PHB titre. In *E. coli*, overexpression of *mreB* using a medium- copy number plasmid in a *mreB*-deleted background, combined with arabinose-induced expression of *sulA*, nearly doubled the PHB titer (Jiang et al., 2015). This demonstrates the potential for enhancing PHB accumulation in *E. coli* by targeting genes involved in cell morphology. MreB, an actin homolog, plays a critical role in peptidoglycan synthesis and maintaining cell shape (Rueff et al., 2014), while *sulA* inhibits FtsZ, leading to filamentous cell formation, increased cell size, and greater intracellular space for PHA accumulation (Bi & Lutkenhaus, 1993; Fenton & Gerdes, 2013). These examples highlight the importance of cell morphology-related genes in PHAs production, emphasizing how cell shape and internal structural space can significantly impact PHAs yields.

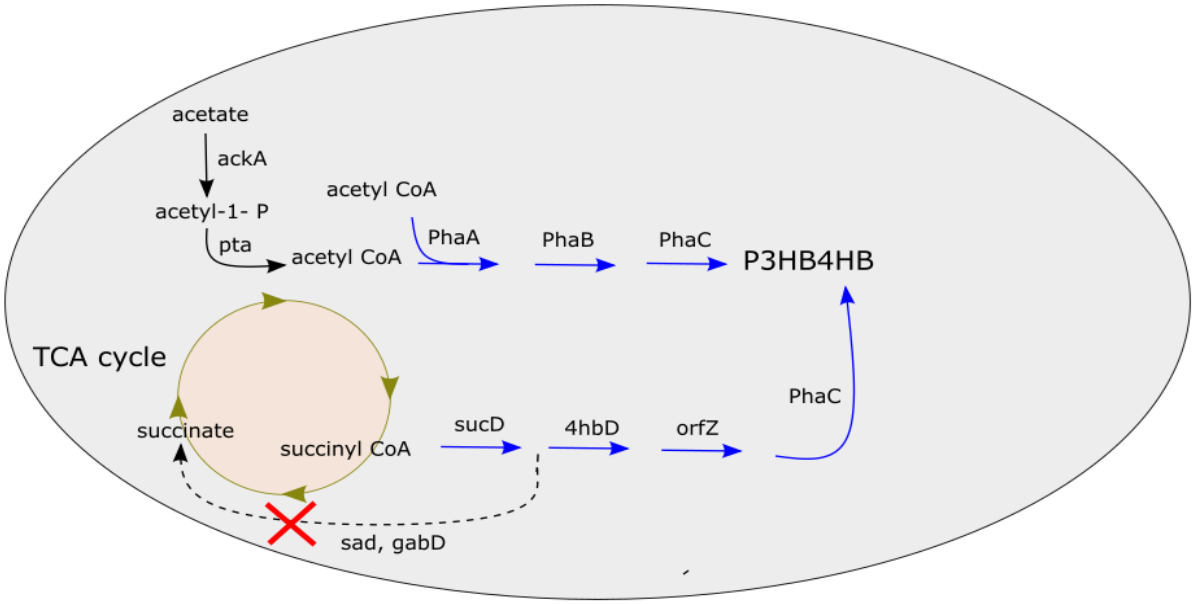

Figure 3: Metabolic engineering of *E. coli* as performed by Chen et al., 2018. Blue coloured lines indicate the steps converted by foreign genes introduced, where genes *sucD*, *4hbD*, *orfZ* originated from *Clostridium kluyveri* and *phaA*, *phaB* and *phaC* are derived from *Ralstonia eutropha*. A red cross mark symbol represents the step deleted as seen for *E. coli* native genes *sad* and *gabD*. Some intermediates are not shown and are denoted with multiple arrows representing multistep reactions. Genes responsible for the expression of respective enzymes are shown above arrows.

Starch based polymers

Another alternative in the biomaterials sector could be starch. Starch is a natural polymer and is thus used as a biodegradable material. Starch is a polysaccharide produced mainly by plants with an aim for energy reservoir system within. Starch granules, which are intracellularly localized, typically exhibit a variety of shapes, with spherical granules being the most common. These granules, ranging in size from 2 to 100 μm in diameter, are primarily derived from edible crops such as corn, rice, and wheat, as well as from tubers like potato and cassava (tapioca) (Chakraborty et al., 2020). Among these, corn, rice, wheat, potato, and cassava starches are the most commercially used due to their widespread availability and functional properties. There are several starch-based products commercialized, such as films, capsules, sheets, foam, etc. available in the market. A review study by Jiang and the co-authors illustrated challenges and potential opportunities for using starch-based materials to meet downstream. One of the key challenges described was moisture sensitivity, which arises due to the inherently hydrophilic nature of starch (Jiang et al., 2020).

