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Formulation of cost-effective agro residues containing potassium solubilizing bacterial bio-inoculants using response surface methodology

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ABSTRACT

Potassium solubilizing bacteria were isolated from soil samples on potassium aluminum silicate amended agar medium. The bacterial strain B15 recorded the highest potassium solubilization in agar (solubilization index = 2.82) as well as in broth (soluble K = 82.012 μg ml^{-1}) medium. It was identified through 16S rDNA nucleotide sequencing as, *Enterobacter hormaechei* 1110BP. The strain besides potassium solubilization was also found to have nitrogen fixation and phosphate solubilization abilities. The production of bio-inoculant with this strain was done using locally available agro residues such as wheat bran, sorghum (jowar) bran, and molasses. The usage of agro residues as a nutrient medium in the production of potash bio-inoculant is a novel approach. It is cost-effective as well as environment friendly and it could help to minimize the pollution problems. In the study, response surface methodology was applied for designing the bio-inoculant medium to attain high cell numbers ($CFUml^{-1}$) and prolonged survival of *Enterobacter hormaechei* 1110BP. A comparison between the expenses on the synthetic nutrient medium and agro-residue nutrient medium of bio-inoculants reveals that synthetic medium incurs nearly 116.13 times excess than agro-residue medium. Thus, the production of multifarious potash solubilizing *Enterobacter hormaechei* 1110BP bio-inoculants using locally accessible agro residues is a highly economical and eco-friendly approach for sustainable agriculture.

1. Introduction

Agriculture is the key sector in the economics of most of the developing countries and it has the greatest challenge to fulfill the exigency of food, as population explosion is stipulating the production of additional food (Mahanty et al., 2017). Thus there is an urgency of escalating crop production without affecting the biosphere. Out of seventeen elements essential for plant growth, potassium is the major nutrient involved in several physiological and metabolic processes in plants (Zhao et al., 2001). It is involved in protein synthesis, photosynthesis, generation of energy (ATP), activation of many growth-related enzymes, etc. (Zhang and Kong, 2014). Its deficiency in plants results in retarded growth, development of weak and unhealthy roots, and poor resistance to pests, and certain diseases (Bahadur et al., 2014). Potassium in the soil is generally classified into four categories; unavailable potassium, fixed potassium, readily available potassium, and soil solution potassium (Parmar and Sindhu, 2013). Unavailable potassium (90–98 %) is found in the form of feldspars, clay minerals, and micas which are part of the soil. Plants are unable to use potassium in this form. Fixed potassium is the potassium that is trapped inside clay materials (1–9 %). It is slowly available to plants over the growing season. Readily available potassium (1–2 %) is present on the surface of clay particles and organic matter in soil and it gets easily released when plants absorb it. An only a small fraction of potassium (1–2 %) is dissolved in the soil solution and plants can readily utilize it. Further, many natural procedures occurring in the environment such as leaching, overflow of water, erosion as well as manmade situations like the introduction of new crop varieties lead to the removal of potassium from the soil at a faster rate (Prajapati et al., 2013). Hence higher crop yield needs an external supply of potassium and mostly it is provided in the form of chemical potash fertilizers (Muriate of Potash) (Dhillon et al., 2019). The chemical fertilizers act as 'fast foods' for plants causing them to grow speedily and vigorously, however, at the same time these chemicals when applied in excess quantity create harmful impacts on living beings and the environment (Shen et al., 2016). Indiscriminate usage of chemical fertilizers is becoming a curse for the ecosystem as it is engendering several troublesome issues like the release of greenhouse gases, global warming, acid rain, abnormal changes in soil pH and salt concentration, etc.

Abbreviations: DOI, Day of inoculation; SI, Solubilization index; Bp, Bio-product.

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(Adesemoye and Kloepper, 2009). Further potash chemical fertilizers are highly expensive and thus increase the cost of crop cultivation (Meena et al., 2014). Thus it is necessary to find out an alternative economical source of potassium for plant growth.

A large number of different types of rhizospheric bacteria have been reported as potassium solubilizers like *Bacillus edaphicus* (Sheng, 2005), *Acidithiobacillus ferrooxidans,* (Sheng and He, 2006), *Bacillus mucilaginosus* (Meena et al., 2014), *Bacillus megaterium* (Zarjani et al., 2013), *Paenibacillus mucilaginosus* (Liu et al., 2012), *Pseudomonas putida* (Bagyalakshmi et al., 2012), *Enterobacter hormaechei* (Prajapati and Modi, 2012; Roslan et al., 2020), *Enterobacter cloacae*, *Enterobacter aerogenes, Klebsiella variicola* (Zhang and Kong, 2014), etc. These microorganisms solubilize unavailable and fixed forms of potassium and make it available to plants. The application of such microorganisms in the field as bio-inoculant to amplify crop production is a profitable and eco-friendly substitute for chemical fertilizers.

The nutrient medium used for the cultivation of bio-inoculant organisms is one of the factors that affect the economics of the bio-inoculant production process. Agricultural residues like bran, husk, and stalks of vegetables, peels of fruits are easily available in plentiful quantities and are highly nutritious (Sadh et al., 2018). A large fraction of most of such wastes is composed of carbohydrates as well as these wastes are ample in nitrogen, phosphorous, different vitamins, and minerals (Sadh et al., 2018). Thus the usage of agricultural residues as a cultivation medium for bio-inoculant production could help to reduce the cost of the production process and also assist to minimize the pollution problems that can arise after its disposal. Thus the present study was aimed to isolate and screen effective potassium solubilizing bacteria and to design the bio-inoculant production medium with the agro-residues available in the local area.

