DISCUSSION

Studies on biodiversity of phosphate solubilizing *Bacillus subtilis* isolated from salinity-affected soil of Amravati and Akola district of Vidarbha was carried out. A total of 362 soil samples were analyzed for screening phosphate solubilizing *Bacillus subtilis*. A total of 176 soil samples were collected from Anjangaon (10), Bhatkuli (62) and Daryapur (104) taluka of Amravati district. Total 186 soil samples were collected from Akola (62), Akot (75), Balapur (24), Murtizapur (13) and Telhara (12) taluka of Akola district. Out of 362 soil samples, 289 (157 from Amravati and 132 from Akola district) samples showed the presence of phosphate solubilizers (Table 5) (Fig. 6, 7). Out of 289 phosphate solubilizers, 135 (65 from Amravati and 70 from Akola district) were belongs to *Bacillus* spp., among these *Bacillus* spp., 50 (23 from Amravati and 27 from Akola district) were the *Bacillus subtilis*. Thus out of 176 soil samples from Amravati, 11 PS *Bacillus subtilis* were isolated from Daryapur taluka, followed by 8 from Bhatkuli and 4 from Anjangaon taluka. From Akola district, total 186 soil samples were analyzed and 10 PS *Bacillus subtilis* were isolated from Akola taluka, followed by 6 from Balapur, 6 from Akot, 3 from Murtizapur and 2 from Telhara taluka (Table 5). In all, 50 PS *Bacillus subtilis* were isolated from 362 soil samples, in which 23 were from Amravati and 27 from Akola district (Fig. 5, 8).

The earlier studies of Bhattacharya *et al.* (1998); Tambekar and Bhokare (2003 and 2006) reported the presence of phosphate solubilizing bacteria in salinity-affected soil of Vidarbha region. The overall objective of the present investigation was to study biodiversity of *Bacillus subtilis* i.e. to identify the strains and to recognize the strains under study were similar to a previously characterized *B. subtilis* or not. Since phosphate solubilization is an inherent function of *B. subtilis*, the study was focused on phosphate solubilizing *B. subtilis* isolated from salinity-affected villages of Amravati and Akola district.

The inspection of master data from physiological diversity (Table 4a) indicated a wide variety of colony type, physiological responses, cell
morphologies etc. It was found that no two strains were alike even from the same local source in this respect. It was reported by Singer (1975). He reported that there was diversity appeared in the physiological characteristics of the strains. Many intermediate varieties were obtained instead of getting a clear discontinuous cluster of strains. This was because bacteria have had the opportunity to diversify genetically for a longer period of time, which has resulted in filling the spaces between the discontinuous clusters of strains. Microbial diversity can be influenced by soil environment and chemical characteristic.

The identification of the genus *Bacillus* was carried out through morphological characteristics and biochemical testing. The genus contains most aerobic, endospore forming rods. All the 50 strains of *B. subtilis* were found to be deviated from each other in cultural and morphological characteristics (Table 4a). Even in well-known and established species of the *Bacilli* there was considerable variation between strains. Thus according to Logan and Berkeley (1984) common test schemes for the identification of species and strains in the genus *Bacillus* using only a few phenotypic characters often do not permit the accurate separation of species or diagnosis of atypical or intermediate strains. Dickinson et al. (1977) stated that Identification of bacteria using a standard diagnostic scheme is of limited value because few strains can be identified beyond the genus level and others not at all. One possible method for overcoming this difficulty is the use of numerical taxonomy (Priest, 1981). A dendrogram showing dissimilarity among the strains tested that results in the clustering of several of the *B. subtilis* strains were generated from this matrix.

As early as 1948, Pikovskaya obtained an organism which was termed as 'Bacterium P' from the soil and phosphorus bearing rocks which was capable of forming water soluble phosphates from tri-calcium phosphate (TCP). Solubilization of TCP by all the strains was examined on Pikovskaya’s agar medium and the diameter of solubilizing zones were measured from 1 to 5 days of incubation. Further the phosphate solubilizing ability was confirmed in liquid medium. Similar study was carried out by
Wani et al. (1979) with Aspergillus awamori, Penicillium digitatum, Pseudomonas striata and Bacillus polymyxa.

