**ISOLATION AND CHARACTERISATION OF INDIGENOUS ZINC SOLUBILISING MICROBES AND THEIR PROBIOTIC POTENTIALITY IN AQUATIC ECOSYSTEMS OF MALAWI**

# **ABSTRACT**

In this study, zinc solubilizing microorganisms (ZnSM) were isolated from the rhizospheres of aquatic plants, water and benthic soils of Malawi aquatic ecosystem with the aim of having cheap and environmental friendly biofertiliser and probiotics in Malawi. Several studies have shown potentiality of indigenous ZnSM in biofertiliser development, but little is known about the presence of indigenous ZnSM and their probiotic potential in Malawi aquatic ecosystem.

Isolates were screened for solubilisation traits using basal medium with three amendments of insoluble zinc (zinc carbonate, oxide and phosphate) supplemented by the characterization of plant-growth-promoting (PGP) and probiotic traits. Molecular characterization using 16S rRNA gene and biochemical test were used in identification and characterisation. Isolates candidacy was based on solubilisation index (SI) of above 1.5 complimented by production by diverse plant growth promoting and probiotic traits.

Eight isolate namely *Serratia marcescens*, *Enterobacter wuhouensis*, *Serratia surfactantfaciens*, *Serratia nematodiphila*, *Alcaligenes faecalis*, *Bacillus altitudinis and Klebsiella pasteurii* were selected as potential candidates for development of zinc based biofertiliser. The study revealed that zinc solubilising microbes have great potential in probiotic development evidenced by production of amylase. The study has revealed and showed that *Bacillus altitudinis* strain had no site specificity and had highest potential in biofertiliser and probiotic development shown by solubilisation index of 4.7, 3.4 and 4 for zinc oxide, carbonate and phosphate respectively. Isolates solubilisation quantification was based on the type of inorganic zinc indicating that some of these microbes may be site specific based on the type of inorganic zinc available. The pH of the culture broth was found to be decreased in the range of 3.33 to 3.35 indicating that solubilisation is based on acid extrudes by the microbes.

The study has given an insight of potentiality of indigenous microbes from aquatic ecosystem in the development of biofertiliser and probiotics in Malawi.

***Keywords****: Biofertiliser; zinc solubilisation; zinc; zinc solubilising microbes; aquatic ecosystem; Plant Growth Promoting traits; Probiotics; Malawi.*

# **INTRODUCTION**

Zinc (Zn) is an imperative micronutrient required by the plant in the ranges of 5 to 100 mg kg-1 but it has crucial implication on plant and animal development, growth, and physiology (Nitu et al., 2020; Prigent-combaret, 2013; Sushil et al., 2012; Swift et al., 2016). Zn deficiency is a precursor of crop susceptibility to heat stress while excessive Zn through the use of zinc based fertilizers has negative impact to humans as it leads to impaired absorption of iron and copper (Sunitha et al., 2014). Zn is a co-factor in the metabolism of more than 300 enzymes and influences essential processes of plant life such as phytohormones production, nitrogen metabolism through protein and nitrogen uptake, carbon anhydrase and chlorophyll synthesis in the process photosynthesis, pollen formation and resistance to stress (Bapiri et al., 2012; Hussain et al., 2015; Rajendran et al., 2003; Saravanan et al., 2004).

Studies have shown that tropical soils are Zn deficient in available (soluble) form which has resulted in application ZnSO4, based inorganic fertiliser (Kumssa et al., 2022). Zn solubility is affected and regulated by soil moisture and pH. ZnSO4 fertiliser contains 33% Zn, out of this less than 8% is used by the crops while the remaining percentage is fixed to become insoluble (Imran et al., 2014; S et al., 2019; Sciences et al., 2015). Studies have proposed the utilization of the fixed Zn form like zinc oxide which contains 80% Zn yet no study has been proposed in Malawi (Kumssa et al., 2022).

Like other tropical countries, Malawi agriculture soils has been documented to contain high reserves of insoluble Zn and other nutrients that has accumulated due to persistent application of zinc based inorganic fertilizers and parent materials (Kumssa et al., 2022). Inorganic fertilizers including zinc based inorganic fertilizers has environmental implication as they are pollutants to aquatic life and rhizosphere which negatively affect plant growth regulatory microbes (PGRM) (Babana et al., 2016; Bhardwaj et al., 2014, 2014; El-Tantawy & Mohamed, 2009)**.** Inorganic chemicals impact on PGRM has an implication on bio-magnification, acidification of the soil and ecosystems. Evolutionally, zinc solubilising microorganisms (ZnSM) solubilise insoluble sources of Zn like (ZnO and ZnCO) (Gontia-mishra et al., 2017; Nandal & Solanki, 2021; Sukhwal et al., 2022)**.** PGRM, including ZnSM are have positive implication in plant growth and nutrient absorption, rood development and pattern and crop responsiveness to stress.

