

*Review Article*

## Advances and Future Prospects on Biotechnological Approaches Towards *Azolla* for Environmental Sustainability

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### ABSTRACT

Environmental sustainability is an integral aspect of living a better life, which will continue to be globally highlighted in the future. Sustainable Development Goals (SDGs) are crucial in most research areas to improve natural resources that will ensure the long-term viability of the environment. The rising population may lead to increased pollution due to extensive anthropogenic activities. Natural resources are being increasingly exploited by an ever-increasing human population and rising per capita consumption. A combination of biotechnological approaches to strengthen environmental sustainability in plant fields has often been used. *Azolla*, an aquatic fern, is a promising candidate for worldwide application and is well established in biotechnology, particularly focusing on

environmental sustainability. This review aims to explore the prospective of *Azolla* using a biotechnology approach. This review highlights current and future research and presents viewpoints on the importance of biotechnology in phytoremediation, genomics, and the animal feed industry.

**Keywords:** Aquatic fern, *Azolla*, biotechnology, environment, sustainability

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## INTRODUCTION

Emerging technologies are increasingly marketed, ensuring long-term advantages along with growth in societies with environmental, economic, and socially responsible societies (Matthews et al., 2019). Biotechnology applications are a powerful strategy for assisting industries in achieving global sustainability, including textile processing (Rahman et al., 2020), animal feed (Kusmayadi et al., 2021), biofuel (Nasir et al., 2018), and agriculture (Adenle et al., 2012; Behera et al., 2021). With their high potential in different industries, it is necessary to use these technologies to aid in the implementation of better sustainability. According to Verma et al. (2011), biotechnology involves using living materials or biological products to produce current innovations for various applications, aiming to benefit humans. Environment biotechnology is helping manage the world's sustainability by reducing adverse effects. Furthermore, it brings forward existing research to be explored further to establish newer opportunities for environmental conservation.

Biotechnology is a fundamental aspect of achieving the Sustainable Development Goals (SDGs) of the United Nations, which will continue to emerge in the future as the global population expands. According to EuropaBio (2018), industrial biotechnology is linked to many SDGs, including SDG Goal 1: end poverty; SDG Goal 2: end hunger, achieve food security, and improve nutrition; SDG Goal 6: availability of water and sanitation sustainability; SDG

Goal 7: ensure everyone access to cheap, dependable, and sustainable energy; SDG Goal 8: promote sustained, inclusive, and sustainable economic growth; SDG Goal 9: build resilient infrastructure and foster innovation; SDG Goal 11: make cities and human settlements inclusive, safe, resilient, and sustainable; SDG Goal 13: take immediate action to address climate change and its consequences; SDG Goal 14: conserve and sustainably use of the natural resources; and SDG Goal 17: re-establishing the global cooperation for sustainable development.

This review focused on an aquatic fern, *Azolla*, which has been exploited as an organic fertilizer to boost rice production in Southeast Asia for over 1,000 years (Lumpkin, 1980). It contributes to sudden global cooling by sequestering atmospheric carbon dioxide (Sessa et al., 2014). Despite its tiny size, it has various benefits for environmental conservation sustainably. The importance of the aquatic fern, *Azolla*, in ensuring ecosystem sustainability has long been established as soil fertilizer, bioremediation, and its part in mitigating greenhouse gas (Kollah et al., 2016). *Azolla* is not just a fern but also a superorganism (Li et al., 2014). Therefore, it has greater potential to achieve future sustainability goals defined by the United Nations through biotechnological approaches in a few main sectors, including animal feed, phytoremediation, and genomes (Figure 1). *Azolla*'s biotechnology approaches have been well established in a variety of studies, such as phytoremediation (Ghorbanzadeh

Mashkani, 2009; Goala et al., 2021; Talebi et al., 2019), enhancement of nutritive value (Brouwer et al., 2018; Costarelli et al. 2021),

phylogenetic analysis (Metzgar, 2007; Reid et al., 2006), and cryopreservation (Brouwer et al., 2014).

