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Optimization of cost effective substrate and media for alkaline phosphatase releasing rhizospheric bacteria isolated from Dehradun, Uttarakhand

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ABSTRACT

The State of Uttarakhand has about 64.79% forest cover, 14.02% under crop cultivation and remaining 21.19% is populated. With limited land under cultivation major focus is on increasing yield and sustaining it for long time. Bacteria residing in the soils, including phosphatase producing bacteria, have potential to improve crop yield and sustaining it as they make phosphates available to plants. We have focused our efforts on isolating bacteria which are high phosphatase producers and standardized optimum media and other conditions for their growth. We isolated fourteen isolates that showed high alkaline phosphatase activity. Isolate ALKP 12 demonstrated highest alkaline phosphatase activity of 600 unit/ml in media 2 (basal media). This isolate was used for further studies. Under optimized conditions of pH 9 and 37°C, alkaline phosphatase activity of this isolate could be induced to very high level using media 1 (fermentation media), ranging from 1706 unit/ml and 1900 unit/ml. Another unique feature of this isolate is that it shows surprisingly high and unusual phosphatase activity of 1002 unit/ml even at pH 4. As this isolate can utilize cost effective molasses and it can even work in both acidic as well as basic soil, it holds potential to improve growth of the plant, increase yield and sustaining it.

Key words: Phosphatase, soil fertility, crop yield, fermentation medium, molasses, Uttarakhand

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INTRODUCTION

The state of Uttarakhand became the 27th state of republic of India on 9th November, 2000 and is located between 28^{0}43'- 31^{0}27'N latitude and 77^{0}34'-84^{0}02' E longitude [1]. The state is divided into two distinct geographical regions – Garhwal and Kumon division. There are 13 districts in the state, ten of them having hilly terrain while three districts are in terai region of Himalaya. The total geographical area of the state is 53,483 sq. km [1], [2]. Forest covers 64.79% area of Uttarakhand. Among the total forest cover, 33% is either rocky/snow covered/glaciated. About 14.02% of the total land is under crop cultivation and remaining 21.19% is populated. Among the cultivated land, more than 55% of agriculture/cultivated land depend on rain fall while only 45% is irrigated [2]. In hilly terrain, the irrigated land is on both side of either a river or small streams while some of the terai districts has canals for irrigation. Beside this the landholdings are small and scattered especially in hilly regions. The average land holding is around 0.68 ha in the hill districts and 1.77 in plain districts [1]. Like most other hill economics, the people of Uttarakhand practice integrated system of farming, forestry and horticulture [1], [2]. Most of the commonly grown crops in hill districts are wheat, rice, barley and sorghum and the chief crops grown in plain districts are wheat and rice.

People in Uttarakhand are primarily engaged in subsistence agriculture. Production and yield of food grains (cereal) production in Uttarakhand is 22.03 Qt/Hectare. The low agricultural yield reflects the small size and scattered land holdings, difficult terrains, dependency on rain as climatic condition for some crops with the state's limitations in land and water resources, alternative sustainable methods for increasing yield are much needed. For sustainable development in agriculture additional investment and scientific interventions are crucial but the cultivated land is also under threat from the construction companies and builders. So with limited land, the only possible scenario that remains is either to increase the yield in a sustainable manner so that the fertility of land is maintained or to improve the quality of specific seeds/crops that are indigenous to Uttarakhand so that the farmers can get a better price for the crop. Previously during green revolution, to increase the yield of crops, excess of chemical fertilizers and pesticides have been used indiscriminately in the pasts and this practice is still being followed [3], [4]. But excessive use of fertilizers over a longer period of time in the same soil and in the same location not only lowers the quality of soil architecture but also leads to overall decrease in the crop productivity. When phosphates are applied in high percentage, sometimes up to 90% fertilizer applied, gets precipitated by metal complexes in the soil [5], [6] and can lead to phosphate accumulation [5], [7]. These then remain as insoluble patches.