PGRM solubilisation process is through acidification by releasing organic acids and chelation (Sun et al., 2020). Acidification has an implication on pH and micronutrient availability hence making the process complex and dynamic while having positive implication in Zn availability (Hussain et al., 2015; Imran et al., 2014). Evolutionally rhizosphere microflora tend change the pH to a manageable extent through organic acids and protons extrusion (Hussain et al., 2015).

In the present study, ZnSM were isolated and characterized for its potential as biofertilisers and probiotics from the aquatic ecosystem of Malawi.

# **METHODOLOGY**

## **STUDY DESIGN**

The study used purposive sampling in a Completely Randomised Design (CRD).

## **STUDY SITE**

Rhizospheric soil from aquatic plants, water and benthic soils of aquatic ecosystem were sampled in the peripherals of protected areas of Malawi with no history of inoculation. These areas were chosen because of high ecological footprint (Nathanailides et al., 2021)

## **SAMPLE COLLECTIONS AND PREPARATIONS**

The samples were collected from the rhizosphere of different aquatic plants, water and benthic soils of aquatic ecosystem no history of inoculation. Rhizospheric benthic soil was preferred due higher CFU of PGPM than rhizoplane and endorhizosphere (Awasthi et al., 2011; Ghevariya & Desai, 2015). Aquatic plants rhizospheric soil were obtained using a method documented by Mwafulirwa, (2023) Li et al., (2017); López-ortega et al., (2013); Walpola & Yoon, (2013) with slight modifications by the following procedure. Root system was separated from the bulk soil by shaking while the remnant soil (rhizospheric soil) was removed by using a brush. Thereafter, the samples placed in polythene bags and placed in iced cooler boxes during transportation and stored in laboratory 4 0C.

## **ISOLATION OF ZNSM**

After homogenisation, 1 g of each soil and water samples were 10 fold serially diluted from neat homogenate to 10-5. Thereafter, 1 ml of 10-3 and 10-5 dilutions was inoculated on basal medium plates at 28 °C incubation temperature within 96 hours. Colonies having clear zones were streaked on new basal medium plates three times. The composition and active ingredient of basal medium was dependent on the three insoluble zinc sources i.e. zinc oxide (ZnO), zinc carbonate (ZnCO3) and zinc phosphate Zn3(PO4)2 as documented by Nandal & Solanki, (2021)

### **QUANTITATIVE ZINC SOLUBILISATION**

Broth each having 0.1 % zinc oxide, zinc carbonate and zinc phosphate was autoclaved in which 50 ml was poured in sterile100 ml Erlenmeyer flask. Each flask was inoculated with 2 mls of isolates in triplicate. The samples were drawn and centrifuged to remove all impurities. Thereafter, available zinc concentration was analysed and expressed in **µg/ml** using atomic absorption spectrophotometer (Goteti et al., 2013).

## **DETERMINATION OF PLANT GROWTH PROMOTING TRAITS**

The determination of Plant growth promoting traits (PGPT) was done for several traits like solubilisation of phosphorous, production of ammonia, solubilisation potassium, IAA, catalase, hydrogen cyanide, siderophores nitrogen-fixing ability etc. (Mwafulirwa et al., 2017)

### **ASSESSMENT OF AMMONIA PRODUCTION BY ISOLATES**

Ammonia production assessment was done by inoculating 1\*109 CFU of individual isolate into a 10 ml tube of peptone water on tryptic digest of casein broth incubated at 35 for 36-60 hrs. for bacteria and fungus respectively. Thereafter, addition of Nessler's reagent (0.5 ml ) to detect the presence of ammonia by development of yellow or brown colour (Ahmad and Khan, 2008; Mwafulirwa et al., 2017).