2. Materials and methods

2.1. Soil samples

Various rhizosphere areas of a variety of crop plants such as sugarcane, wheat, sorghum, groundnut, etc. from different regions of Maharashtra (India) were screened for collection of samples. Soil samples from a depth of 10–15 cm were collected in zip-lock polyethylene bags and bags were labeled properly and brought to the laboratory.

2.2. Enrichment and isolation of potassium solubilizing microorganisms

One gm of each soil sample was inoculated in a separate 100 ml sterile broth medium containing 1 % glucose, 0.05 % yeast extract, and 0.5 % potassium aluminum silicate and incubated at 28±2°C at 120 rpm in an orbital shaker incubator (Remi, India) for 1 week. The enriched sample from each flask was inoculated on sterile Aleksandrov's agar medium (1% glucose, 0.05 % magnesium sulfate heptahydrate, 0.0005 % iron (III) chloride, 0.01 % calcium carbonate, 0.2 % calcium phosphate, 0.5 % potassium aluminum silicate, and 2 % agar, pH 7.0–7.5) (Hu et al., 2006) and a modified Aleksandrov's agar medium (Rajawat et al., 2016). This medium additionally consists of a pH indicator bromothymol blue (BTB, 0.025 %). All the plates were incubated at 28±2°C for 2–4 days (Prajapati and Modi, 2012). The well-isolated colonies showing clear zones of potassium solubilization on Aleksandrov's agar and yellow zones on modified Aleksandrov's agar were selected and based on colony morphology; colonies were assigned as B, Y, and F for bacteria, yeast, and fungus respectively. The purity of isolates was checked by staining characteristics. The impure isolates were purified by serial transfers on Aleksandrov's agar plates.

2.3. Screening of isolates for potassium solubilization ability

The most potent strain was identified by estimating the potassium solubilization ability of all isolates through qualitative assay by determining solubilization index and quantitative assay by flame spectrophotometry (Hu et al., 2006; Zhang and Kong, 2014).

2.3.1. Qualitative estimation of potassium solubilization

Each pure isolate (3×10^8 CFUml^{-1}) in 10 µl quantities was spot inoculated on Aleksandrov's agar. The diameter of the colony and the total diameter of the solubilization zone was measured with the help of scale after incubation at 28±2°C for 5 days and the solubilization index (SI) was calculated for each isolate as expressed in Equation (1) (Parmar and Sindhu, 2013). The experiment was conducted in triplicate for each isolate and the average values of total zone diameter and colony diameter were used to calculate the solubilization index (Equation (1)) of each isolate.

ꢀꢁ = ꢂꢃꢄꢅ ꢆꢇꢈꢉꢊꢋꢌ ꢍꢎꢏ ꢐꢑꢒꢓ (ꢆꢔꢄꢅꢕꢓ + ꢖꢗꢄꢅꢕ ꢐꢑꢒꢓ) / ꢘꢙꢄꢅꢕꢓ ꢆꢇꢈꢉꢊꢋꢌEquation 1

2.3.2. Quantitative estimation of potassium solubilization

Twenty-five ml sterile Aleksandrov's broth containing potassium aluminum silicate as an insoluble source of potassium was inoculated with 2 % (v/v in sterile saline) fresh suspension (3×10^8 CFUml^{-1}) of each isolate separately. All inoculated flasks were incubated at 28±2°C for 5 days at 120 rpm. Subsequently, all flasks were centrifuged at 10,000 rpm for 10 min. One milliliter supernatant was withdrawn and soluble potassium in the supernatant was estimated using flame spectrophotometry (Zhang and Kong, 2014). Potassium chloride solution was used to prepare the standard curve (Hu et al., 2006). The experiment was repeated three times for each isolate. The results are reported as mean ± standard deviation for each isolate.

2.4. Identification of the most effective strain

The most effective isolate was examined for its morphological and biochemical characteristics. The 16S rDNA of the isolate was sequenced and submitted to NCBI. The sequence was compared with available standard sequences using the BLAST program. The phylogenetic tree was constructed with MEGA X software. The Maximum Composite Likelihood method was used to compute evolutionary distances amongst 18 (nucleotide sequences) strains. A pairwise deletion option was selected to eradicate ambiguous positions for each sequence pair. The final dataset included a total of 1110 positions.

2.5. Evaluation of plant growth promotion properties of the potent isolate *Enterobacter hormaechei* 1110BP

2.5.1. Phosphate solubilization

Pikovaskaya's agar medium containing tricalcium phosphate as a source of insoluble phosphate was spot inoculated with freshly grown potent isolate *Enterobacter hormaechei* 1110BP and the plate was incubated at 28±2°C. Phosphate solubilization was detected by the formation of a halo zone around the growth (Babu et al., 2017). The efficiency of phosphate solubilization was represented qualitatively in the form of a solubilization index (Equation (1)) (Pande et al., 2017). The phosphomolybdic acid blue (Mo blue) colorimetric method (Zhang et al., 2019) was used for the quantitative assessment of the phosphate solubilization ability of the strain.