Microbes form an integral part of the soil. Various types of microbes participate in various cycles of organic and inorganic matter in the soil. One among the important components of soil fertility besides nitrogen is phosphate [8]. It plays an indispensable role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and many other processes in living plant. It helps plants to survive winter rigors and also contributes to disease resistance in plants [9]. Availability of phosphate in the soils of some regions of Uttarakhand are as follows: Chamoli Garhwal is 6.66 ppm and Pauri Garhwal is 7.20-14.30 kg/ha , where as in Uttarkashi Garhwal is 6 kg/ha. If we compare these values with the average phosphate content in soils of other states, then the average phosphate content in the soil of Uttar Pradesh is about 41 ppm, Madhya Pradesh-15 ppm, Karnataka- 16 ppm, which is relatively higher as compared with Uttarakhand soil and it is important for any plant's optimum productivity [10]. It is estimated that about 98% of Indian soil contain insufficient amounts of available phosphorus; available phosphates are those phosphates which are available to the plants for their growth [9]. Phosphate requirement for any plant is approximately 30 μM [11] but as phosphate is sequestered in soils in various forms, it becomes difficult for plants to use it result in phosphate deficiency. Below this requirement there is a reduction in the crop productivity. Replenishing soil phosphates to enhance and maintain soil and plant productivity has been long recognized by agronomist in phosphate deficient agricultural soil [12].

Insoluble phosphorus compounds, particularly organic phosphorus compounds, can be mineralized and metabolized by soil enzymes particularly phosphatase. Phosphatase enzyme, specifically acid and alkaline phosphatase (E.C 3.1.3.1), is in the class hydrolase and has pH optima at acidic and/or alkaline condition [20]. This enzyme is responsible for removing phosphate groups (de-phosphorylation) from many types of organic molecules including nucleotides, proteins and alkaloids present in the plant debris like leaf, stem, dead microbes and insects in the soil. In the soil bacteria that occupy the rhizosphere, alkaline phosphatase is usually located in their periplasmic space and this enzyme is secreted into the soil to generate free phosphate groups for the uptake and use by the plants as well as the bacteria [11]. It has been suggested that microbial alkaline phosphatase is comparatively resistant to degradation and inactivation and also has a higher rate of activity [13].

Gains due to increase in agricultural output due to fertilizer would be short term gains as application of fertilizer will increase the yield for a certain time but eventually the soil quality will degrade, as seen during the green revolution [3]. But if a proper scientific biotechnological intervention using microbes and additional traditional methods is practiced, sustainable and increased crop yield can be maintained over a long duration of times. Plant growth-promoting microorganisms are mainly soil and rhizosphere-derived organisms that are able to colonize plant roots in significant numbers (10^5-10^7 CFU per gram of fresh root) and influence plant growth in a positive manner under certain environmental and soil conditions and has been used to increase the crop yield successful [14]. Apart from *Allorhizobium, Azorhizobium Bradyrhizobium, Mesorhizobium and Sinorhizobium* are reported as the potent biofertilizer [15]. *Bacillus, Pseudomononas*, *Enterobacter, Alcaligens, Arthrobacter, Azotobactor, Enterobactor, Serratia* also been tried [16], [17], [18]. Developing a microbial intervention for any region is unique for every region and is region specific. The microbial intervention would also help in developing organic farming and if the program is developed, it would be beneficial for entire Uttarakhand.

The aim of the study was to isolate soil bacteria capable of producing high levels phosphatase enzymes using cost effective substrates from the agricultural rhizopheric soils of Dehradun region of Uttarakhand.

EXPERIMENTAL SECTION

Sampling sites

Soil samples were collected from rhizosphere region from five different location of Dehradun district Uttarakhand, India. The soils were having pH range from 7.2 to 8.5. Briefly, intact soil cores (5 cm diameter x 7 cm deep) were collected near the rhizospheric region of various plants and trees (litchi, tomato and barley). Isolation of microbes from these samples and experiments with the microbes was carried out in Department of Biotechnology, Graphic Era University, Uttarakhand, India.

Isolation of bacteria from the soil rhizosphere of crop plants and trees

The collected soil sample was serially diluted up to 10^{-7} dilutions using sterile saline as a blank and the diluted samples were plated in to the sterile nutrient agar plates using spread plate method. The plates were incubated at 37^0C. The isolated colonies were further purified by streak plate method using sterile nutrient agar medium. Isolated bacterial strains were re-checked within one week interval and were maintained on 15% glycerol at -85^0C for further tests.