### **ASSESSMENT OF IAA PRODUCTION BY ISOLATES**

IAA assessment was done by inoculating 1\*109 CFU of individual isolate (in nutrient and SD broth amended with 100µg/ml tryptophan in rotating incubator at 35 °C for 48 hours at 120 rpm bacteria and fungus respectively. Thereafter the media was centrifuged and supernatant was collected. The supernatant was inoculated with two drops of o-phosphoric acid, and four mls of Salkowski reagent. Appositive was confirmed by development of pink colour (Ahmad et al., 2019; Ashry et al., 2022; Leontidou et al., 2020).

### **ASSESSMENT OF HYDROGEN CYANIDE PRODUCTION BY ISOLATES**

Hydrogen Cyanide production was confirmed in-vitro by inoculating isolates in nutrient broth and sabouraud dextrose broth supplemented with glycine for bacteria and fungus respectively as documented by Mwafulirwa, (2023).

### **ASSESSMENT OF NITROGEN FIXING ABILITY BY ISOLATES**

Nitrogen fixing traits was confirmed using Liu et al. (2016) with manipulation of the pH. Winogradsky's N-free medium was used as a selective media because it has no nitrogen in its ingredients. isolates ability togrow was an indication of its ability to fix nitrogen (Aquilanti et al., 2004; Doroshenko et al., 2007, 2007; Navarro-noya et al., 2012).

### **ASSESSMENT OF SOLUBILISATION POTENTIAL FOR PHOSPHOROUS AND POTASSIUM BY ISOLATES**

Isolates were assessed on their ability to solubilise phosphorous and potassium using Pikovskaya’s (PVK) Medium using a method documented and described by Karpagam & Nagalakshmi, (2014), and Aleksandrov medium (Rajawat et al., 2016; Mubarik et al., 2014; Saha et al., 2016; Vishwavidyalaya, 2012) for phosphorous and potassium respectively. A modified Pikovskaya medium was formulated using different concentrations of bromophenol blue.( Gupta et al.,1994)

### **ASSESSMENT CATALASE PRODUCTION BY ISOLATES**

The isolates potentiality to production of catalase was done by dropping 3% of hydrogen peroxide on the colony. gas bubbles formation was considered as isolates ability to production of catalase.

## **DETERMINATION OF ISOLATES POTENTIALITY IN BIOREMEDIATION**

### **ASSESSMENT OF LACCASE ENZYME PRODUCTION BY ISOLATES**

Isolates ability to be involved in bioremediation was assessed using production of laccase enzyme. This was assessed by inoculating isolates Sabouraud Dextrose Agar which had 1% 2, 2’-azino-bis3-ethylbenzothiazoline-6-sulphonic acid. Colour change to purple or dark green was used as a positive indicator (Mwafulirwa et al., 2017; Mwafulirwa, 2023).

## **DETERMINATION OF ISOLATES POTENTIALITY AS PROBIOTIC STRAINS**

### **SCREENING FOR AMYLASE PRODUCTION**

Isolates ability to produce amylase were observed by halo zone (hydrolysis zone) after pouring Gram’s iodine on starch agar plates inoculated with isolates colonies using the method documented by Bhatt, Rajiv and Vyas, (2014).

### **QUALITATIVE AMYLASE ASSAY**

Each and every isolate producing hydrolysis zone was inoculated on new SA plates incubated at 32 °C. Thereafter Lugol’s iodine solution was flooded on the SA plates to detect extracellular amylase shown by presence of halos around the colonies. The halos diameter was measured using a caliper and those showing largest halos were candidates for quantitative analysis (Andriani & Pratiwy, 2020; T. J. Hossain et al., 2020; Mawardi et al., 2023; Taheri et al., 2009).

Amylolytic index = DB-DK/ DK

Where; AI is Amylolytic index (mm); DB is Clear zone diameter (mm), and DK is Colony diameter (mm) (Firmani, 2022).

### **QUANTITATIVE AMYLASE ANALYSIS**

The selected isolates from qualitative assay were inoculated separately in 30 mls of TSB media for 12 hrs. at 32 °C. Thereafter 1 ml aliquot from TBS was inoculated on starch medium (broth) while maintaining the temperature. Supernatant solution which harbors enzyme was isolated via centrifugation and be stored in 20ml eppendorf at -80 °C. Analysis was done using DNS as described above with slight modifications where by 50 μl of supernatant was mixed with 25 μl starch solution (1% w/v) and 25 μl of Tris-HCl buffer. The diluted mixture was taken for OD test at 550 nm absorbance. This is based on reduced sugar in which maltose was used as a standard. Enzymatic activity was quantified as a unit (U) and that 1 U is the amount of enzyme equivalent to release one mg of reducing sugar per minute.

