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Full Length Research Paper

Synergistic growth of lactic acid bacteria and photosynthetic bacteria for possible use as a bio-fertilizer

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In order to promote the growth of photosynthetic bacteria (PB) for use as biofertilizers in organic saline paddy fields, a co-culture of selected PB and lactic acid bacterium strains was investigated. Using MRS agar medium (de Man Rogosa and Sharpe), a total of 46 LAB were isolated from the same source as the PB. The source was soil samples from organic saline paddy fields in 14 provinces of the Northeast region of Thailand that had been treated with straw to obtain soil and straw products (SSPs) and their average salinity was equivalent to 0.25% NaCl, about 4 dS/m. Over a 24 h strain L35 showed the highest ability to grow in MRS broth with 0.25% NaCl added, which was selected for co- culture. The PB strain used was *Rhodopseudomonas palustris* tk123 that had been previously selected for its ability to produce 5-aminolevelinic acid (ALA). Cultures were grown in Tryptic Soy Broth (TSB) medium with and without supplementation of 0.25% NaCl under aerobic dark and microaerobic light conditions. Both organisms grew best with microaerobic light conditions; however, a significant inhibition by the extra NaCl on growth was only observed with strain tk123, grown with microaerobic light conditions. This inhibition was removed by a co-culture with L35 and the significant increase of pH was observed when strain tk123 was grown by itself. This was also removed when co-cultured with L35. This synergism between LAB and PB occurred in a commercial complex medium (TSB) with high salinity. It is therefore most likely that a co-culture in rice straw medium will be possible for use as a tool for producing bio-fertilizers for use in organic saline paddy fields.

Key words: Photosynthetic bacteria (PB), lactic acid bacteria (LAB), co-culture, aerobic dark conditions, microaerobic light conditions

INTRODUCTION

At present, the demand for increased food production has normally been met by increasing the use of chemical fertilizers in agriculture. It is known that these fertilizers can cause many problems such as soil compaction and pollution with toxic residues such as heavy metals (Borlaug, 2007). This is a serious problem when production and consumption of food is considered on a global scale. In addition, unregulated or unscrupulous additions to agricultural system has reduced crop productivity and caused unfavorable environmental conditions such as soil salinity and loss of essential plant nutrients which with the onset of drought has created huge disasters. Recently, increasing demands for products from 'organic farming' has encouraged farmers to use biofertilizers instead of chemicals. For instance,

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application of cultures of effective microorganisms (EM) to some agricultural lands in Asia countries, including Thailand, has been reported to improve soil quality, crop growth and enhance crop yield (Ncube et al., 2011). An EM preparation consists of a consortium of so called 'regenerative' naturally occurring microorganisms (Higa, 1996) such as photosynthetic bacteria (PB) yeasts, fungi and different lactic acid bacteria (LAB). These can increase the microbial diversity of soils and plants to assist regenerative reactions. They have now been recommended for use with livestock, gardening, composting (Daly and Arnst, 2005) and for bioremediation (Miyajima et al., 2001).

Amongst the microbes present in EM preparations, PB has been claimed to be essential because of its ability to fix N_2 (Raymond et al., 2004) and produce plant growth promoting substances such as 5-aminolevulinic acid (ALA), indole 3-acetic acid (IAA) (auxins) and cytokinins (Sasikala and Ramana, 1995; Koh and Song, 2007). ALA has been used in agriculture as a herbicide, an insecticide and a growth-promoting factor (Nagadomi et al., 2000) including an ability to encourage salt and cold temperature tolerance in plants (Watanabe et al., 2000). PB has been extensively used for promoting growth of plants because it can grow as photoautotrophs/ photoorganotrophs under anaerobic/microaerobic-light conditions or as chemoorganotrophs under aerobic dark conditions (Imhoff and Triiper, 1989; Cheng et al., 2000). It is able to utilize a broad range of organic compounds as carbon and energy sources during either photoorganic or chemoorganic growth (Kim et al., 2004; Kantachote et al., 2005). In addition, there are claims that PB can remove environmental contaminants such as H_2S, and develop mechanisms of resistance to high concentrations of toxic metals such as Hg, As, V, U, Cd, Cu, Pb, Se, Te and Zn (Ercal et al., 2001; Panwichian et al., 2010b) including Na (Panwichian et al., 2010a). Therefore, the use of PB in paddy fields could be an attractive process to remove heavy metals and as a consequence, for example, reduce accumulation of heavy metals in rice grains.