2.5.2. Nitrogen fixation

The freshly grown potent isolate was spot inoculated on nitrogen-free 0.0025 % bromothymol blue (w/v) agar medium and the plate was incubated at 28±2°C for 7 days. The color change of the medium from green to blue around growth indicates nitrogen fixation by the isolate (Nakbanpote et al., 2014). Further quantitative estimation of nitrogen fixation was done by Kjeldahl's method. Sterile nitrogen-free

Ashby's broth was inoculated with a fresh inoculum of *Enterobacter hormaechei* 1110BP and incubated at 28±2°C on a rotary shaker at 120 rpm for 7 days. The uninoculated broth was used as a 'control'. The inoculated broth after incubation was centrifuged at 5000 rpm for10 min to exclude the biomass and the quantity of Total Kjeldahl Nitrogen (TKN) in both the flasks was estimated (Kumar et al., 2014).

2.6. Seed germination bioassay of Enterobacter hormaechei 1110BP

The effect of the most potent isolate, *Enterobacter hormaechei* 1110BP on the root and shoot elongation of seed was studied by plate assay (Kumar et al., 2012). The experiment was conducted on chickpea seeds (*Cicer arietinum*).

Enterobacter hormaechei 1110BP was inoculated in the sterile nutrient broth and it was incubated at 28±2°C for 24 h. Chickpea seeds were surface sterilized and subsequently rinsed with sterile distilled water 5 times. Seeds were then placed in nutrient broth tubes previously inoculated with *Enterobacter hormaechei* 1110BP. After 30 min seeds were removed from tubes and kept on moist filter paper in sterile Petri plates. Subsequently, plates were wrapped in black paper and incubated. The surface-sterilized seeds placed in sterile distilled water were used as 'control' seeds. Vigor index (Ghorbanpour and Hatami, 2013) of both the 'inoculated' and un-inoculated 'control' seeds was calculated as described in Equation (2) after 5 days of incubation. The experiment was conducted in triplicate.

Equation 2

2.7. Formulation of bio-inoculant production medium

2.7.1. Agro-residues as a nutrient medium

Different agro residues like vegetable wastes, potato peels, orange peels, wheat bran, sorghum bran, and molasses were examined as nutrient media for the cultivation of *Enterobacter hormaechei* 1110BP. Vegetable waste materials such as stalk, and leaves of coriander, fenugreek, cabbage, beetroot, and shell of pods were collected from the kitchen and ground to obtain a paste. It was mixed with distilled water to prepare a 10 % solution (w/v). Subsequently, the vegetable slurry was filtered through a muslin cloth, and pH was checked by a pH meter and adjusted to neutrality. Potato peels of raw potatoes and orange peels were collected from small-scale food industries. Peels were washed thoroughly under tap water to remove extraneous materials and ground. A water-based slurry (10 % w/v) of the paste was prepared. It was then filtered to obtain a clear extract. The pH of both was checked and adjusted to neutrality. Wheat and sorghum (jowar) bran were mixed independently with distilled water to prepare a 10 % (w/v) solution. Further bran solutions were boiled and then filtered to obtain clear bran hydrolysate. The pH was examined and adjusted to neutrality. Liquid molasses was collected from the sugarcane industry and diluted with distilled water to attain a 2 % final sugar concentration. It was then added with 0.5 % sodium chloride. Subsequently, all agro residue media (vegetable wastes, potato peels, orange peels, wheat bran hydrolysate, sorghum bran hydrolysate, and molasses) were sterilized by autoclaving and then used as cultivation media for *Enterobacter hormaechei* 1110BP.

2.7.2. Determination of viable cell count of Enterobacter hormaechei 1110BP in agro residue nutrient media

The sterilized media were inoculated with 2 % (v/v in sterile saline) suspension (3×10^8 CFUml^{-1}) of potent strain *Enterobacter hormaechei* 1110BP and incubated at 28±2°C at 120 rpm. The viable cells in the broth media were counted by standard plate counting procedure (Shahravy et al., 2012). Different tenfold serial dilution of inoculated broth media was prepared using sterile saline as diluents. Each dilution in 0.1 ml quantity was inoculated on a separate sterile nutrient agar plate. Colonies of *Enterobacter hormaechei* 1110BP from each plate were counted after 24 h incubation. The colony-forming unit (CFU) was then calculated for each plate with standard formula and the final average value of CFUml^{-1} was converted into log values. The viable cell density in the form of log CFU (Shahravy et al., 2012) values was determined on the 0th day as well as at periodic intervals like 7th day, 15th day, and 30th day of incubation to understand the survival rate of the isolate. The actual viable cell number on the nth day of the incubation period was denoted as "Bio-product" (Bp) and calculated as depicted in Equation 3

Equation 3

where; X_n=log$_{10}$CFUml^{-1} at nth day, X_0=log$_{10}$CFUml^{-1} at 0th day.

2.7.3. Investigation of optimum concentration of best suitable agro residues

The three agro residues media wheat bran, sorghum bran, and molasses showing the highest cell densities (Bp value) and longer survival for *Enterobacter hormaechei* 1110BP were selected for further study. The various concentrations of wheat bran and sorghum bran (5, 10, 20, and 30% each) were prepared in distilled water to obtain bran hydrolysates. Molasses was diluted with distilled water to acquire a final sugar concentration of 3, 5, 7, and 9 %. All media after sterilization were inoculated with 2 % (v/v in sterile saline) suspension (3×10^8 CFUml^{-1}) of *Enterobacter hormaechei* 1110BP. The colony-forming unit was determined on the 0th day and 7th day of incubation and further bio-product values were determined (Equation (3)). The concentrations of media showing maximum bio-product values were selected for statistical analysis to discover the medium composition which can give a high cell number of *Enterobacter hormaechei* 1110BP.