Alkaline phosphatase activity screening

Fourteen colonies were picked for each soil sample collected from the rhizosphere of crop plants and these colonies were cultured and checked for extracellular phosphatase activity. Two kinds of media were used for checking phosphatase activity. Medium 1 was complex fermentation medium (Dhaked et al. 2005) that contained peptone (0.5%), dextrose(0.5%), ammonium sulphate (3g/l), calcium chloride(0.2 mM), sodium chloride (0.08 mM), potassium chloride(0.02M), ammonium chloride(0.02 M), magnesium sulphate (1 mM), zinc sulphate (0.004%), sodium phosphate (200 µM), calcium nitrate (50 µM) & the other simple basal media (medium 2) is slightly different from that of nutrient broth. This medium contains glucose (0.2%), peptone (0.5%), calcium chloride(0.2 mM), sodium chloride (0.08 mM) & sodium di hydrogen phosphate (0.3%). For the initial screening of the best phosphatase producers, both the media was screened and then medium 1 was used further for the different optimization study. The pH for initial screening was maintained at pH 7.0. Flasks were cotton plugged & autoclaved at 15 psi/cm^2 for 20 minutes. The sterile media was cooled to room temperature & inoculated with 500 µl of the culture for high growth of bacteria along with the phosphatase secretion. The flasks were incubated in an orbital cooled shaking incubator at 37°C with 150 RPM. The bacterial biomass was separated from culture after a sequential interval of 2 hours incubation period through centrifugation & further analysis of alkaline phosphatase activity was carried out from the culture broth (Medium 2). The alkaline phosphatase activity and bacterial growth was determined and monitored in triplicates (Dhaked et al. 2005) from the culture broth of all the grown bacterial strain within a gap of two hour interval for about 48 hours. This was done to check the effect of time period on the enzyme activity. 1.5 % glucose, 1.5% fructose, 1.5% galactose, 1.5% starch, 1.5% molasses & 1.5% date syrup were used separately as different carbon sources in the Media 1 (fermentation media) to monitor both the alkaline phosphatase activity and bacterial growth at 680 nm. To assess the best nitrogen sources (1.5% peptone, 1.5% tryptone, 1.5% yeast extract, 1.5% sodium nitrate, 1.5% potassium nitrate & 1.5% ammonium chloride) were used separately in the Media1. Alkaline phosphates activity and bacterial growth kinetics in the medium were then measured separately. Alkaline phosphatase activity and bacterial growth pattern was further observed at different pH of medium 1 (fermentation media) in the range 4 to 11 (pH was changed by using 0.1 N HCl and 0.1 N NaOH to desired level). Effect of different temperature (30°C to 60°C) of incubation on bacterial growth and alkaline phosphatase activity was investigated to optimize the temperature where the bacteria would show maximum growth and also give highest alkaline phosphatase activity. Finally the concentration of best carbon sources and nitrogen sources were optimized in the fermentation media and alkaline phosphatase activity and the bacterial growth were checked at each of the concentration in the medium 1 (fermentation media)

RESULTS AND DISCUSSION

Fourteen bacterial isolates (from the rhizospheric soils of Dehradun district in Uttarakhand) were finally screened for their ability to release high alkaline phosphatase into the media. Out of fourteen, six isolates named ALKP 1, ALKP 3, ALKP 4, ALKP 8, ALKP 9, ALKP 12 isolates showed high enzyme activity as compared with their corresponding controls in medium 2 (Fig 2). ALKP 12 isolate was selected for detailed investigation. This strain showed best growth after 24 hours in the medium 1 at 37°C and our data suggests that this isolate is capable of growing in the pH range of 4 to 11 [21]. The isolate showed unusually high, three times the normal phosphatase activity at pH 9 and 37^0C in the medium 2. It was usual to see a very high activity even at pH 4 which was twice the

basal activity in this kind of fermentation media. This surprisingly high activity is somehow due to the specific components in the fermentation media in low concentration because when we used sodium phosphate (200 µmol) and calcium nitrate (50 µmol), this low amount triggers or up regulates the gene responsible for the extracellular secretion of alkaline phosphatase with a high enzyme activity. Hence this isolate can be important as the enzyme secreted by this isolate can work very efficiently in varied pH conditions. Our data suggests that these isolates have unique feature that they can have potential for promoting plant growth and crop yield.

Maximum enzyme activity of this isolate was found after 24 hours. The effect of different carbon sources, Fig 6 a: (1.5% glucose,1.5% galactose,1.5% fructose,1.5% starch,1.5% molasses & 1.5% date syrup) on the microbial growth was determined. It is shown in figure (6 b and c) that the strain can grows well & gives high alkaline phosphatase activity when grown on 1.5 % molasses at 37^0C and at pH 9 and after 24 hours of incubation in comparison to other carbon sources in the media 1. Molasses is a cost effective carbohydrate source which can be a substitute for starch. Figure 7a and 7 b shows the effect of inorganic nitrogen sources (0.5%) on alkaline phosphatase activity and bacterial growth respectively. It is noted that the maximum alkaline phosphatase activity was measured when bacterial isolates were grown in a medium containing 1.5 % sodium nitrate & 1.5% molasses**.** Efforts are underway to determine the identity of this isolate using molecular techniques involving sequencing. Usefulness of this isolate in promotion of plant growth is also being checked.