A significant challenge in the starch industry is the extensive modification required for plant-based starch to meet industrial needs. Various physical and chemical treatments are employed for starch modification (Kaur et al., 2022), with the most common chemical modifications including esterification, etherification, or oxidation of hydroxyl groups. However, the application of genetic engineering techniques for starch production in microorganisms remains limited. Additionally, since starch is an insoluble polysaccharide at room temperature, it poses considerable challenges for enzymatic treatment and limits access to its functional properties. As a result, native starch is often unsuitable for direct industrial use. This limitation can be addressed by modifying starch to create starch derivatives, which can be tailored to meet specific industrial processing requirements. Microorganism has immense importance due to the versatility of enzymes obtained from them. One such microbial enzyme is a thermostable pullulanase from *Bacillus naganoensis*, having the ability to hydrolyze amylopectin to amylose in starch (Chang et al., 2016). This highlights a promising alternative to traditional physical and chemical methods, leveraging large-scale production of recombinant enzymes through genetically modified microorganisms for starch processing and modification. Such approaches offer innovative solutions to overcome the challenges associated with conventional starch modification techniques.

One example in the published literature is utilizing the bacterium *Klebsiella* enzyme cyclodextrin glycosyl transferase to be expressed in potato tubers to generate cyclodextrins (Oakes et al., 1991). Starch or starch derivatives as a source acted upon by cyclodextrin glycosyl transferase produce cyclodextrin. This starch-derived cyclodextrin is described for its advantageous application in food, pharmaceuticals, cosmetics, chemicals, agriculture, etc. (Del Valle, 2004; Szente & Szejtli 2004). Hence, enzymes related to starch modification or starch derivatives production hold significant promise in biotechnology for nutritional applications.

Polylactic acid synthesis

Another polymer shown to be produced microbially and has a high industrial application is named - Polylactic acid, mainly used to form biodegradable plastic, hence, addressing the environmental concerns. Review literature from Shah and coworkers reports the different biopolymers that can be used for bioplastic synthesis, such as starch, cellulose, PHAs, and polylactic acid (Shah et al., 2021). Among a range of biodegradable plastics, one of the most commercially used and successful bioplastics is polylactic acid (PLA), which has good processability and mechanical properties.

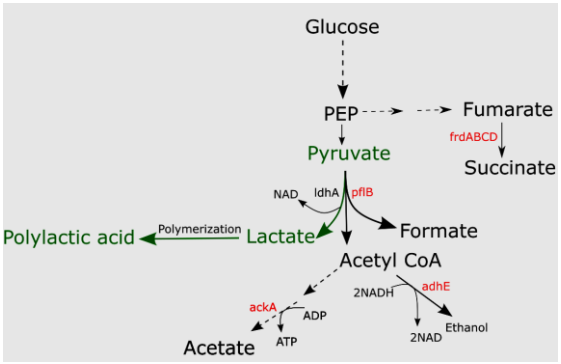

Figure 4: Metabolic pathway for production of polylactic acid in *E. coli*. Monomer lactic acid is produced via pyruvate with the enzyme lactate dehydrogenase (*LdhA*). Competing branches encoded by genes such as *frdABCD*, *ackA*, *adhE*, *pflB* are deleted to accumulate more pyruvate, a precursor of polylactic acid. Letters in red (*frdABCD*, *ackA*, *adhE*, *pflB*) indicates deleted genes. The solid arrows represent a one-step reaction, and the dotted arrows with multiple arrows indicate a multistep reaction. This figure is modified from Zhou et al., 2003.

Apart from the ability of some microbes and plants to produce PLA naturally (Paswan et al., 2023; Guo et al., 2011; Marzo-Gago et al., 2023; Lahtinen et al., 2011), some genetic engineering insights show promising approach for increasing the productivity. Polylactic acid is made up of its monomer lactate, which is produced from pyruvate by the action of lactate dehydrogenase (LDH) using NADH as a cofactor (Figure 4). Different competing branch to over-accumulate pyruvate was experimented on to increase the lactate yield (as shown in Figure 4). PflB directs some pyruvate towards formate as a byproduct. And, *frd* genes product catalyzes the last step in formation of succinate from PEP as a main source. Similarly, AdhE converts the pyruvate derived acetyl CoA to ethanol. The formation of acetate from acetyl-CoA is carried via *ackA*. All of these genes catalyze a reaction that competes with the accumulation of pyruvate, as this is the main precursor of lactate. Via knocking out of these genes (*pflB, frdABCD, adhE* and *ackA*) together with the replacement of native *E. coli* LDH (lactate dehydrogenase) with the heterologous gene L-LDH