2.7.4. Manufacturing of bio-inoculant production medium having high CFU by response surface methodology

Wheat bran (10 %) hydrolysate, sorghum bran (20 %) hydrolysate, and molasses (5 % sugar concentration) were used as media components for statistical analysis. The effects of the interaction of these three components in different combinations on the viable counts of *Enterobacter hormaechei* 1110BP were studied statistically by response surface methodology to optimize the medium composition for maximum yield of biomass (Han et al., 2014). A Box-Behnken design developed by the Design-Expert software, version 12 (8.0.7.1) (Stat Ease Inc. Minneapolis, USA, trial version) was used for this purpose. The different concentrations of wheat bran (5, 10, and 15 %), sorghum bran (15, 20, 25 %), and molasses (3, 5, 7 % sugar) were used in the Box-Behnken design to generate different fusions of media composition. Thus the Box-Behnken design created 17 combinations (runs) of media. All 17 runs were inoculated with 2 % (v/v in sterile saline) suspension (3×10^8 CFUml^{-1}) of *Enterobacter hormaechei* 1110BP. The viable cell count of each run was determined on the 0th day and 7th day of inoculation. The responses of the experimental runs were recorded in the form of bio-product (Bp) (log$_{10}$CFUml^{-1}) after 7 days of incubation. The statistical reliability of the model was evaluated through analysis of variance (ANOVA) (Han et al., 2014).

Finally, the authentication of the experimental design was verified by repeating the experimental set with a medium composition (experimental run) having the highest bio-product value identified by the Box Behnken design. (Yun et al., 2018). The experimental bio-product value obtained was compared with the Box Behnken response value.

2.8. Comparative study of the expenses on novel agro residues medium with synthetic medium

The expenses on agro residues bio-inoculants medium designed by response surface methodology (24.793 % sorghum bran, 14.882 % wheat bran, and 6.66 % molasses) were compared with the synthetic medium (Glucose 10 g L^{-1}, Yeast extract 6 g L^{-1}, Mannitol 10 g L^{-1}, and Ammonium sulfate 0.5 g L^{-1}) used for the production of potash

solubilizing bi-inoculants (Ghadge et al., 2020). The nutrient medium cost of the 1-L synthetic medium was calculated by taking into consideration the cost of each component according to the catalog of Hi-media and it was compared with the cost of a 1-L agro-residue medium.

3. Results

3.1. Isolation and screening of most potent potassium solubilizer

By enrichment technique, different bacterial, yeast, and fungal potassium solubilizing strains were successfully isolated from the soil samples. The solubilization indexes of all isolates calculated by measuring colony diameter and clear zone diameter after 5 days of incubation are represented graphically in Fig. 1. The results of quantitative estimation of potassium solubilization are represented graphically in Fig. 2. Qualitative and quantitative estimation both revealed that the bacteria are efficient potassium solubilizers in comparison to yeast and fungal isolates. The bacterial isolate labeled as B15 reported a solubilization index of 2.82 on agar medium and recorded nearly 82.012 ± 1.51 μg ml^{-1} of soluble potassium in the broth medium which is the highest quantity of all isolates and thus this bacterial strain was identified as the most potent potassium solubilizing strain.

3.2. Identification of the most superior potassium solubilizing strain

The potent strain B15 was found to be Gram-negative, short rods, singly arranged, and motile. It can utilize several sugars and showed positive tests for acetoin production, citrate utilization, and nitrate reduction. Urease activity, arginine hydrolysis tests were positive after 48 h incubation while isolate showed negative tests for H_2S production, indole production, methyl red test, phenylalanine deaminase activity, and lysine decarboxylase activity. PCR amplification of 16S rDNA sequencing was employed to identify bacterial strain B15 at the genomic level. The 16S rDNA sequence of isolate B15 was compared on the NCBI BLASTN tool based on percentage similarity, E-value, and query coverage. It was deposited to the NCBI GenBank database and the unique accession number was retrieved. The study disclosed that B15 is *Enterobacter hormaechei* 1110BP with NCBI GenBank accession number **MH057393**. The evolutionary relationship of the isolate was reconstructed using the neighbor-joining method to create the phylogenetic tree (Fig. 3).

Enterobacter hormaechei (KSB-8) isolated by Prajapati and Modi (2012) has recorded a solubilization index of 1.57 after 7 days of incubation at 37°C on Aleksandrov's agar medium. Similarly, Roslan et al. (2020) isolated three *Enterobacter* strains named *Enterobacter hormaechei* 15a1, *Enterobacter hormaechei* 40a, and *Enterobacter cloacae* 38 were reported to release 72.90 μg ml^{-1}, 65.95 μg ml^{-1}, and 71.15 μg ml^{-1} soluble potassium respectively from potassium aluminum silicate after 5 days of incubation. Thus owing to these reports, *Enterobacter hormaechei* 1110BP (solubilization index 2.82 and soluble K 82.012 ± 1.51 μg ml^{-1}) can be concluded as a superior potassium solubilizing strain.