### **SCREENING FOR CELLULASE PRODUCTION**

Cellulase production by isolates was assessed using CMC (for endoglucanase) and modified MM (for exoglucanase) medium using a methods as described by Bhagat & Kokitkar, (2021); Jayasuyra et al., (2020); Upadhyaya et al., (2012) with slight modifications because gut contents were not used. Isolates were repeatedly streaked on CMC and MM agar plates for verification of endoglucanase and exoglucanase. 1% Congo red was poured on colonies grown on CMC agar followed by de-staining with 1M NaCl. The efficiency of hydrolysis was formulated by subtracting the Colony diameter from Clear zone diameter then dividing with Colony diameter then multiplying by100. Growth on MM medium indicated appositive indicator.

### **SCREENING FOR PROTEASE PRODUCTION**

Protease production by isolates was assessed using gelatine agarusing methods documented by Sony and Potty, (2016) Mary et al., (2017) and Balaji et al., (2012) incubated at 35ºC under aerobic condition for 72-192 hours with slight modifications because gut contents were not used. The gelatine agar medium contained (g/l) glucose 1.0, K2HPO4 2.0, Peptone 5.0, gelatin 15.0, and agar 15 and. The presence of halos around the colony was used as appositive indicator. Halos diameters will be measured using gelatine clear zone method as described by Abomughaid, (2020) which adds mercuric chloride solution to the brim of the plates.

### **SCREENING FOR LIPASE PRODUCTION**

Lipase production by isolates was assessed using lipolytic agar medium using a methods as described by Jini et al., (2011) Bakteria and Pekasam, (2018) with slight modifications because gut contents were not used. Lipolyitic agar contained 50ml/l tributyrin 50g/l NaCl, 10g/l peptone, 0.1g/l CaCl2 .2H2O, and 20g/l g agar. The presence of halophilic colonies with an opaque zone around the colony was used as appositive indicator.

### **SCREENING FOR LACTIC ACID PRODUCTION**

Lactic acid by isolates was assessed using Man Rogosa Sharp (MRS) medium (incubated at 37˚C +/- 2) as documented by using Chowdhury and Islam, (2016), Dowarah et al., (2018) and Maji et al., (2016) methods. colonies showing halo-zone indicated appositive result.

### **SCREENING FOR BILE SALTS HYDROLASES (BSH) PRODUCTION**

The qualitative BSH assessment was done as described by Shehata et al., (2016) where isolates were inoculated on specific media that has been supplemented by CaCl2 (0.037%) and taurodeoxycholic acid sodium salt (0.5% {w/v}). Precipitate formation around the colonies after 96 hrs. anaerobic incubation at 37 °C indicated appositive result.

### **ASSESSMENT OF ISOLATES TO DIVERSE PH AND BILE SALT (BS)**

Isolates tolerance to diverse pH was be assessed using pH of 2.0-4.5 and 7.0-8.0 for acidic and basic conditions in growth medium using HCl and NaOH as documented by Dowarah et al., (2018) and Maji et al., (2016). To assess tolerance bile salt levels, microbes were inoculated in modified Man Rogosa Sharp broth at diverse concentration ranging from 0.075-1%. The growth was measured using optical density method at OD 580 nm for pH and 620 nm BS.

### **ASSESSMENT OF ISOLATES FOR PHYTASE PRODUCTION**

The phytase production of isolates was assessed by inoculating on specific phytase enzymatic medium as documented by Dowarah et al., (2018). The phytase enzymatic medium contained 0.5% NH4NO3, 1.5% glucose, 0.05% MgSO47H2O, 0.5% calcium phytate, 0.02% MnSO47H2O, 1.5% agar, 0.001% FeSO47H2O and 0.05% KCl with pH adjusted to 7.0.

### **ASSESSMENT OF ISOLATES ADHESION ON SURFACE HYDROPHOBIC ASSAY**

The microbial adhesiveness to epithelium surface was done using hydrocarbons modeling, xylene, toluene and hexadecane according to Chaia and Gonza, (1999) and Sánchez-Ortiz et al., (2015). Five mls of the isolate with CFU of 107/ml was harvested by centrifuge from which 2 mls was deliberately made into contact with 0.6 mls of each of the hydrocarbons for 15 minutes. Thereafter they were mixed thoroughly by vortex for 2 minutes. The OD of the vortexed mixture was obtained at 600nm. The percent hydrophobicity (H) was calculated based on the formula as documented by Chaia and Gonza, (1999). H(%)=[(AA0)/A]x100.