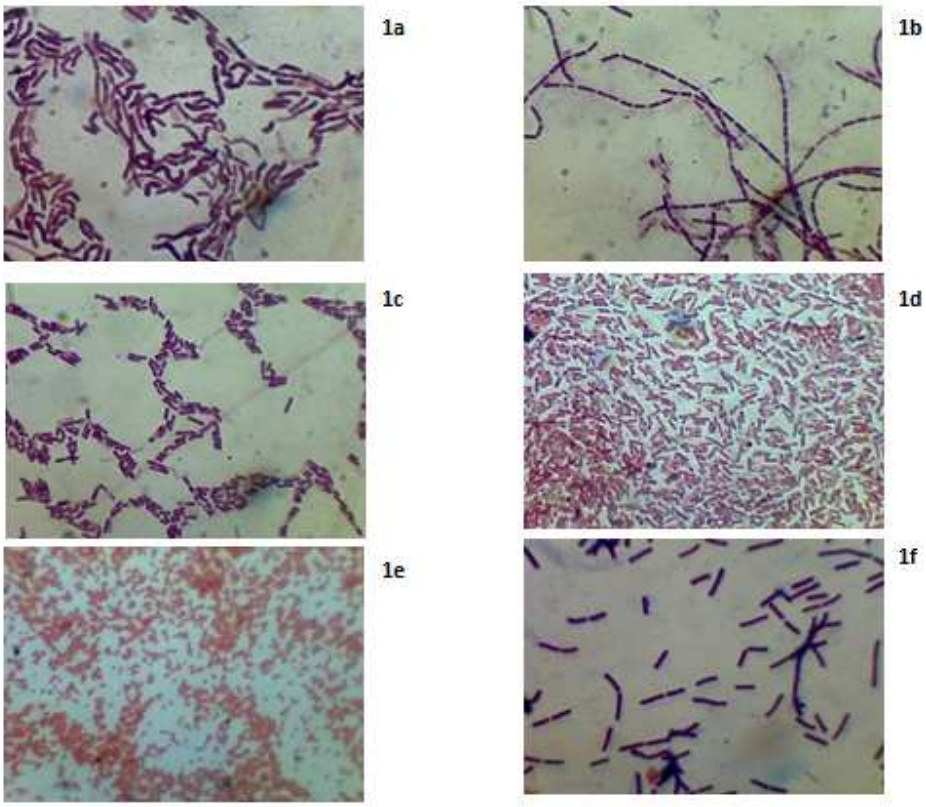

Fig 1. Gram Staining pictures of different isolates isolated from soil samples confirming presence of bacteria. 6 samples are represented here out of total 14 isolates. The characters of these isolates are: a) ALKP 1: Thick, comma shaped, gram positive bacteria b) ALKP 3: Thin, non-linear gram-positive rods, many chains forming a network c) ALKP 8: Thin, gram positive rods, forming a definte shape when clustered together d) ALKP 4: Thin, numerous and scaterred gram negative rods e) ALKP 9: Round shaped, numerous and clustered gram negative bacteria f) ALKP 12: Thick rod shaped, both the bacteria joined together