All the strains showed solubilizing zones in the range of 2-20 mm. Among the 50 strains, PS-16 and PS-19 showed highest zone (Table 4d- 4g). Similar observations of solubilization of phosphorus in this range have been known for bacterial isolates (Veena et al., 2002). The amount of tricalcium phosphate solubilized in the Pikovskaya's broth ranged from 5-60-mg/l broth whereas the single strain (PS-22) released soluble phosphorus 64-mg/l in presence of galactose as a carbon source(Table 4h- 4k).

It was found that almost all of the variables considered were characterized by varying phosphate-solubilizing (PS) activity in relation to their site of isolation. In addition, the strains from the same taluka showed marked differences in these activities, as indicated by the highest PS activity and minimum to moderate ranges (Fig. 11, 12, 13). Out of 50 B. subtilis, almost strains appeared alike, there was a diversity of characteristics soon appears. Some strains appeared to cluster and have common characteristics. (Fig. 9). Variations were noted in the ability of these strains to utilize the various substrates. Some general phenotypic characteristics of the B. subtilis through the use of biochemical characteristics were suggested by Gordon et al. (1973); Knight and Proom (1950), who mainly examined the use of nitrogen and carbon sources. The key differentiating tests for the strains of Bacillus were the utilization of arabinose, glucose, lactose and mannose sugars, production of indole, hydrolysis of starch, hydrolysis of casein, hydrolysis of gelatin, reduction of nitrate to nitrite, Voges-Proskauer test (Table 8) (Fig. 11).

**PS Activity Under the Influence of Various Carbon Sources:**

Appropriate carbonaceous substrates, nutrients and proper conditions are important parameters for maximum phosphate solubilization. Since these microorganisms are heterotrophs and solubilized insoluble phosphates by secreting organic acids, the role of carbon sources in this context was well understood (Bardiya and Gaur, 1974).
On the basis of phosphate solubilization results, glucose was found to be the best source of energy followed by sucrose, galactose, mannitol and fructose. When glucose was replaced by sucrose in the medium, PS-19 released the highest soluble phosphorus (52 mg/l). In presence of galactose, PS-22 released 64 mg/l, in presence of mannitol PS-29 and in presence of fructose PS-1 released 50 mg/l of soluble phosphorus (Table 4h). Previous studies of Gaur (1990) with Pseudomonas striata as well as studies of Tambekar and Bhokare (2006) also reported similar findings of effective carbohydrates in the order listed. The phosphate solubilization was found to be influenced by the types of carbon and nitrogen sources used. It was observed that TCP solubilizing Bacillus subtilis utilized a variety of carbon compounds as energy sources, but the amount of phosphate solubilized varied significantly with different sources of energy.

The extent of solubilization of insoluble phosphates depends upon the production of organic acids by microorganisms, which in turn was influenced by concentration of carbon substrate in the medium. Increasing glucose levels lead to produce acidity in all of the isolates as demonstrated by decline in pH of the medium with release in glucose concentration (Singh and Kapoor, 1958).

**PS Activity Under the Influence of Various Nitrogen Sources:**

While studying the influence of nitrogen sources, ammonium sulphate was found to be the best source utilized by maximum number of strains which is the constituent of Pikovskaya's medium, followed by ammonium nitrate, potassium nitrate, sodium nitrate, calcium nitrite and urea (Table 4i), whereas PS-34 released maximum amount of phosphorus (52 mg/l) in presence of ammonium nitrate, PS-4 (45 mg/l) in presence of potassium nitrate. The strain PS-21 released (50 mg/l) phosphorus in presence of sodium nitrate, PS-23 and PS-9 released (50 mg/l) soluble phosphorus in presence of calcium nitrite and PS-22 released (45 mg/l) soluble phosphorus in presence of urea. Sodium and potassium nitrates were preferred to ammonium nitrate as a nitrogen source (Table 4i). But amongst the ammonium salts, ammonium
sulphate was preferred as compared to ammonium nitrate (Haque and Dave, 2002). The solubilization of calcium and iron phosphate in liquid medium by *Bacillus subtilis*, *B. mycoides*, *B. megatherium* and *B. mesentericus* with ammonium sulphate as the nitrogen source was reported by Sen and Paul (1957).