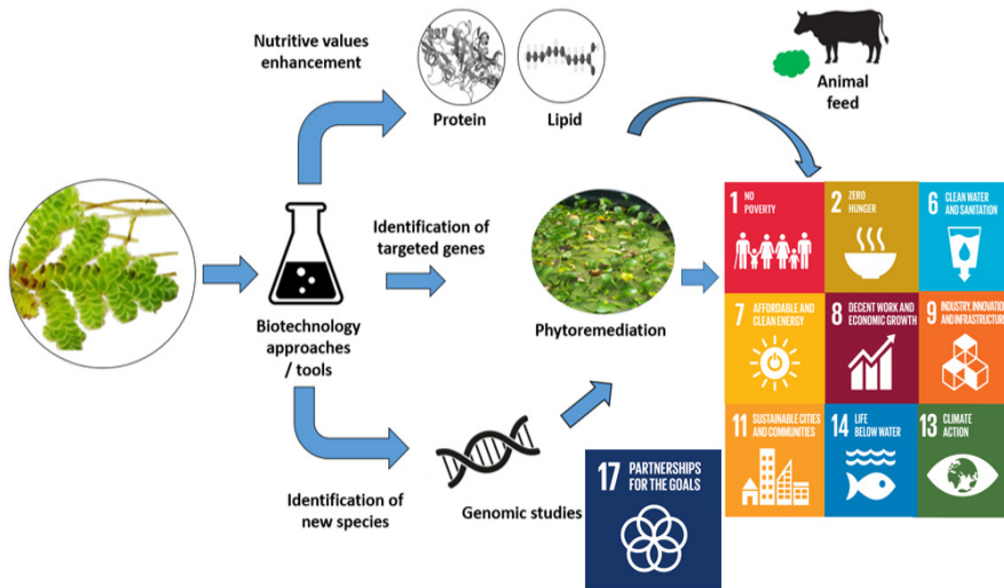


Figure 1. Biotechnological approaches in several sectors for *Azolla*

## LITERATURE REVIEW

A literature search was conducted using Scopus, Web of Science, and PubMed databases with keywords of *Azolla* OR aquatic fern OR aquatic macrophytes AND biotechnology AND sustainability. The literature search was limited to a specific period, i.e., 2000–2021. It included research articles and a book chapter; only titles and abstracts were screened to remove irrelevant articles. This search also examined the reference list of the retrieved articles. Original research articles on the biotechnological application of

*Azolla* published in English were included. The use of molecular tools to identify *Azolla* began in the early 90s, although its full genome was discovered in 2018. The first genomic data from ferns have been used to generate and understand the mechanisms that govern the evolution of plant genes and gene families (Li et al., 2014). According to PubMed, Google Scholar, and ScienceDirect databases, the total number of publications under the keywords of “biotechnology” and “*Azolla*” increased in 2020 compared with 1988 (Figure 2).