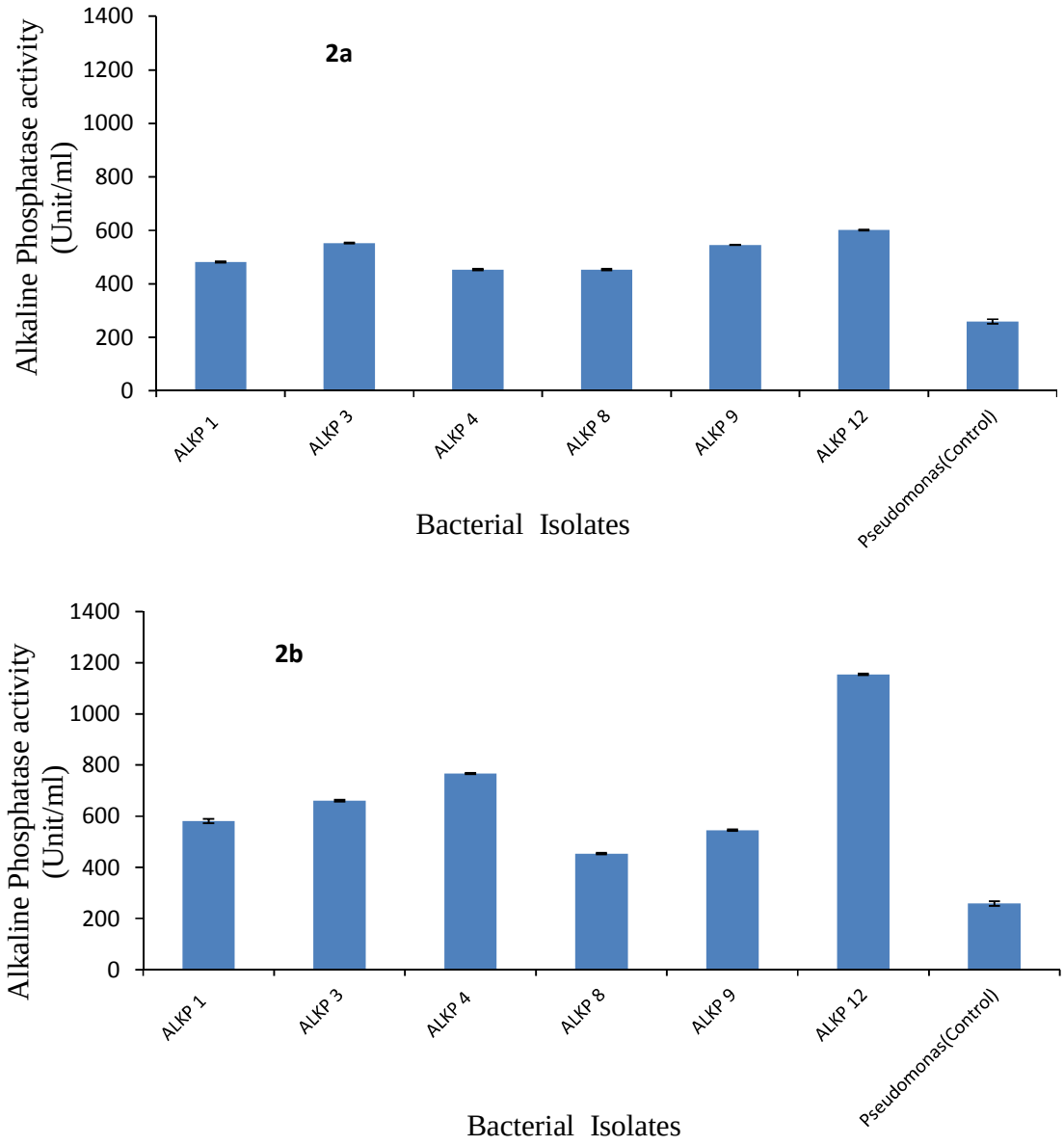

Fig. 2. Different bacterial isolates were grown in a basal medium containing defined concentration of sodium phosphates (200mM). Activity of phosphatase secreted into the medium was measured after 24 hours. Bacterial isolate named ALKP 12 shows maximum activity out of the other phosphatase releasing bacteria. This isolate was used in all further studies