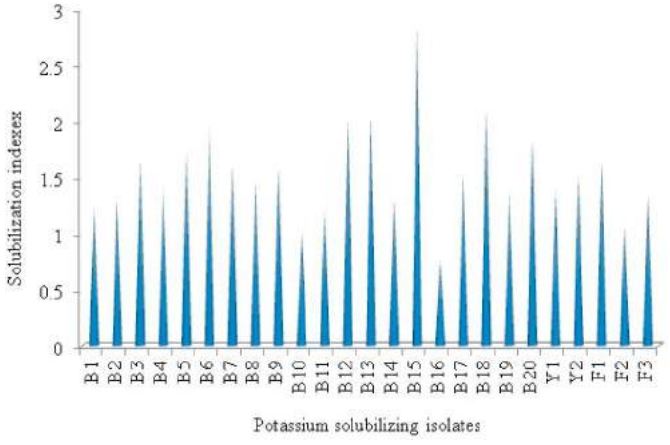

Fig. 1. Potassium solubilization indexes of the microbial strains isolated on Aleksandrov's agar; B1 to B20: Bacterial strains; Y1 & Y2: Yeast strains; F1, F2, & F3: Fungal strains.

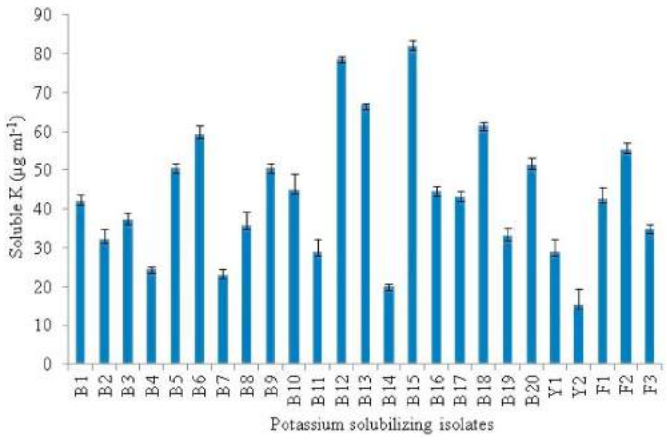

Fig. 2. Quantification of potassium solubilization of isolates. Each bar represents a mean of three replications. Error bars designate the standard deviation.

3.3. Evaluation of plant growth promotion properties of Enterobacter hormaechei 1110BP

Enterobacter hormaechei 1110BP showed high phosphate solubilization activity and it was recorded in the form of solubilization index (8.6) on Pikovaskaya's agar medium. The quantitative estimation of phosphate solubilization by *Enterobacter hormaechei* 1110BP in the Pikovaskaya broth was analyzed by phosphomolybdic acid blue (Mo blue) colorimetric method. The isolate inoculated Pikovaskaya broth recorded nearly 317 ± 2.65 μg ml^{-1} soluble phosphorous in 5 days of incubation.

The nitrogen fixation ability of the isolate was studied by streaking the isolate on a nitrogen-free bromothymol blue agar medium. The isolate exhibited a blue color zone around growth. Bromothymol blue is a pH indicator and it is green in neutral condition and blue in alkaline condition. Thus the appearance of blue color in the medium is an indication of ammonia production which is a product of nitrogen fixation activity. Simultaneously, Kjeldahl's method used for the estimation of the nitrogen fixation process revealed that *Enterobacter hormaechei* 1110BP fixes nearly 0.352 μg N ml^{-1} of broth.

3.4. Seed germination bioassay of Enterobacter hormaechei 1110BP

The study of the effects of *Enterobacter hormaechei* 1110BP on chickpea seed development (Fig. 4A) in comparison with the uninoculated control seed (Fig. 4B) revealed its highly encouraging role as a bio-inoculant. The results of different parameters of inoculated and uninoculated seeds are depicted in Table 1. In the experiment, seeds inoculated with *Enterobacter hormaechei* 1110BP have shown a 3.75 times increase in the vigor index as compared to uninoculated seeds. More vigor index in inoculated seeds designates that the isolate could help for active, healthy, well-balanced growth of plants. The previous studies have shown significant enhancement of germination percentage and seedling vigor in seeds and seedlings of plants inoculated with the potassium solubilizing microorganisms (Meena et al., 2014). The study conducted with Okra (*Abelmoschus esculentus*) has shown improvement in seedling vigor index in seeds inoculated with *E. hormaechei* and *E. cloacae* (Roslan et al., 2020).