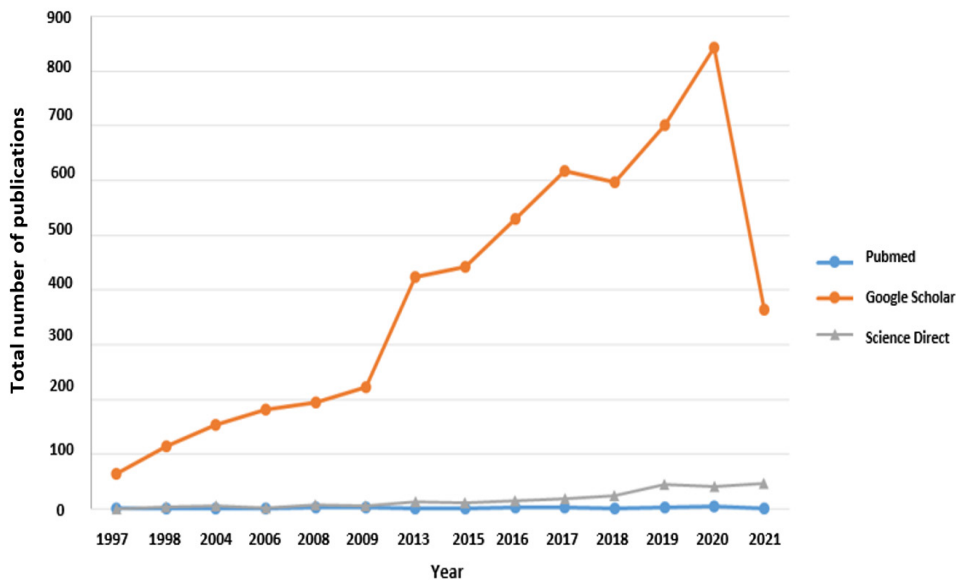


Figure 2. Research trends of biotechnology application in *Azolla* based on Science Direct, Google Scholar, and Pubmed databases

## CURRENT STATUS OF BIOTECHNOLOGY IN *AZOLLA*

*Azolla* biotechnology approaches emerge rapidly and will continue to increase in the future. The biotechnology approach's effectiveness has mostly been focused on increasing the genetic diversity of *Azolla*'s species, phytoremediation, and improving nutritive value. This approach strategy had much success with this aquatic fern species. Additionally, one of the important aspects that have been considered in the development of molecular genetics, cellular, and genetic engineering, as well as genome editing, is to increase the efficiency in *Azolla*. Basic genetics and genetic engineering research also need to be utilised to increase the content of nutritive value and secondary metabolites in aquatic plants.

## PHYTOREMEDIATION WITH BIOTECHNOLOGY TOOLS IN *AZOLLA*

Plants may be used to counteract the adverse effects of emissions and other human influences on the environment. The aquatic fern, *Azolla*, has an amazing ability as a phytoremediator to protect the environment from pollution (Naghypour et al., 2018). *Azolla* successfully detoxified some heavy metals, including arsenic (Zhang et al., 2008), ferrum, aluminium (Bianchi et al., 2020), plumbum, zinc, and cadmium (Khosravi et al., 2005). The genes related to phytoremediation in *Azolla* have been isolated and stated in Table 1 to understand the mechanism contributing to *Azolla*'s accumulation and detoxification effect. In a previous study, Schor-Fumbarov et al.

(2005) identified the efficiency of *Azolla* as a phytoremediator at the molecular level, where AzMT2 was linked to genes associated with *Azolla filiculoides*. The overexpression of phytoremediation-related genes in the molecular analysis may prove the effectiveness of plants as well-established phytoremediators (Nedjimi, 2021). According to Talebi et al. (2019), the expression of metallothionein and phytochelatin (MT2) and (PCS1), respectively, by *Azolla* was significantly induced by heavy metal treatment.

As part of the response to achieve environmental sustainability by reducing climate change, biochar is used to improve soil's physical, chemical, and biological properties, thereby reducing greenhouse gas emissions and sequestering carbon (Rawat et al., 2019). *Azolla* has been used as a primary organic source of biochar (Sadegh Kasmaei et al., 2019) along with other biochar sources (Dewi et al., 2018). Biochar is a high-carbon organic material produced via heat biomass without oxygen (pyrolysis) using biotechnology tools (Kimani et al., 2020). Table 1 lists several genes which are related to phytoremediation.