from *Pediococcus acidilactici* (ldhL), authors were able to elevate the titer of D-lactic acid by ~ 2.5 times with the engineered *E. coli* strain named SZ63, and the product achieved 99% optical purity (Zhou et al., 2003). Similarly, in another prokaryotic engineering endeavor, the metabolic engineering of *Corynebacterium glutamicum* was undertaken, where the organism was engineered to utilize cellobiose and xylose through the expression of cellobiose and xylose utilizing genes. This engineered strain demonstrated the ability to consume cellobiose and xylose, resulting in the production of approximately 450 mM lactic acid with the consumption 29.3 mM cellobiose, 134 mM xylose, and 222 mM glucose (Sasaki et al., 2008).

In *Saccharomyces cerevisiae*, heterologous expression of the *ldh* gene from *Leuconostoc mesenteroides* resulted in an improved D-lactic acid titer of 2.3 g/l. To enhance the titer and minimize side product formation, efforts were made to increase the availability of pyruvate, a precursor for lactic acid, while reducing carbon loss. This was achieved by deleting genes *GPD1*, *GPD2*, and *ADH1*, which are responsible for glycerol and ethanol production. The engineered strain with these deletions exhibited improved lactic acid production, showing a 6.3-fold increase in titer. Notably, Adh1 in yeast catalyzes the conversion of acetaldehyde to ethanol, while *GPD* genes are involved in converting the glycolytic intermediate dihydroxyacetone phosphate (DHAP) to glycerol (Sornlek et al., 2022).

In another study, metabolic engineering was conducted in cyanobacteria by heterologous expression of *ldh* from *Bacillus subtilis* and co-expressing a transhydrogenase, resulting in an improved yield of lactic acid production. Expression of *ldh* derived from *Bacillus subtilis* in the cyanobacterium *Synechocystis* led to the accumulation of approximately 0.7 M lactic acid. Furthermore, the additional expression of transhydrogenase showed a five-fold increase in titer (Angermayr et al., 2012). In addition to the rational approaches mentioned above for increasing PLA (lactic acid) yield, some studies also explore non-rational approaches such as evolutionary engineering for strain improvement, aiming to achieve high-purity yields. Tian and his group report attaining highly pure lactic acid (99.1%) in a temperature-resistant strain capable of withstanding up to 45 °C. It was achieved through an adaptive laboratory evolution approach applied to an engineered strain of *Lactobacillus paracasei* (Tian et al., 2021). These improved strains can offer significant advantages for the polymer industries.

Taken together, these examples illustrate the potential of both rational and non-rational strain engineering methods to achieve high production of polylactic acid or its monomers. This represents a sustainable approach to biopolymer production using biotechnology and reveals the future of biotechnology in the production of biodegradable plastic as demanded by our modern society.

The biodegradable plastic market is projected to grow USD 20.9 billion by 2028, as noted by Biodegradable Plastics Report (2023); it reflects a substantial expansion of degradable plastic industries driven by the escalating demand for environmentally friendly materials. Similarly, in the market research survey 2022, the Fact.MR (Fact.MR, 2023) reports a significant increase in the value of biodegradable package materials, expected to rise from 88 billion dollars in 2022 to 169 billion dollars over the next decade. Key players from various countries, including Germany, the US, and Japan, are leading the innovation and adoption of biodegradable materials, fostering economic growth and job opportunities. Notably, industry giants like Coca-Cola and PepsiCo have committed to adopting 100% biodegradable and recyclable packaging materials by the end of 2025, further propelling the market forward. Metabolic engineering stands as a crucial driver in this bioplastic revolution, optimizing microbial pathways to efficiently produce biopolymer precursors through genetic modifications. The diverse array of bioplastic monomers, such as polylactic acid (PLA), polyhydroxyalkanoates (PHAs), and Polyethylene terephthalate (PET), highlights the versatility and potential of bioplastics in addressing environmental concerns and advancing sustainable development on a global scale.

Conclusion

To conclude, this review elucidates the significant potential of microbes in addressing environmental concerns through the production of biodegradable polymers. Examining various microbial species and their reported biopolymers, alongside genetic manipulation techniques such as heterologous gene

expression, gene knockout, and overexpression, reveals promising avenues for enhancing product yields. By showcasing the applicability of genetic engineering in elevating high-value product production, this review highlights its pivotal role in industry and environmental sustainability. Looking forward, optimizing microbial strains and refining fermentation technologies can offer promising directions for advancing biopolymer production.

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