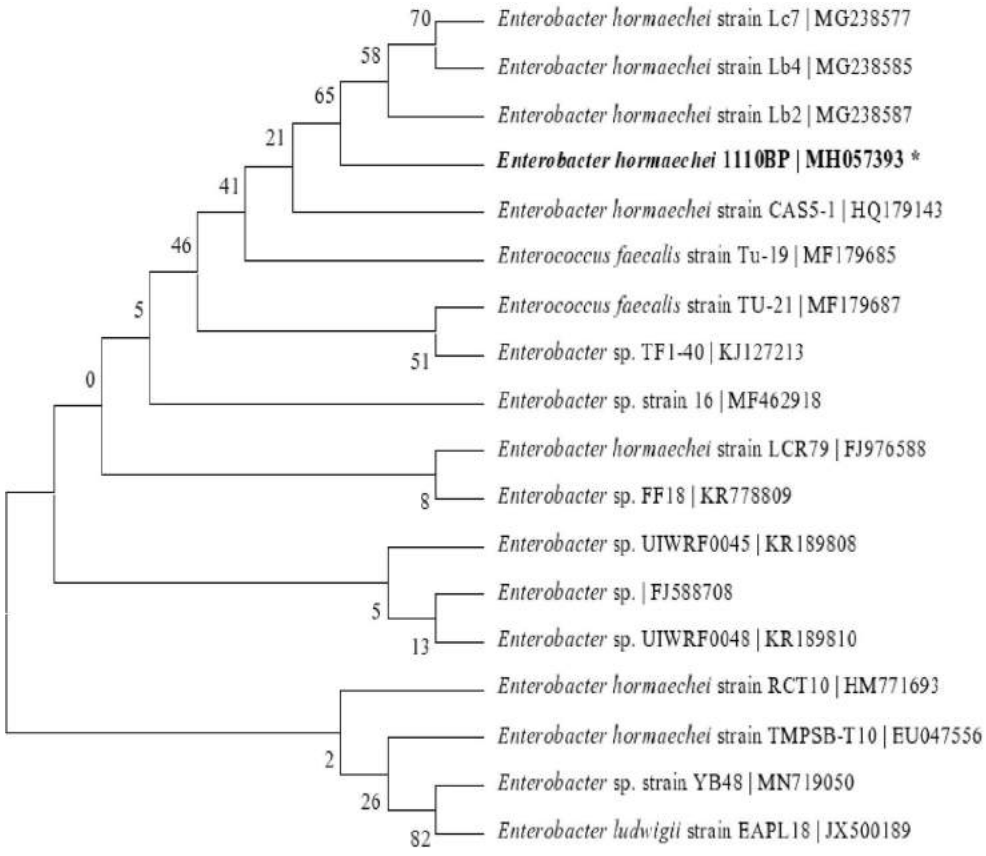

Fig. 3. Phylogenetic tree of *Enterobacter hormaechei* 1110BP constructed with 16S rDNA nucleotide sequence using the Neighbor-Joining method. The numbers represented at the branching point of the phylogenetic tree indicate the percentage of replicate trees in which the related taxa were grouped in the bootstrap consensus tree test of 1000 replicates.

Fig. 4. Chickpea seed germination bioassay A) Effect of *Enterobacter hormaechei* 1110BP on chickpea seed germination and elongation; B) Chickpea seed development in absence of *Enterobacter hormaechei* 1110BP

3.5. Screening of agro-residues for the cultivation of Enterobacter hormaechei 1110BP and investigation of optimum concentration of best suitable agro residues

Agro residues are treated as waste materials and their disposal causes environmental pollution. However, the good nutritional value of agro residues could make use of it as a nutrient medium. Hence in this research study, different locally available agro-residues such as fruit wastes, bran, and molasses were tried as a cultivation medium for the bio-inoculants microorganism. The quality of agro residue as a nutrient medium was decided based on the viability and survival of the isolate. Thus the results indicated that sorghum bran is the best raw material for

Table 1
Chickpea seed germination bioassay of *Enterobacter hormaechei* 1110BP.

Parameters	Inoculated seeds	Un inoculated seeds
Radicle length (cm)	5.80 ± 0.10	1.53 ± 0.06
Plumule length (cm)	1.30 ± 0.25	0.37 ± 0.12
Vigor index	713.33 ± 35.12	190 ± 17.32
No. of lateral roots on the main root	13 ± 0.10	3 ± 0.15

the cultivation of *Enterobacter hormaechei* 1110BP as it has recorded the highest cell density (Bp=17.20 on the 30th day). Besides it, wheat bran and molasses were also proved good for the growth of the isolate. In contrast, log CFU values (Bp) of vegetable wastes, potato peel waste, and orange peel waste suggest that these are not very suitable for growth. These wastes showed an initial increase in the cell density for nearly 15 days but later it observed decreasing, thus it indicated poor survival of the isolate for a longer period. The continuous and tremendous increase in the cell density (Bp values) for sorghum bran hydrolysate, wheat bran hydrolysate, and molasses (Table 2) suggests the longer survival rate for the *Enterobacter hormaechei* 1110BP in these media. Hence, these three agro residues were used to design the cultivation medium of *Enterobacter hormaechei* 1110BP in the bio-inoculants production process.

The optimum concentration of wheat bran, sorghum bran, and molasses was spotted out by inoculating various concentrations of these raw materials with *Enterobacter hormaechei* 1110BP. As depicted in Table 3, wheat bran, sorghum bran, and molasses have shown the highest log CFU values (Bp) as 9.01, 9.64, and 9.11 at 10 %, 20 %, and 5 % (sugar) concentration respectively. Thus, agro residues in these concentrations are most suitable for attaining a high count of *Enterobacter hormaechei* 1110BP.

3.6. Manufacturing of bio-inoculant production medium having high CFU by response surface methodology and further confirmation of results

The most suitable agro residues wheat bran, sorghum bran, and molasses were combined in different concentrations and further standardization was done through response surface methodology. Box Behnken design devised 17 combinations of these three components which are presented in the form of actual and coded values in Table S1. The different concentrations of these components were mixed in 1:1:1 proportion and viable cell count in the form of bio-product value was determined for each combination. Box Behnken's design interpreted the interactions between the three factors and evaluated the high cell density growth medium.

The interactive effects of medium components concluded by standard analysis of variance (ANOVA) are depicted in Table S2. ANOVA of the second-order regression model demonstrated that the model is significant with a low P-value of 0.02. Lack of fit (>0.05) is not significant indicating a good fit of the model. As predicted by the model, the most significant term is molasses having a p-value of 0.0019. The R^2 (determination coefficient) of the regression equation obtained from the analysis of variance was 0.8672 (a value > 0.75 indicates the applicability of the model), which indicates that the model explains 86.72 % variability in the response. "Adeq Precision" measures the signal-to-noise ratio. A ratio of more than 4 is desirable. In this model, the ratio of 8.989 indicates an adequate signal, and the model can be used to navigate the design space. Based on the quadratic regression analysis, the Box Behnken design demonstrated the following second-order polynomial equation.