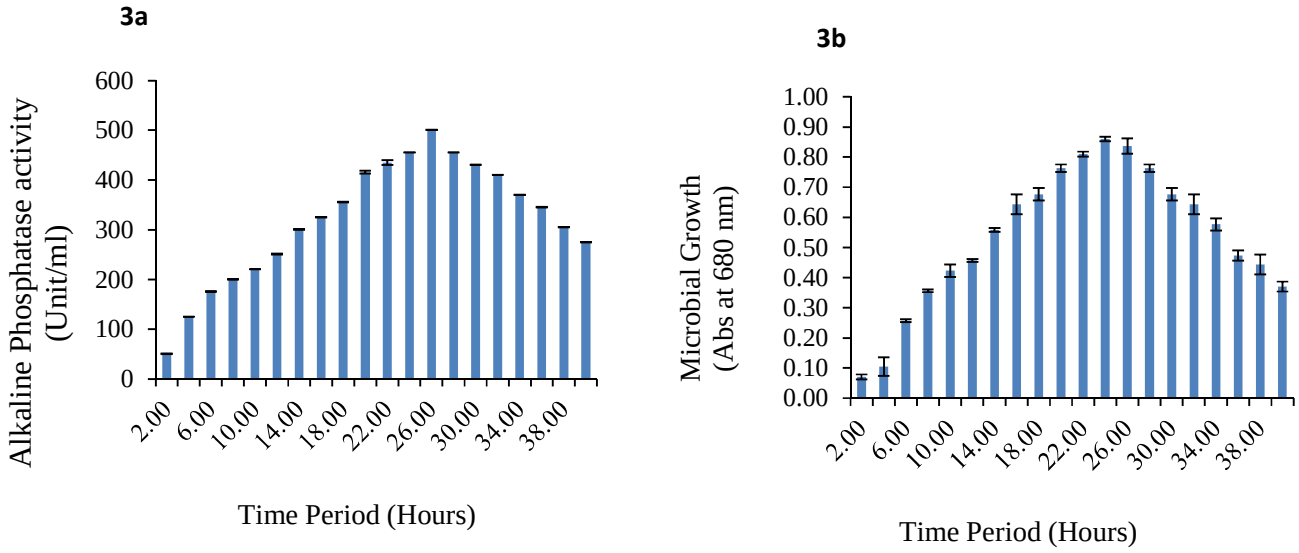

Fig. 3. Standardization of optimum growth time and attainment of highest alkaline phosphatase activity time in medium 2 for ALKP12. a. Measurement of enzyme activity at different time interval. b. Observations on growth at different time interval. Data shows that 26 hours was sufficient growth time for ALKP12 isolate for growth and maximum phosphatase in media also peaked during within this time. Time duration of 26 hours was used for cultures and enzyme assay from media in all further studies

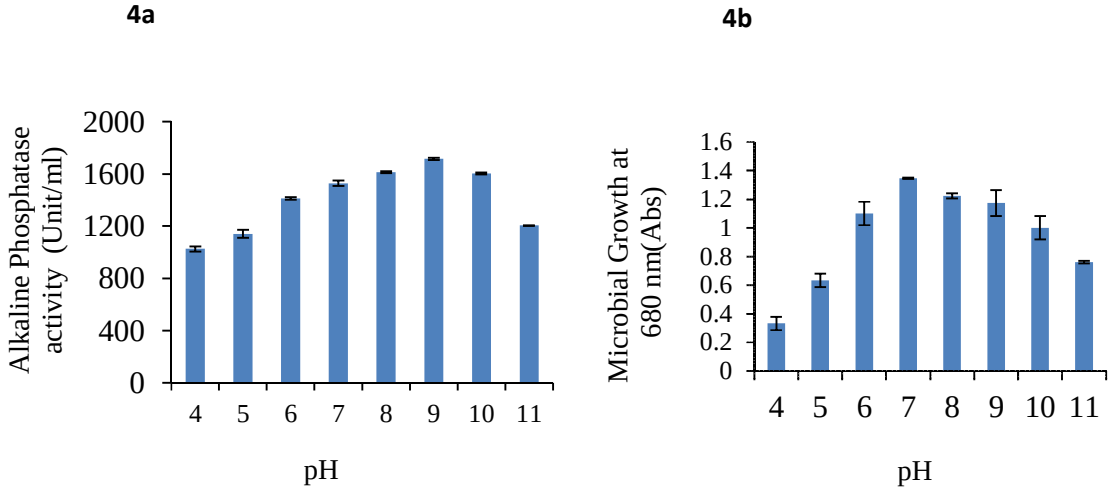

Fig. 4. Optimization of pH for ALKP12 isolate. Fermentation Media was used in this experiment which has been shown to induce more phosphatase secretion. a. Phosphatase activity of ALKP12 isolate at different pH. b. Growth of ALKP12 isolate at different pH

In fermentation media, ALKP12 isolate showed phenomenal increase in phosphate activity. Maximum activity at optimum pH of 9, activity of alkaline phosphatase was almost 3 times the basal level. Interestingly, even at pH 4, alkaline phosphate activity was twice the basal level (as seen in Fig. 2).

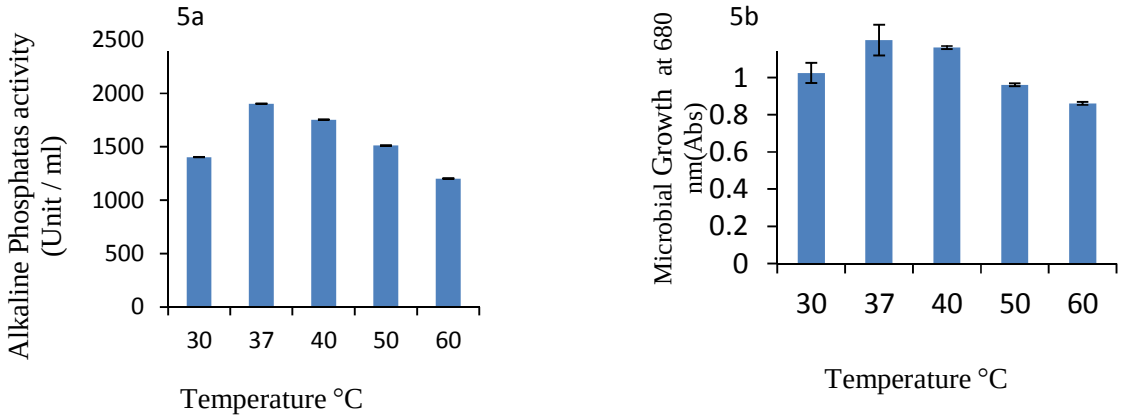

Fig. 5. Optimization of growth temperature for ALKP12 isolate. a. Different temperatures were used for incubation of cultures and growth of isolate along with alkaline phosphatase activity was observed. Data showed that at 37° C, alkaline phosphatase recorded highest activity (1900 Unit) b. Maximum microbial growth was observed at 37°C