**PS Activity under the Influence of Different pH Levels:**

The growth and activity of the microorganisms can be affected by pH of the medium. The experiments dealing with optimization of pH revealed that *Bacillus subtilis* showed varying degree of TCP solubilization. This might be due to bacterial activity in the medium which turn pH of the medium to acidic pH. This was supported by the fact that solubilization was mainly due to production of organic acids in the growth medium. Highest TCP solubilization was recorded by the strain PS-19 and PS-20 (52mg/l) with pH 6, PS-20 (45mg/l) with pH 5 and PS-34 (45mg/l) with pH 7 (Table 4k).

Thus effect of pH on tricalcium phosphate solubilization showed that pH 6 was the most favorable pH level with maximum number of strains, followed by pH 5, pH 7 and pH 8. The findings were in support of Ahmad and Jha (1968); Bajpai and Rao (1971) who reported optimum pH 6 for bacteria and pH 4.0 for fungi for maximum phosphate solubilization. Whereas the results were in contrast with the findings of Wani et al. (1979), they found that, for maximum solubilization of tricalcium phosphate in Pikovskaya’s medium, the best pH was around 6 for fungi (*A. awamori* and *P. digitatum*) and 7 to 8 for the bacteria (*P. striata* and *B. polymyxa*).

Out of 50 strains, phosphate solubilization as well as the growth of *B. subtilis* was negatively influenced at pH level 8 i.e these strains did not grow and solubilize phosphorus at pH 8. These results were similar in concordance with the results of Ahmad and Jha (1968).

The fall in pH clearly indicated the production of organic acids, which was considered to be the sole mechanism responsible for solubilization of insoluble inorganic phosphates (Dave et al., 2002; Bajpai and Rao, 1971).
Increase in incubation time period of PS *B. subtilis* showed decreased in pH of the medium (Table 4k) and the pH range was between 4 and 5.

**PS Activity Under the Influence of Various Temperatures:**

Temperature is the vital factor for the growth and activity of microorganisms. The best temperature for PS activity by maximum number of PS *B. subtilis* was proved to be 30°C followed by 35°C, 40°C, 45°C and 50°C. (Table 16, 17). The activity of these microorganisms was rapidly reduced at higher temperatures (45°C and 50°C). At higher temperature, the culture medium was found less acidic. In this context Gaur and Sachar (1980) obtained very similar findings who reported that 28°C-30°C was the best temperature for PS by *Bacillus* spp. Wani et al. (1979) reported that 30°C temperature was optimum for tricalcium phosphate solubilization by *Pseudomonas striata, Aspergillus awamori* and *Penicillium digitatum,* whereas 35-40°C temperature favored phosphate solubilization by *Bacillus polymyxa.*

Absence of solubilization zones on agar plates, above pH 8 and 45°C could be limitation in production of organic acids by the *B. subtilis* strains (Table 20). Phosphate solubilizing *B. subtilis* strains those were found with PS zone below 15-20 mm range on solid medium showed considerable phosphate solubilization in liquid medium. This might be due to the fact that more time is required for diffusion of organic acid secreted during the process of phosphate solubilization, because the substrate (phosphate) in solid Pikovskaya’s agar medium is in solid phase and for the conversion of solid phase into liquid phase more time is required. Louw and Webley (1959) reported that many isolate which did not show any zone of solubilization on agar plates were found to released phosphate in liquid medium. A similar investigation by Ahmad and Jha (1968) reported that during the process of phosphate solubilization, a part of the phosphate might be assimilated by the concerned organism, which would be released after the autolysis of the aged cells. These findings were also supported by the work of Tardieux-Roche (1966); Agnihotri (1970).
Influence of Various Carbon, Nitrogen Sources, pH and Temperatures on PS B. subtilis With Respect To Incubation Period:

When different carbon sources were incorporated in the growth medium, solubilization of P by 10% of the strains isolated from Akola district showed highest peak on 1st day with a decrease on 2nd and 3rd day but again increased on 4th day. The 4% of strains from Amravati district showed maximum activity on 2nd day with decreased on 3rd day and 4th day but again increased on 5th day. While one of the 2 strains from Akola district showed maximum activity on 1st day with decrease on 2nd and 3rd day and again increased on 5th day and the other one had given maximum activity on 2nd day with decreased on 3rd day with further increased on 4th day (Table 4h).