Table 1

*Genes synthesized by Azolla species related to different types of heavy metals*

Genes	Primers sequences	Heavy metals detected	Outcomes	<i>Azolla</i> species	References
Metallothionein-2 (MT2)	F GCAAGAGGAGCTTCGATGAGACC R CGCAAGAGCTATCGAACCCACAG	Copper (Cu), zinc (Zn), nickel (Ni), and cadmium (Cd)	Heavy metal treatments dramatically increased MT2 and PCS1 gene expression patterns	<i>Azolla filiculoides</i>	Talebi et al. (2019)
Phytochelatin synthase-1 (PCS1)	F TCCAATTCTCATCATTGCAGGAC R TCCAATTCTCATCATTGCAGGAC				
Actin	F TTGCTGATCGTATGAGCAAGGA R GATCCTCCAATCCAGACACTGTA				
nifB	5'-ATCCCTGCTACAGCGAAGAA-3' 5'-CCAGCAATCCCAGAACTGT-3' 5'-ATGTCTTGCTGTGGAGGAAA-3' 5'-TTAGCATCGGCAAGGATT-3'	Zn, Cu, Cd, and Ni	Inhibit biomass growth in <i>Azolla</i>	<i>Azolla filiculoides</i>	Khosravi (2005)

## GENOMIC STUDIES ON *AZOLLA* USING BIOTECHNOLOGY TOOLS

The complete genome sequence of *A. filiculoides* has been explored in a study by Li et al. (2018), which states that the genomic resources of *Azolla* are the most significant feature of future biotechnology research. Genetic diversity and phylogeny analysis for the identification of *Azolla* species was first established in 1993 by Van Coppenolle et al. (1993). Molecular tools are needed for the identification process in *Azolla* as their reproductive structure is rarely available. Although a vegetative structure can be used, it is unreliable (Madeira et al., 2016).

Therefore, eliminating cyanobacteria from *Azolla* is the most crucial step for DNA extraction in molecular marker techniques. Cyanobiont (AzCy) was extracted from erythromycin-treated *A. filiculoides*, and its gene expression pattern was compared with that of the wild type (AzCy+) using several molecular techniques (Li et al., 2018). Using erythromycin, a type of antibiotic, before DNA extraction methods may improve the removal of cyanobacteria, thus allowing only the extraction of DNA from plants. Table 2 summarizes various molecular techniques for detecting *Azolla* and their DNA sequences.

Table 2

*Molecular technique, DNA sequences, and the phylogenetic analysis in Azolla species*

Molecular techniques	DNA fragments and sequences	<i>Azolla</i> species	References
Intergenic spacer	TrnL- (CGA AAT CGG TAG ACG CTA CG) trnF - (ATT TGA ACT GGT GAC ACG AG) trnLD - (GGG GAT AGA GGG ACT TGA A) trnLE - (GGT TCA AGT CCC TCT ATA CC)	<i>Azolla filiculoides</i>	Madeira et al. (2016)
	trnL-trnF region (the trnL intron and trnL-trnF intergenic spacer [IGS]), the atpB-rbcL IGS of the chloroplast, internal transcribed spacer (ITS) region of nuclear ribosomal DNA		Reid et al. (2006)
Random amplified polymorphic DNA (RAPD)	rbcL, atpB, rps4, and rps4-trnS RAPD markers (OPA - OPF)	<i>Azolla pinnata</i> R. Br.	Chang et al. (2020) Pereira et al. (2011)

Table 2 (Continue)

Molecular techniques	DNA fragments and sequences	<i>Azolla</i> species	References
Sequence defined amplified region (SCAR) marker	F: GCCTAAGTCCAAGCTTACTCATCTTA R: ATTTAGGCTTAGGCCACAGATAGAAG F: CAATACCTTGTTTCAGTGTCGTAGG R: TGGCAATGTACCATGAAGTAGAATA F: AGATGGTTAGAAGTGACAGCATATCTTT RAT: TTCGC	<i>Azolla rubra</i> , <i>Azolla pinnata</i> , <i>Azolla filiculoides</i> , <i>Azolla microphylla</i>	Abraham et al. (2013)
Restriction fragment length polymorphism (RFLP)	CYA359F and 1051R	<i>Azolla rubra</i> , <i>Azolla microphylla</i> , <i>Azolla filiculoides</i> , <i>Azolla pinnata</i>	Sood et al. (2008)

The random amplified polymorphic DNA (RAPD) technique was performed using markers to identify *Azolla* species in the family of Azollaceae at the molecular level. Further, vegetative characteristics were used to identify *Azolla* species (Pereira et al., 2011). The findings revealed that the Shannon Index was higher (2.276) than vegetative characteristics (0.054), implying that molecular techniques are more reliable than morphological characteristics for species identification. Additionally, Dong et al. (2010) found greater genetic diversity in China's aquatic fern species *Ceratopteris*.