### **HAEMOLYTIC ACTIVITY TEST**

Isolates were assessed for hemolysis using sheep blood (SP) agar. A loopful of isolate culture was inoculated on SP agar plate and incubated for 24 h at 37 ºC and observed for haemolytic zones around the colonies.

## **PHYLOGENETIC ANALYSIS**

Isolates were identified using genetic characterisation by sequencing the 16S rRNA genes using both forward and reverse strands. BioEdit software was used to have the consensus sequences which was used in the BLAST algorithm query against the public nucleotide sequence in National Center for Biotechnology Information (NCBI) database to find the closely related strains (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>.). Results on analysis will be based on the maximum identity score in which high similar sequences will be downloaded and aligned with isolate sequences in BioEdit. All isolates sequences were deposited in the GenBank and accession numbers were given.

The evolutionary history was inferred using the Neighbor-Joining method to define the datasets because it establishes relationships between sequences according to their genetic distance (a phenetic criterion), without considering an evolutionary model (Kuan et al., 2016). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analysis and phylogenetic tree construction was done using MEGA11 software.

## **DATA ANALYSIS AND STATISTICS**

Data was analyzed and subjected to descriptive statistics, t-test and analysis of variance (ANOVA) using the GENSTAT.

# **RESULT AND DISCUSSION**

Three hundred and twenty zinc solubilising microbes were isolated from 420 samples from 8 districts in Malawi.

**Table 1: Sites where samples were collected**

|  |  |  |
| --- | --- | --- |
| **District** | **River/Lake**  | **Protected and Conserved areas**  |
| Chikwawa  | Shire river  | Elephant marsh |
| Nsanje  | Shire river  | Shrine and Ndindi marsh |
| Zomba  | Shire river  | Lake Chirwa  |
| Machinga  | Shire river | Liwonde national park  |
| Mangochi  | Shire river and Lake Malawi  | Lake Malawi national park  |
| Nkhotakota  | Lake Malawi, Bua river and Chia lagoon  | Nkhotakota game reserve |
| Nkhatabay  | Lake Malawi and Lweya river | Shrine |
| Karonga  | Chimbiriri river and Lake Malawi  | Chitende shrine and Nyika national park |



Figure 1.Map of study sites

**Table 2: Sites where isolated strains were collected**

|  |  |
| --- | --- |
|  | Location  |
| Isolates lab No | Chikwawa  | Nsanje | Zomba | Machinga | Mangochi | Nkhotakota | Nkhatabay | Karonga |
| CK14 | + | + | - | + | - | - | - | + |
| SA19 | - | - | - | + | + | + | + | - |
| MZ22 | - | - | + | - | - | + | + | - |
| KA6 |  + | + | + | + | + | + | + | + |
| KA9 |  + | + | + | + | + | + | + | + |
| CP12 | + | + | + | + | + | + | + | + |
| CK22 |  + | + | + | - |  + | + | + | + |
| CP54 | - | - | - | + | + | + | + | - |

These isolates were isolated based on zinc solubilisation on three insoluble zinc compounds in basal medium. Selected best 8 isolates used in the study were able to solubilise zinc in three insoluble zinc compounds by showing halo zone and had solubilisation index (SI) of above 2 which is above the standard of 1.5. The sizes of halo zones and amount of soluble zinc in broth and soil produced were dependent on inorganic zinc and isolate.

All the isolates showed potentiality to be used as probiotics (amylase producing microbes) based on starch hydrolysis on a starch agar plate shown by halozone formation around the microbial colony upon the addition of Lugols Iodine solution. Diameter of colony and solubilisation zone formed by isolates on basal medium supplemented with insoluble zinc compounds were recorded as shown in table 1.