In case of nitrogen sources all the strains (6%) isolated from Akola district showed same trend of solubilization of phosphorus (solubilization increased upto 3rd day with decreased on 4th day and again increased on 5th day.) which were supported by the findings of Dave et al. (2003). This can be explained by the fact that, during the process of phosphate solubilization, the organism might assimilated a part of the phosphate (Tardieux-Roche,1966; Agnihotri,1970). The 10% of the strains, all except one, were isolated from Akola district reported with increased in PS activity upto 2nd day with decreased on 3rd day and again increased on 4th day (Table 4i). Single strain that was isolated from Amravati district located not far a distance from these villages of Akola district. Similar results with 22% strains were noted but the further increased was on 5th day. Rest of the strains once attained, their maxima, and reduced phosphate solubilization on the next day, which might be due to the availability of soluble form of phosphate that reduced the phosphate solubilization (Narsian and Patel, 2006).

The 12% of strains at different pH levels showed large amount of soluble phosphorus on 1st day. PS activity decreased or remained constant on 2nd day, 3rd day and again increased upto 4th day. Maximum activity on 2nd and 3rd day was reported with 4% of strains with decreased on 3rd day and
again increased on 4th and 5th day. Phosphate solubilization with 6% of strains showed that after 1st day of incubation the amount was reduced on 2nd day with increased on 3rd day (Table 4k). Rest of the isolates showed a linear increase in P released with the advancement of incubation period upto 5th day. Similar trend has been reported in *Penicillium* spp. by Asea *et al.* (1998); with *Pseudomonas* spp. by Deepa (2003).

The mechanism of phosphate solubilization has been a subject of analysis for a long time and still a matter of curiosity. The different mechanism so far hypothesized has been reviewed herein. Organic acids have been implicated to chelate the cationic counterpart of P ion and might released soluble P in the medium (Katznelson and Bose, 1959). The amount of acids liberated by these microbes roughly was more than 5 percent of the carbohydrate consumed (Banik and Dey, 1982). Fall in pH of the liquid medium was observed during the growth of phosphate solubilizing organisms. The change in pH value showed an inverse correlation with the soluble P concentration (Gerretsen, 1948; Kim *et al.*, 1997), whereas Illmer and Schinner, (1992) reported that solubilization of Ca₃(PO₄)₂ is known to occur even in the absence of available form of organic acids.

The size of solubilization zone in diameter was found to increase with time. The diameter of the zones was found to range from 15 mm to 20 mm considered as maximum. Thus phosphate solubilization in terms of maximum zone reported that 52% of strains showed such zone on 5th day, 14% on 4th day and only 2% on 3rd day of incubation period.

**Diversity of Phosphate Solubilizing *B. subtilis***:

During the enumeration of phosphate solubilizers, 50 phosphate solubilizing isolates of *B. subtilis* were obtained. Amongst the Gram-positive *B. subtilis*, 42 were short rods singularly arranged, 6 were in chains, 1 was long rod singularly arranged and 1 was in chain. The frequency of variations in cultural and morphological characteristics was shown in table 7.
Biochemical study statically analyzed by SPSS software package revealed that out of 50, 41 strains showed typical tests when studied for indole production. Nine strains were found to hydrolyze tryptophan to indole and were indole positive. Thirty-nine, out of 50 were unable to produce acid and thus unable to change methyl red indicator to yellow while 8 strains were able to change methyl red indicator to yellow. Forty-five out of 50 were capable to produce acetyl-methyl carbinol and 5 were unable to produce. Further, out of 50, 44 strains were able to utilize citrate as their carbon source and a single strain was delayed in utilizing citrate, while 5 were unable to utilize it (Table 8) (Fig. 10).