Additionally, the molecular approach of a specific sequence-defined amplified region (SCAR) was established using the nucleotide sequence of specific RAPD markers. According to Abraham et al. (2013), compared with RAPD, the SCAR approach has a slight advantage because RAPD markers detect many nonspecific fragments, whereas SCAR markers

only identify specific RAPD fragments. Furthermore, the SCAR approach has been applied by Oyange et al. (2020) to evaluate the difference at the molecular level for a few *Azolla* species in Kenya.

Inter Simple Sequence Repeat (ISSR) markers have not been used in *Azolla* species, even though these approaches are commonly used in other aquatic fern species. Dong et al. (2007) applied the ISSR marker in *Ceratopteris pteridoides*, an aquatic fern species whose level of genetic diversity could provide valuable baseline data for conservation strategy. According to Wang et al. (2016), DNA barcoding is an emerging approach to identifying closely related fern species using short and standard sequences. As a method for species identification, DNA barcoding entailed the sequencing of a standard DNA region. According to this review, no studies on the DNA barcoding method were found for *Azolla* species.

## BIOTECHNOLOGICAL APPLICATION OF *AZOLLA* FOR THE PRODUCTION OF ANIMAL FEED

As the human population is increasing worldwide, an alternative animal feed is one of the most important aspects to consider. According to Alemneh (2019), the role of biotechnology in the animal feed industry is crucial in two aspects, improving nutrient content in animal feed and production of animal feed. The recommended nutrient requirements for livestock are based on the evaluation by nutritionists (protein, amino acids, fatty acids, vitamins, and crude fibres) (Tona, 2018).

Several biotechnology-based methods were used to determine the nutritional value and phytochemical properties of *Azolla*, which livestock can consume. For example, the alkaline protein extraction method has

been applied in *Azolla* and other aquatic plants of *Lemna*, *Spirodela*, and *Wolffia* to increase the crude protein levels by reducing condensed tannins (CTs) through protein precipitation (Brouwer et al., 2019). Further, the integrated biorefinery is one of the most recently developed methods to increase the amount of lipid, protein, and phenolic contents extracted from *Azolla* (Dohaie et al., 2020). Ultrasound-assisted water, sodium hydroxide extraction, and hot trichloroacetic acid extraction have been sequentially applied for protein extraction within an integrated biorefinery method. Advanced technology can also extract various primary nutritional values as part of the methodology. The technology advancements used in *Azolla* to improve the key nutritional values for animal feed production are listed in (Table 3).

Table 3

Several advanced technologies involve improving the main nutritive value in *Azolla* species

Technologies advancement	<i>Azolla</i> species	Nutritive value	Main findings	References
Elemental analyzer	<i>Azolla filiculoides</i>	Crude protein	20.6% of DW of protein content	Brouwer et al. (2019)
Fleurence method	<i>Azolla filiculoides</i>		12.6% of DW of protein content	Dohaie et al. (2020)
Kjeldahl method	<i>Azolla filiculoides</i>		12% of DW of protein content	Tran et al. (2020)
	<i>Azolla pinnata</i>		17% of DW of protein content	



Table 3 (Continue)