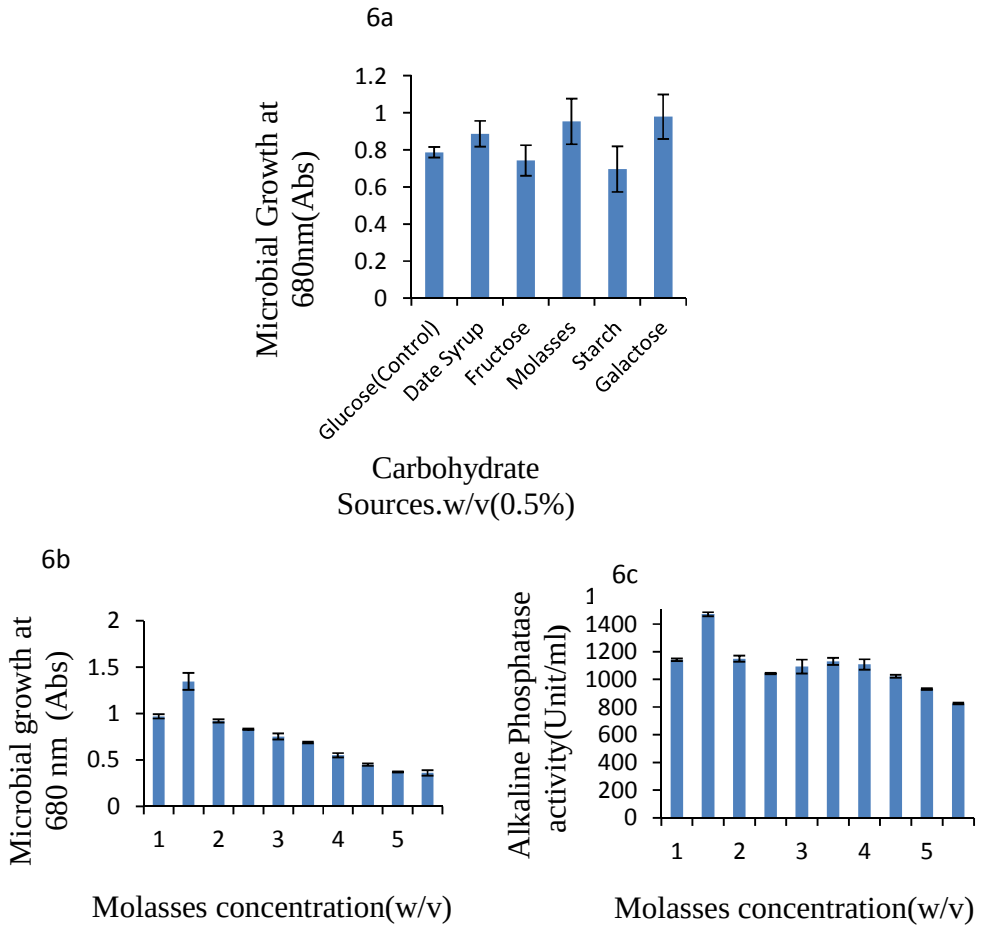

Fig. 6. Optimization of carbon source for ALKP 12 isolate. a. Different carbon sources were used in the fermentation media along with other components and bacterial growth pattern was monitored as against each sources. Similar growth was observed when Galactose and Molasses were used. b. Optimization of Molasses concentration for best growth of ALKP12 isolate. 1.5% Molasses showed maximum growth. c. Phosphatase activity of ALKP12 isolate with 1.5% molasses concentration