The strain PS-32 was found to be deviated in 6 characters from being typical B. subtilis. This strain was recorded with highest PS activity in both solid as well as liquid medium. The metabolic and physiological differences among the 5 strains of B. subtilis and their ability to utilize the carbon sources might have influenced phosphate solubilization (Wani et al. 1979). Table 6 summarizes the data relative to the typical, intermediate and atypical strains isolated from Amravati and Akola district. All the 3 typical strains were recorded with highest phosphate solubilization (41-60 mg/l) in liquid medium while only 1 strain isolated from Daryapur taluka showed highest PS zone (15-20 mm) on solid medium during the incubation period of 1-5 days. Remaining 2 strains were unable to give highest zone. These results were in conformity with the earlier findings of Louw and Webley (1959).

From Amravati district 3 strains were found to be efficient phosphate solubilizers. Two strains isolated from Sonkhas (PS-30) and Bhamod (PS-41) of Anjangaon and Daryapur taluka respectively were 100% typical in characteristics of B. subtilis. Phosphate solubilization recorded by the strain PS-30 and PS-41 was highest in presence of environmental and nutritional factors apart from the optimum conditions required for phosphate solubilization. The strain, isolated from Hirapur (PS-42) was recorded with the highest phosphate solubilization in liquid as well as on the solid medium. The marked PS activity in liquid medium was noted for environmental and
nutritional parameters while on solid medium it was only for optimum conditions (Table 18).

Out of 10 PS B. subtilis isolated from Akola district, 7 were found to be efficient phosphate solubilizers although the salinity of this taluka is the highest as compared to other taluka of Akola district. The strain isolated from Apatapa village (PS- 1) was 100% typical in characteristics of B. subtilis and also found to be efficient phosphate solubilizers. Thus the morphological and physiological functions were not found to be altered with increase in salinity. However the strain isolated from Khel-Deshpande village (PS- 27) was very least typical in characteristics of B. subtilis (Table 6), in spite of this it was reported with marked PS zone on solid Pikovskaya's medium. Thus the altered morphological features might be responsible for highest PS activity. When PS activity was studied under the influence of various environmental and nutritional factors, the strain showed highest PS activity for 8 parameters out of 20 parameters (Table 20, 21). The villages Apatapa and Khel- Deshpande are located very close to the Purna river basin (Fig. 6) and the strains isolated from these villages were found to be most ecologically adapted for saline conditions.

Diversity in Phosphate Solubilizing Activity by B. subtilis:

The findings of the present study highlighted the prevalence of phosphate solubilizers even in the saline soil. These findings were similar in concordance with the results of Tambekar and Bhokare (2006) who have isolated 39 strains of Bacillus megatherium, B. subtilis, Pseudomonas striata, Fusarium spp., Penicillium digitatum, Aspergillus flavus and A. awamori from saline belt of Amravati and Akola district. Bilolikar et al. (1996) also reported the prevalence of microorganisms such as fungi, bacteria, actinomycetes, algae and mycorrhizae in saline alkali soils of Marathwada region. Further they have reported that these organisms play an important role for establishment of sustainable plant community in such soils. In spite of semi-arid nature of Gujarat soil, Haque and Dave (2006) isolated Pseudomonas spp., Bacillus spp., Saccharomyces spp. and Aspergillus niger.
Detailed hydrogeological investigation carried out by GSDA of the Purna basin has identified three zones. The upper northern zone of boulder alluvium extends over a distance of 120 km from Buldana district to Amravati district with a width ranging from 8-13 kms (least saline). This zone is followed by sandy alluvium in the form of discontinuous patches, which extends over an area of 2500 sq.km (moderately saline). Further, the central section which mostly comprises of clay and is saline in nature has an estimated width varying from 15-20 km occupies an area of 3000 sq. km (highly saline) and is characterized by saline ground water (Adyalkar, 1969).