**Table 3. List and capabilities of zinc solubilising microbe isolated in diverse lentic and lotic water bodies of Malawi**

|  |  |  |  |
| --- | --- | --- | --- |
| Isolate  | Zinc oxide  | Zinc carbonate  | Zinc phosphate |
|  | **Colony diameter**  | **Halozone** | **SI** | **Colony diameter**  | **Halozone** | **SI** | **Colony diameter**  | **Halozone** | **SI** |
| CK14 | 4 | 15 | 3.8 | 5 | 12 | 2.4 | 3 | 9 | 3 |
| SA19 | 2 | 8 | 4 | 5 | 15 | 3 | 3 | 8 | 2.7 |
| MZ22 | 8 | 15 | 1.9 | 3 | 14 | 4. 7 | 4 | 12 | 3 |
| KA6 | 6 | 12 | 2 | 4 | 14 | 3.5 | 7 | 16 | 2.2 |
| KA9 | 4 | 10 | 2.5 | 2 | 6 | 3 | 5 | 9 | 1.8 |
| CP12 | 3 | 14 | 4.7 | 5 | 17 | 3.4 | 2 | 8 | 4 |
| CK22 | 7 | 15 | 2.1 | 3 | 12 | 4 | 5 | 8 | 1.6 |
| CP54 | 9 | 34 | 3. 8 | 4 | 9 | 2.3 | 4 | 12 | 3 |

**Table 4. Characterization of isolates for plant growth regulatory and bioremediation traits.**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Serial Number** | **Ammonia production** | **IAA production** | **Catalase production** | **Nitrogen fixation** | **Cyanide production** | **ACC deaminase** | **P solubilisation**  | **K solubilisation**  |
| **CK14** | + | + | + | + | - | + | + | - |
| **SA19** | - | + | + | + | - | + | - | - |
| **MZ22** | - | + | + | + | - | + | - | + |
| **KA6** | + | + | + | + | - | + | - | + |
| **KA9** | + | + | + | + | - | + | + | - |
| **CP12** | + | + | + | + | - | + | + | - |
| **CK22** | + | + | + | + | - | + | + | - |
| **CP54** | - | + | + | + | - | + | + | - |

**Table 5. Quantification of isolates for potentiality for solubilisation of different types of insoluble inorganic zinc.**

|  |  |  |
| --- | --- | --- |
| Isolate  | Soluble zinc content (µg/ml broth) | Available Zn in the soil  |
|  | **Zn phosphate**  | **Zn carbonate**  |
|  | **Zn levels**  | **Ph. values**  | **Zn levels**  | **Ph. values**  |  |
| CK14 | 4.5 | 3.9 | 2.91 | 5.2 | 2.5 |
| SA19 | 3.5 | 4.0 | 2.57 | 5.1 | 2.4 |
| MZ22 | 4.1 | 4.2 | 2.56 | 5.8 | 1.8 |
| KA6 | 3.9 | 4.5 | 2.69 | 5.6 | 2 |
| KA9 | 4.1 | 4.0 | 2.98 | 5.2 | 2.3 |
| CP12 | 4.3 | 4.1 | 3.73 | 5.1 | 2.5 |
| CK22 | 3.89 | 3.9 | 3.4 | 5.4 | 1.89 |
| CP54 | 3.79 | 3.9 | 3.52 | 5.1 | 2.1 |
| Uninoculated | 2.34  | 7.0 | 2 | 7.0 | 1.2 |

**Table 6. isolates Amylolytic potentiality**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Isolate  | Location  | River/lake  | Types of fishes GIT found | DK | DB | Amylolytic Index (SI) |
| CK14 | Nsanje  | S  | All  | 2 | 8.2 | **3.1** |
| SA19 | Nkhotakota | M & Ch | All  | 4 | 10 | **1.5** |
| MZ22 | Karonga  | M, W & C | All  | 4 | 15 | **2.8** |
| KA6 | Mangochi | M & S | All  | 1 | 4 | **3** |
| KA9 | Mangochi | M & S | All  | 3 | 10 | **2.3** |
| CP12 | Liwonde  | S | All  | 3 | 14 | **3. 7** |
| CK22 | Nkhotakota | M & Ch | All  | 4 | 14 | **2.5** |
| CP54 | Nkhatabay | M & L | All  | 4 | 14.4 | **2.6** |
| KA 34 | Karonga  | M, W & C | All  | 2 | 8.2 | **3.1** |

All the eight isolates were cultured in two basal broth medium containing zinc phosphate and zinc carbonate. All the broth with diverse isolates showed a shift in pH after 8 days of incubation. The average decline ranging from 7.0 to 3.9 - 4.5 and 7.0 to 5.1-5.8 with zinc phosphate and zinc carbonate, respectively. These results are in tandem with other studies that reported that microbes involved in solubilisation of Zn produce organic acid production (Nandal & Solanki, 2021; Sukhwal et al., 2022).