In a previous study, soil and straw products (SSPs) were developed in our laboratory using rice straw, water and soil samples collected from organic saline paddy fields. They were then used to isolate and select PB with an ability to produce ALA (Kantha et al., 2010). However, cell proliferation of PB in non sterile SSPs is limited although they were being grown under microaerobic light conditions (our unpublished data). The two main reasons for poor growth may be because of competition from other microbes in the SSPs and also the effect of saline conditions particularly from NaCl. It is well recognized that LAB plays an important role in rice straw silage due to its ability to utilize water-soluble carbohydrates to produce lactic acid that decreases the pH for example in silage (McDonald et al., 1991, Ennahar et al., 2003). The rapid decrease in pH inhibits the growth of undesirable anaerobic microorganisms such as enterobacteria and clostridia (Limin et al., 2009). Moreover, there is evidence that the LAB produces organic acids that can be used as carbon substrates for promoting the growth of PB (Kantachote et al., 2010). The use of short chain fatty acids (SCFA) such as acetic acid, propionic acid and butyric acid as the carbon source and as potential feedstocks for polyhydroxyalkanoate (PHAs) production by *Rhodopseudomonas capsulatus* has been reported by Verlinden et al. (2007). Hence, adding LAB into SSPs may provide conditions that will better support the growth of PB in SSPs for possible use as a source of biofertilizers in paddy field under salt stress which is one of the main problems in Thailand's agriculture. Therefore, the aims of this study were to isolate and select LAB strains that will tolerate salt (NaCl). In addition, their ability to enhance the growth of PB by co-culture with the selected LAB strain under salt stress with various incubating conditions was investigated.

MATERIALS AND METHODS

Photosynthetic bacteria (PB)

Rhodopseudomonas palustris strain tk123 was isolated from soil and straw products (SSPs) from Surin Province of Thailand. It was selected because it produced the highest amount of ALA (2.96 mM) in sterile SSP after 4 weeks incubation (Kantha et al., 2010). However, when grown in non sterile conditions SSP strain tk123 produced little ALA probably because it had a low cell density and poor growth perhaps due to an effect of salinity (our preliminary work). This bacterium was maintained in G5 medium and subcultured every month. To prepare an inoculum, strain tk123 was stab inoculated into G5 agar and incubated at 35°C under a light intensity of *ca.* 3,000 lux using an incandescent lamp for 48 h and then one loopful of culture was grown in G5 broth under microaerobic light conditions for 48 h. The cell suspension was then centrifuged at 8000 rpm (Sorvall RC 5C Plus) for 15 min and cell pellet washed twice with sterile 0.1% peptone water, and then suspended in the medium used (TSB) for obtaining a cell suspension. The cell suspension turbidity was adjusted to 0.5 McFarland units (roughly 3 x 10^8 cells/ml) before being used as an inoculum. Previous research indicated that PB grows better in microaerobic light conditions than with aerobic dark conditions (Panwichian et al., 2010a and b); therefore, the former condition in the light was established as the method for preparing the inoculum.

Lactic acid bacteria (LAB)

Seventy SSPs samples that had been incubated under microaerobic light conditions for isolating PB in our previous study (Kantha et al., 2010) were also used to isolate LAB with the aim to use them for co-culture. One loopful of suspension from each SSP was streaked on MRS (de Man Rogosa and Sharpe) agar containing 0.04% bromocresolpurple as an indicator of acid production. All plates were incubated at 35°C and colonies that changed color to yellow (acid was produced) were chosen for purification by re-streaking on the same medium. Gram staining and catalase tests were performed as a preliminary isolating method for LAB as LABs are Gram-positive and catalase negative (Axelsson, 1998; Chen et al., 2005).

Selection of lactic acid bacteria

The average concentration of NaCl found in organic saline paddy fields in the northeast region of Thailand was roughly 4 dS/m (0.25%); therefore 0.25% NaCl was added into MRS broth for screening of salt resistant LAB. Generally, the total amount of dissolved ions in the water with electrical conductivity values of more than 2dS/m is toxic to rice (Department of Agriculture, 2005). One loopful of isolated LAB was transferred into the MRS medium containing 0.25 NaCl and incubated under static condition at 35°C for 24 h. Bacterial growth was measured by a spectrophotometer at a wavelength of 660 nm. Sterile MRS broth containing 0.25% NaCl was also used as the blank. The LAB isolates that showed the best growth were selected for further testing.