All the 3 isolates of *B. subtilis* isolated from Murtizapur taluka showed highest phosphate solubilizing activity. The salinity of Murtizapur taluka is very less as compare to other taluka of Akola district and the inherent functional activity of *B. subtilis* isolated from Murtizapur taluka was highest (Fig.11).

Considering with the severity in salinity, amongst the 50 phosphate solubilizing *B. subtilis*, 10% of the strains isolated from highly saline soil showed highest phosphate solubilizing activity. These strains were isolated from Daryapur taluka of Amravati district. According to GSDA, all the 5 villages from which these highest phosphate solubilizers isolated come under the highly saline belt of Purna river (Fig.11). Similarly 8% of the strains showed PS activity in moderate range (Fig.12). These results indicated the relationship between soil salinity and phosphate solubilizing activity of *B. subtilis*. These strains showed marked tolerance towards soil salinity. On the other hand, 4% of the strains were recorded with weak phosphate solubilizing activity (Fig.13). These strains were isolated from the village Sangwi of Akola district and from the village Mhaispur of Amravati district. The strains were ecologically not adapted to salinity. The suppressed PS activity by the strains was not influenced by salinity alone but also with many agronomic, climatic and soil factors.

Out of 50 phosphates solubilizing *B. subtilis*, 22 showed highest phosphate solubilizing activity, 25 in moderate range and 3 *B. subtilis* showed weakly phosphate solubilizing activity. Out of 22 *B. subtilis*, 5 strains were isolated from highly saline Daryapur taluka of Purna river basin (PRB), 5
*B. subtilis* (2 from Akot, 1 from Anjangaon Surji, 2 from Telhara taluka) were isolated from moderately saline soil of PRB. Out of 12 *B. subtilis* isolated from least saline villages of PRB, 5 were isolated from Balapur taluka, 3 from Murtizapur, 2 from Bhatkuli, 1 from Daryapur and 1 from Akot taluka (Fig.11).

Out of 25 *B. subtilis* showing moderate PS activity, 4 (2 from Akola and 2 from Daryapur taluka) were isolated from highly saline village of PRB. The 7 strains (3 from Anjangaon Surged, 3 from Akot and 1 from Akola taluka) were isolated from moderately saline villages of PRB and the 14 (6 from Akola, 5 from Bhatkuli, 1 from Akot, 1 from Balapur and 1 from Daryapur taluka) were isolated from least saline belt of PRB (Fig.12).

The weak PS activity was recorded with 3 *B. subtilis*. Out of 3, 2 (1 from Akola and 1 from Bhatkuli taluka) were isolated from highly saline belt of PRB and 1 strain was isolated from moderately saline village Akola taluka (Fig.13).

Thus the salinity in these regions did not adversely affected phosphate-solubilizing activity. In some cases it adversely affected the morphological characters but showed highest phosphate solubilization. These isolates could be a choice as phosphatic biofertilizer for soil of saline region of Amravati and Akola district of Vidarbha region, as they have been isolated from such ecosystem.

The above study suggested that all *Bacillus subtilis* strains isolated from Amravati and Akola district in Purna river basin showed marked phosphate-solubilizing activity. A number of factors influenced the growth and phosphate solubilizing ability of *B. subtilis*. These isolated strains most ecologically adopted and have optimum PS activities at various nutritional and environmental conditions. Hence biofertilizers produced from these specific strains can help to derive maximum benefits in agricultural production. The use of such microbial inoculants may become helpful in increasing phosphorus availability to the plants from the soil as well as added phosphates. Use of PSM would be of course, be cost effective and compatible with local ecology, they're by obviating the increasing demand of chemical fertilizers.
The extensive and intensive efforts world over in understanding the diversity of extremophilic microbes has indicated that what we know today is just the tip of the iceberg. Many more novel and useful extremophilic microbes are expected to be discovered in the near future. Only sporadic attempts have been made to isolate extremophiles from extreme Indian environments. Extensive funding and collaborative efforts are needed for understanding the diversity of these microbes from extreme environments of the Indian subcontinent and their exploitation.

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