Technologies advancement	<i>Azolla</i> species	Nutritive value	Main findings	References
Acid hydrolysis using ion-exchange liquid chromatography	<i>Azolla filiculoides</i> and <i>Azolla pinnata</i>	Amino acid	The biomass's total amino acids (AA) ranged from 208 to 244 g kg <sup>-1</sup> DW, and the AAs composed 82–88% of the total nitrogen.	Brouwer et al. (2018)
Ninhydrin colorimetric method	<i>Azolla pinnata</i>		39% higher in free amino acid levels as compared to control	Chen et al. (2017)
Gas chromatography-mass spectrometry GC-MS	<i>Azolla filiculoides</i> and <i>Azolla pinnata</i>	Fatty acids	Monounsaturated fatty acids (MUFA) make up just 10–12% of total FAMES in <i>Azolla</i> species and are dominated by palmitoleic (C16:1) and oleic (C18:1) acids	Miranda et al. (2018)
High performance thin-layer chromatography (HPTLC)	<i>Azolla microphylla</i> and <i>Azolla caroliniana</i>	Vitamin (β – carotene)	471.73 mg/g in <i>A. microphylla</i> and 354.57mg/g in <i>A. caroliniana</i>	Azhar et al. (2018)

Note. DW = Dry weight

## PROSPECTS OF BIOTECHNOLOGY IN *AZOLLA*

Further biotechnology application has shown *Azolla* has a unique feature of sustainable development through biotechnology, unlike any other higher plant. Its role in feeding the world's rapidly increasing population helps solve a significant issue for sustaining food security. A variety of biotechnological approaches can improve a plant's nutritional value and productivity. For example, the use of enzymes to boost the nutritional value and secondary metabolites can be introduced in *Azolla* plants. According to Le et al. (2016), methionine (MET) synthesis has been

manipulated in genetic engineering research to boost MET content in plant proteins.

Using a bioreactor to cultivate *Azolla* can reduce the *Azolla*'s growth period and save cost. A study by Sobhani et al. (2020) showed that applying a low-cost disposable bioreactor shows the mass production of the *Hypericum perforatum* L. within four weeks. Duckweed is an aquatic plant with a strong potential for being produced on a large scale in a bioreactor (Coughlan et al., 2022; Yang et al., 2021). Moreover, the hybridization procedure should be performed to improve the valuable traits in *Azolla*.

Based on *Azolla*'s increasing nutritional value yield, it can be declared as an alternative animal feedstock for livestock, aquaculture, and poultry to declare *Azolla* as an alternative animal feedstock for livestock, aquaculture, and poultry industries. Hemalatha et al. (2019) suggested that the structure of *Azolla*, which is a self-sustained closed-loop, tremendously benefits the environment. The use of alternative feedstock derived from aquatic plants and the adoption of biotechnology have been statistically demonstrated to aid in the sustainability of agricultural sectors. The effects of using *Azolla* as an animal feedstock for improving nutrient absorption efficiency in the gut metabolite system in the cattle and aquaculture industries should be further investigated. The complete genome sequences of *Azolla* species have provided several opportunities to investigate various phases and determine the genetic variation in *Azolla*'s species. DNA barcoding and cleaved amplified polymorphic sequence (CAPS) are the two molecular marker technologies that can be used in the future for *Azolla* species. Loss of genetic diversity in particular plant species is mostly due to environmental changes and demographic fluctuations in the short and long term (Khan et al., 2012). Therefore, this technology is critical for conserving the genomic DNA of uncommon and endangered plant species, particularly for aquatic water fern, *Azolla*, which is considered to have existed since the Carboniferous Period. The cost of handling the most significant hurdle to continuing integration of *Azolla* in

several biotechnology techniques; therefore, *Azolla* should be widely employed in underprivileged nations. According to Lencucha et al. (2020), in developing countries, the government's responsibility is to provide farmers with incentives and support programs that are often needed to encourage sustainable agriculture practices.

## CONCLUSION

Biotechnological approaches in *Azolla* have provided several opportunities for this unique aquatic fern to be further investigation of this unique aquatic fern for various environmental reasons. These approaches combine conservation programs, environmental sustainability, and food security systems under one tool. Several molecular techniques, such as intergenic spacer, RAPD, SCAR marker, and RFLP, have been explored, which can be further used in different *Azolla* species. However, more studies should investigate the current method in *Azolla* to better understand its role in the alternative for environmental sustainability, particularly as a phytoremediator, biopesticides, and biofertilizer to fulfil the increasing demand.

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