The present findings revealed that inoculation of microbes increased soluble zinc content in zinc phosphate and zinc carbonate broth as compared with uninoculated control. In case of zinc phosphate, the soluble zinc concentration increased with inoculation in the ranges of 3.5-4.5 µg/ml broth) as compared with 2.34 µg/ml in uninoculated control while zinc carbonate the values ranged from 2.56-3.52 µg/ml broth) as compared with 2.0 µg/ml in uninoculated control. Isolates solubilisation of zinc was dependent on type of insoluble zinc source. The data has revealed that inoculation of isolates increased soluble zinc in broth supplemented with zinc phosphate to a greater extent as compared with zinc carbonate which is in consonance with other studies (Sushil et al., 2012).

**Table 7. Characterization of isolates for other probiotic traits**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Serial Number** | **Cellulase production** | **Protease production** | **Lipase production** | **lactic acid production** | **phytase production** | **BSH production** | **Haemolytic activity** | **surface hydrophobic assay** | **Chitinase production** | **Pectinase production** | **Amylase production**  |
| CK14 | ++ | - | - | + | + | + | - | + | **-** | **+** | + |
| SA19 | + | + | - | + | - | + | - | + | **-** | **+** | + |
| MZ22 | - | + | - | - | - | + | - | + | **+** | **+** | + |
| KA6 | + | - | - | - | - | + | - | + | **+** | **+** | + |
| KA9 | - | - | + | + | - | + | - | + | **+** | **+** | + |
| CP12 | + | - | + | + | - | + | - | + | **+** | **+** | + |
| CK22 | + | - | - | + | + | + | - | + | **+** | **+** | + |
| CP54 | + | - | - | + | + | + | - | + | **+** | **+** | + |

**Table 8: Isolates and their BLAST related species and GenBank deposit accession numbers**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Lab Serial No** | **Related Species(Homologs)** | **Nucleotide Identity %** | **Given Accession NO** | **Strain Name** |
| CK14 | *Serratia marcescens* | 99.97 | PQ676097 | LOGO24 |
| SA19 | *Enterobacter wuhouensis* | 99.53 | PQ676098 | LOGO26 |
| MZ22 | *Serratia surfactantfaciens* | 99.60 | PQ676099 | LOGO27 |
| KA6 | *Serratia nematodiphila* | 98.52 | PQ676100 | LOGO28  |
| KA9 | *Alcaligenes faecalis* | 98.77 | PQ676101 | LOGO30 |
| CP12 | *Bacillus altitudinis* | 99.33 | PQ676102 | LOGO31 |
| CK22 | *Serratia marcescens* | 99.80 | PQ676103 | LOGO32  |
| CP54 | *Klebsiella pasteurii* | 99.80 | PQ676132 | LOGO69     |

**Figure 2: Phylogenetic tree based on 16S rRNA gene sequence showing the position of indigenous diverse fungal probiotic strains in Malawi compared with those available in GenBank of NCBI.**

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model conducted in MEGA11 with a total of 1410 positions in the final dataset. The tree with the highest log likelihood (-7924.31) was taken. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 24 nucleotide sequences.

Therefore, the present study indicated that indigenous microbes in Malawi have greater potential to solubilize zinc phosphate oxide and carbonate. It was observed that all isolates changed the ph. of broth, indicating that solubilisation is based on acid production. The mechanisms of acquisition of the zinc by isolates from insoluble zinc compounds might be a consequence of production of organic acids of microbial origin leading to solubilisation of zinc and thereby influencing the bioavailability of zinc (Gontia-mishra et al., 2017; Hussain et al., 2015; Kamran et al., 2017). Such solubilisation of zinc compound mediated through production of organic acids by microbes and subsequent release of zinc in the external environment and bioaccumulation of zinc inside cells of Bacillus species has been reported by earlier workers (Ahemad & Kibret, 2014; Kamran et al., 2017; Krithika & Balachandar, 2016; Sukhwal et al., 2022).