Table 2
Viable cell count of *Enterobacter hormaechei* 1110BP in different agro residues.

Agro residues media	Bp ($log_{10}CFUml^{-1}$)		
	7th DOI	15th DOI	30th DOI
Vegetable wastes	7.82	9.88	8.75
Potato peels	7.89	8.46	8.15
Orange peels	6.74	7.51	6.53
Wheat bran	8.66	9.94	11.38
Sorghum bran	9.21	9.45	17.20
Molasses	8.93	10.64	11.75

DOI: Day of inoculation; Bp: Bio-product.

Table 3
Viable cell count ($log_{10}CFUml^{-1}$) of *Enterobacter hormaechei* 1110BP at various concentrations of agro residues.

Medium	Concentration (%)	Bp ($log_{10}CFUml^{-1}$)
Wheat bran hydrolysate (w/v)	5	8.90
	10	9.01
	20	8.60
	30	8.40
Sorghum bran hydrolysate (w/v)	5	8.90
	10	9.25
	20	9.64
	30	9.00
Molasses sugar (v/v)	3	8.67
	5	9.11
	7	8.95
	9	7.66

$$Y = 9.39 + 0.645A + 0.087B + 0.984C + 0.025AB + 0.025AC - 0.065BC + 0.079A^2 + 0.628B^2 + 0.045C^2 \quad \text{Equation 4}$$

Where, Y=Predicted response; A=Sorghum bran; B=Wheat bran; C=Molasses.

Comparisons of experimental value and predicted value of the regression model in equation (4) are shown in Table S1. Data shows that the agreement was satisfactory.

The fitted polynomial equation was further expressed in the form of contour plots and 3D plots where interaction between two factors keeping one factor constant to the optimum level is pictured. Fig. S1A and S1B represent the interaction between sorghum bran and wheat bran. The circular nature of the contour plot indicated no significant interaction between the two variables. Fig. S1C and S1D described the interaction between sorghum bran and molasses in the form of an elliptical contour plot. It indicates that the reaction between two factors is significant and dependent on each other. Fig. S1E and S1F showing the interaction between molasses and wheat bran summarized that log CFU value is directly proportional to the concentration of molasses but in the case of wheat bran as concentration increases initially log CFU increases but later it becomes dependent on the concentration of molasses.

Thus, the optimum medium composition found for getting maximum log CFU was 24.793 % sorghum bran, 14.882 % wheat bran, and 6.66 % (sugar) molasses. The predicted maximum log CFU value for this combination was 9.721 on the 7th day of incubation. To validate the predicted results, the experiment was repeated in triplicate using the above optimal medium composition. The log CFU (Bp) value of 9.758 ± 0.78 obtained during the experiment corresponds near to the value predicted by the model (9.721). The bio-product value (Bp) was further calculated on the 30th day of incubation and it was found to be 12.67 ±1.23. The survival of the culture in this novel medium was also monitored in terms of CFU at periodic intervals after 30 days and it was found to increase till six months of manufacturing and further CFU remained constant till eight months.

3.7. Comparative study of the expenses of novel agro residues medium with synthetic medium

At an industrial level, the usage of synthetic chemicals during production increases the economics of the whole process which finally affects the price of the product. The manufacturing cost of the bio-inoculants production is completely affected by the expenses on the nutrient medium used for the cultivation of bio-inoculants organisms. The standard nutrient media used in the commercial production of

potash biofertilizer consist of costly chemicals like dextrose, mannitol as a source of carbon and yeast extract, peptone as a source of nitrogen, and growth factors. The usage of such high-value chemicals increases the final price of bio-inoculants and it makes products unaffordable for farmers especially in countries like India. In the case of carrier-based bio-inoculants, peat is the best carrier material, however, it is not universally available and thus its usage is limited (Kaljeet et al., 2011). In this context, the exploitation of locally available agricultural wastes as a nutrient medium can be profitable. The agro wastes bio-inoculants medium devised in this study is consisting of wheat bran, sorghum bran, and molasses. All these wastes are good sources of carbon. Wheat bran and molasses are good sources of amino acids, different vitamins, and minerals. Moreover, all these wastes are locally available free of cost or at a very minor price.

To understand the cost-effectiveness of agro residues in the production of bio-inoculants, a comparative study between the synthetic medium used in the production of potash bio-inoculants and agro residue medium designed by response surface methodology was done. The study indicates that expenses on the components of a 1-L synthetic medium are nearly 116.13 times more than 1-L agro residues medium components (24.793 % sorghum bran, 14.882 % wheat bran, and 6.66 % (sugar) molasses) (Table 6). Thus in consideration with industrial production, usage of agro residues could save very huge expenses on the preparation of nutrient medium as well as help to combat the pollution problems.