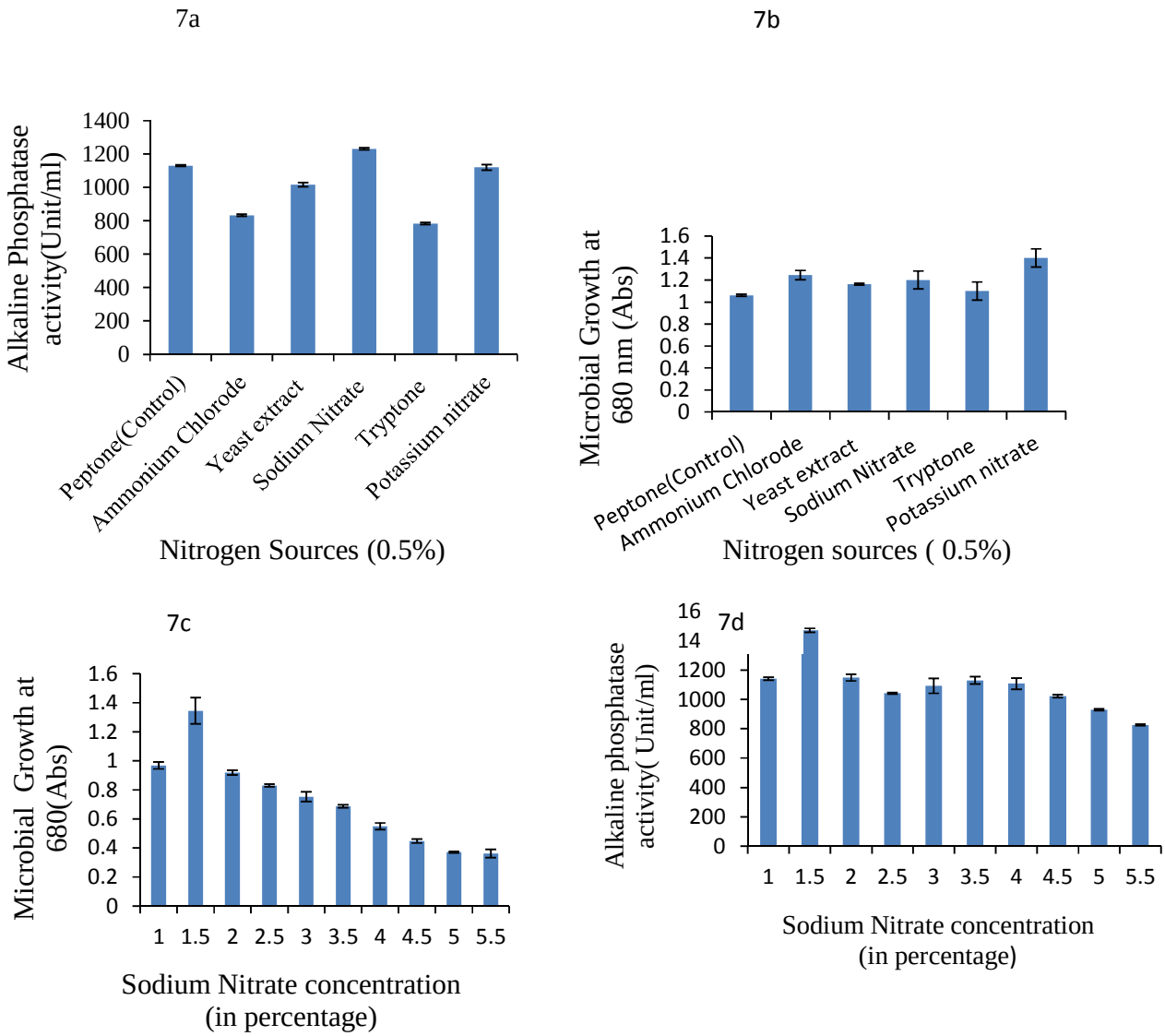

Fig 7. Optimization of nitrogen source for ALKP 12 isolate. a. Different nitrogen sources were used in the fermentation medium and growth was monitored. Potassium nitrate showed maximum activity but as Sodium Nitrate was used as it is more cost effective. b. Optimization of concentration of sodium nitrate for maximum growth. 1.5% sodium nitrate showed best growth of ALKP12 isolate. c. Phosphatase activity at different concentration of sodium nitrate

CONCLUSION

Rhizospheric bacterial isolates (ALKP 12) isolated from Dehradun district have tremendous potential to release phosphatase enzyme in the medium 2 which is a fermentation media as compared with basal media. Best phosphatase activity of ALKP12 isolate is seen at 37°C and pH 9, however significantly high phosphate activity is seen even in the acidic pH (pH 4). This isolate can utilizes 1.5% molasses as the carbon source. It can also utilize 1.5% sodium nitrate as nitrogen source to give maximum product. This isolate has huge potential to improve plant growth as phosphatase enzyme mineralize and catalyze organic phosphorous compounds present in the soil and release soluble inorganic phosphate. These phosphates can be made available to the plants and ultimately plant-microbial interaction will improve plant growth. This work is ongoing.

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