Zinc is one of the key micro nutrient at small concentration but lethal at elevated concentration. Continuous application of fertilizers principally in zinc also becomes eccentric because of the transformation into unavailable fractions soon after application and accumulation in the soil. Thus identification of an elite strain capable of transforming unavailable forms of Zn into available forms will be an alternative tool to alleviate zinc deficiency in plants (Jerlin et al., 2017; Kamran et al., 2017; Suseelendra Desai, 2012). The study has revealed the potentiality of indigenous microbes involved in zinc solubilisation to be used as in biofertiliser and probiotic development. The plant growth promoting zinc solubilizing microbes are proved to be good alternative of chemicals for increasing the plant growth and yield and help reduction in the use of hazardous agro-chemicals and used for inoculants (Suseelendra Desai, 2012). Further studies are at progress to use the microbial strains as inoculants for zinc availability and growth stimulation to overcome the problem of stunted crop growth of in Malawi.

One of the criterion for selecting a potential probiotic is its ability to have diverse traits which is based on evolutional adaptation. This doesn’t exclude transient microbes which most of the time produce a positive influence on the host. All isolates are autochthonous microbiota, shown by no previous history of probiotic usage and development in Malawi (C. Li et al., 2019; Rohman et al., 2021). Characterisation of indigenous isolates for α-amylase production is a worthy and practical parsing achievement in the feed production and field of industrial biotechnology. The isolation and characterization of suitable strains is critical and essential for the development of raw material for specific strains (Borges et al., 2020; Jha et al., 2020; Wu et al., 2021).

Besides the biological (strains) physical and chemical composition of medium or feed e.g. carbon sources, nitrogen sources, salt, pH and temperature affect the starch hydrolysis enzyme-like. Slight changes in biological, physical and chemical have an adverse effects on the omics of microorganisms as well as on the productivity of α-amylase and have an implication on the development of adjuncts (El-Fattah et al., 2013; Mwafulirwa & Kanyada, 2023).

In most studies, GITMs are isolated using conventional culture-based methods which are said to be time-consuming, not representing the whole diversity in the GIT even using diverse media and are not accurate (Das et al., 2014; Dey et al., 2016). However, the use of conventional culture-based methods by employing a specific substrate containing selective media is justifiable, as the major aim of the present study is to detect different extracellular enzyme-producing GITM. In the present study, the isolates are to be used in the development of probiotics while using genetic analysis it may capture GITM which cannot be cultured or multiplied to be used as a probiotic inoculum (Hossain et al., 2022; Sumathi & Priya, 2011).

In most studies, microbes are isolated using conventional culture-based methods which are said to be time-consuming, non-accurate and not representing whole diversity in the GIT even while using diverse media (Das et al., 2014; Dey et al., 2016). However, the use of a conventional culture-based methods by employing a specific substrate containing selective media is justifiable, as the major aim of the present study is to detect and isolate different extracellular enzyme-producing microbes which are cultured for development of biofertilisers and probiotics. In the present study, the isolated are to be used in development of probiotics while using genetic analysis may capture microbes which cannot be cultured or multiplied to be used as a biofertiliser inoculum (Hossain et al., 2022; Sumathi & Priya, 2011).

The extracellular enzyme-producing isolates of the present study were all identified genetically from their 16S rDNA gene analysis which was further supported by their morphological and biochemical properties. The isolates appeared taxonomically diverse at the genus level each belonging to a separate phylotype. All isolates therefore seem to be autochthonous to location. Moreover, these site-specific GITM strains other possess extracellular beneficial properties, and are, therefore, considered as probiotic candidates for all fishes.

Phylogenetic analysis based on the NJ method revealed that diversified divergent genera and species as illustrated and evidenced by formation of unique site specific clades. Genus *Serratia* dominated in terms of diversity at species and strain. These isolates were *Serratia marcescens*, *Enterobacter wuhouensis*, *Serratia surfactantfaciens*, *Serratia nematodiphila*, *Alcaligenes faecalis*, *Bacillus altitudinis and Klebsiella pasteurii.* All these isolates mainly those in the genus *Serratia* have been already found to be zinc solubilizing bacteria as well as ingredients in probiotic development.

# **CONCLUSION**

The study explored and found indigenous zinc solubilizing microbes that have potential for development of zinc biofertilisers. The study also found that the isolated zinc solubilizing microbes are all amylase producing microbes which puts a preposition of further investigation on association of zinc solubilizing and amylase producing genes. The study also revealed new strain of zinc solubilisation and the study puts proposition that its presence in fields is a result of abundance of insoluble zinc due to natural selection pressure. Study gaps on field performance investigations are required for these aquatic ecosystem microbes on various crops as biofertilisers as well as livestock as probiotics

**DISCLAIMER**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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