4. Discussion

The literature survey indicates the beneficial effects of inoculation of seeds or seedlings with potassium solubilizing microorganisms on the growth of plants. The study conducted on a variety of crop plants like cotton and rape (Sheng, 2005), wheat (Sheng and He, 2006), sudangrass (Basak and Biswas, 2009), etc. evidenced the significant enhancement in the seedling vigor, germination percentage, potassium uptake by plants and yield of the crop under both pot and field conditions. Prajapati and Modi (2016) showed the positive effect of potassium solubilizing *Enterobacter hormaechei* (KSB-8) on the growth and yield of cucumber (*Cucumis sativus*). A strain of potassium solubilizing *Enterobacter hormaechei* also showed improvement in the vegetative growth of okra seedlings (Prajapati et al., 2013; Roslan et al., 2020). Similarly, Roslan et al. (2020) have documented nitrogen fixation and phosphate solubilization abilities of *Enterobacter hormaechei* and *E*nterobacter *cloacae*. *E. hormaechei* isolated by Ruangsanka (2014) has also demonstrated phosphate solubilization ability. *E. hormaechei* 1110BP isolated in the study is a strong potassium solubilizing isolate and hence can fulfill the potassium requirement of plants. Moreover, the strain also can furnish the plants with nitrogen and phosphorous. *E. hormaechei* despite having powerful plant growth promotion abilities is the least studied organism (Roslan et al., 2020). In this entire context, *Enterobacter hormaechei* 1110BP could have an optimistic approach to be exploited as a bio-inoculant for intensive farming.

Table 6
Production costs of agro residues bio-inoculants medium designed by response surface methodology and the synthetic medium used in the industrial-scale production of potash bio-inoculants.

Medium	Component	Quantity per lit medium	Cost (Rs.)
Agro-residue medium designed by RSM	Sorghum bran (24.793 %)	240.793 gm	–
	Wheat bran (14.882 %)	140.882 gm	–
	Molasses (6.66 % sugar) (Rs.7500/tonne)	130.032 ml	0.974
			Total cost = 0.974
The synthetic medium	Dextrose[a]	10.0 gm	25.60
	Mannitol[a]	10.0 gm	37.15
	Ammonium sulfate[a]	0.5 gm	02.22
	Yeast extract[a]	6.0 gm	48.14
			Total cost = 113.11

[a] Cost of the components according to Hi media.

The literature review also points out that, none of the researchers have concentrated on the optimization of the growth medium of potassium solubilizing microorganisms to achieve maximum yield of viable cells with a longer survival rate. Further, no literature is available concerning the usage of agro wastes for the production of potash solubilizing inoculants. A very huge amount of agro-based residues come out from different industries. Simultaneously a very large quantity of vegetable wastes, fruit wastes, bran is also dumped through hotels, restaurants as well as from home kitchens into municipal wastes every day. The disposal of these wastes in the environment without proper treatment causes environmental pollution together with negative impacts on human and animal health. Reports have proven that agro-based raw materials are rich sources of different nutrients and hence can be used as growth media for microbes (Sadh et al., 2018). There are huge references concerning the application of wheat bran as a fermentation medium (Katileviciute et al., 2019), however, usage of sorghum bran as a nutrient medium is least documented in works of literature. Maharashtra is the largest producer of sorghum (jowar) in India. Just as wheat bran, sorghum bran too is a rich source of proteins, carbohydrates, vitamins, and minerals. Hence it is a self-sufficient medium for the growth of microorganisms. Cane molasses is a by-product of the sugar cane industry. Maharashtra is the second most grower of sugar cane in India. It is a good source of carbon, nitrogen, minerals such as Ca, Mg, Fe, Zn, Mn, and vitamins like inositol, pantothenic acid, thiamine, biotin, pyridoxine, nicotinic acid, etc. (de Oliveira Lino et al., 2018). Thus it can be a good substitute for peptone and yeast extract in the medium. So the utilization of locally accessible agro residues is a costless or cheap replacement for synthetic chemicals making the production process profitable together with the benefit of a safe environment.

5. Conclusion

At present agronomists are becoming aware of the utilization of biofertilizers especially nitrogen fixers and phosphate solubilizers for sustainable agriculture. But very less attention is given towards the usage of potassium solubilizing microorganisms as bio-inoculants. Along with phosphorous and nitrogen, potassium too acts as a macronutrient for plants and is equally important for the growth and development of the plant and thus, in turn, enhancing the crop yield. *Enterobacter hormaechei* 1110BP is not only an efficient potassium solubilizer but also has multiple plant growth promotion characteristics like nitrogen fixation and phosphate solubilization. The seed germination bioassay study of *Enterobacter hormaechei* 1110BP has also shown a highly significant positive effect on the germination and seed development of chickpea seeds. This study indicated and confirmed the ability of *Enterobacter hormaechei* 1110BP as a powerful bio-inoculant for plant development.

A very little study has been done to date on the commercial production of bio-inoculant using cheaper raw materials as nutrient sources, alternative to costly synthetic chemical nutrients. The use of agro-wastes is an eco-friendly approach for sustainable agriculture; simultaneously it is economical for manufacturers and henceforth users, especially in developing countries where farmers can't afford huge expenses on chemical fertilizers. With this respect, the production of *Enterobacter hormaechei* 1110BP as a bio-inoculant using agro residues is a highly promising approach for viable agriculture to positively enhance crop yield.

CRediT authorship contribution statement

Savita D. Mali: Methodology, Software, Writing – review, and editing. **Yasmin C. Attar:** Conceptchualization, Visualization, Review.

Declaration of competing interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2021.102113.

Appendix

[illegible]

[illegible]

[illegible]
Where; X_n = $\log_{10}$CFUml^{-1} at nth day, X_0 = $\log_{10}$CFUml^{-1} at 0th day

[illegible]
Where, Y=Predicted response; A=Sorghum bran; B=Wheat bran; C=Molasses.

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