Effects of NaCl and incubating conditions on the growth of pure cultures and a co-culture

As TSB medium is a complex medium suitable for the growth of many fastidious organisms including LAB (Cote, 1999; Cheng and Yin, 2007), it was selected as being most suitable for investigating the possibility of a co-culture between LAB and PB before proceeding with a rice straw medium. TSB contains in g/L of distilled water 17 g casein, 3 g peptone from soymeal, 2.5 g D (+)-glucose, 5 g NaCl, and 2.5 g K_2HPO_4. This experiment has 2 aims, first to establish the effect of NaCl and incubating conditions of aerobic dark and microaerobic light on pure cultures of LAB and PB. Thus 4 sets of conditions were used: cultivation of strain L35 and strain tk123 in TSB broth supplemented with 0.25% NaCl (total 0.75% NaCl) together with control sets with no extra NaCl. Then 4 sets were separately incubated under aerobic dark and micro-aerobic light conditions. Secondly, the effect of salt stress on a co-culture under different incubating conditions was also investigated by conducting 2 more sets: cultivation of strains L35 and tk123 with a ratio of 1:1 in normal TSB as control and in TSB containing extra 0.25% NaCl. Then repeat using aerobic dark and microaerobic light conditions. Both incubating conditions were used because PBs can grow in both aerobic dark and microaerobic light conditions, although they grow better in the light.

An active culture of strain L35 after subculturing twice was used to prepare a one loopful inoculum into MRS broth and incubating at 35ºC for 24 h. This culture was centrifuged (8000 rpm, 15 min) and the cell pellet twice washed with 0.1% peptone water, before resuspension in TSB to produce a cell suspension equal to a 0.5 McFarland standard (roughly $3x10^8$ cells/ml). Strain tk123 was prepared as previously described. In all sets including the co-culture a total 1% inoculum was applied and this made an initial cell density in each set of roughly 3×10^6 cells/ml. For a co-culture set, each culture was added as a 0.5% inoculum so the same number of total initial microbes ($3x10^6$ microbes/ml) was present in all sets.

All sets were separately incubated under two growth conditions (aerobic dark and microaerobic light) at 35°C for 72 h and culture broths were sampled at 6 h intervals for measuring bacterial growth (OD_{660}nm) and pH value. Viable cell counts were determined at zero time and the first point after the culture reached its maximum growth (30 h) by a standard plate count method and incubating conditions dependent on their previous history of incubation. Microaerobic light conditions were established by placing culture plates in anaerobic jars each with the same light intensity. Maximum specific growth rate (μ_{max}) was calculated by fitting the obtained growth curve data to the modified Gompertz equation (Zwietering et al., 1990). Aerobic dark was obtained by leaving a half space of the test tubes empty (20x150 mm, total volume 30 ml) after adding culture and inoculum and finally cultures were incubated under continuous dark in a closed box with no light penetration. To achieve microaerobic light conditions, total amounts of medium and inoculum for each culture were added to almost fill screw cap test tubes (28 ml, total volume 30 ml), which had a little space at the very top of the test tubes and finally cultures were incubated under continuous light with incandescent lamps (*ca.* 3000 lux).

Statistical analysis

All experiments in this study were conducted in triplicate. The data were analyzed using analysis of variance and means were compared by the least significant differences (LSD) at $p < 0.05$.

RESULTS

Isolation and selection of LAB from soil and straw products (SSPs)

A total of 46 isolates of LAB from soil were collected from organic saline paddy fields treated with SSPs from the northeast region of Thailand. All 46 isolates of LAB were confirmed as LAB: growth with MRS medium producing acid, Gram positive and catalase negative. Following their growth on MRS supplemented with 0.25% NaCl they could be divided into 3 groups. The 7 isolates in group 1 had poor growth, (OD_{660}nm < 0.5). Group 2 had 30 isolates showing moderate growth, (OD_{660}nm > 0.5 and ≤ 0.8) and the 9 isolates in group 3 had good growth, (OD_{660}nm > 0.8). Isolate L35 from group 3 (Figure 1) produced the best growth and was chosen as a representative LAB strain for co-culture testing.

Effects of NaCl and incubating conditions on the growth of pure cultures and a co-culture

Strain L35, cultured in TSB medium with microaerobic light conditions, had the highest specific growth rate (μ_{max}) of 0.39 h^{-1}. This decreased in the presence of extra 0.25% NaCl but no significant difference. With aerobic dark conditions the growth rate in TSB was less (0.36 h^{-1}) but the extra 0.25% NaCl had no significant effect (see details in Table 1). After 30 h (maximum growth) with TSB under microaerobic light conditions strain L35 showed the highest number of viable cells of 9.14 log cfu/ml and this was statistically significantly different from other treatments (8.95-9.11 log cfu/ml) (Figure 2a). There was no significant difference in the initial pH value for all cultures (6.40-6.57) but after 30 h of growth the pH values with strain L35 under microaerobic light decreased to 5.15 and in the presence of extra 0.25% NaCl to 5.27 while with aerobic dark conditions the pH fell to 5.73 and 5.77, respectively (Figure 2b). This corresponded well with the observed differences in growth (Figure 2a).

The selected PB strain tk123, cultured in TSB medium with microaerobic light conditions, had the highest growth rate of μ_{max} (0.72 h^{-1}), and this was significantly reduced in the presence of 0.75% NaCl (0.67 h^{-1}) but was still

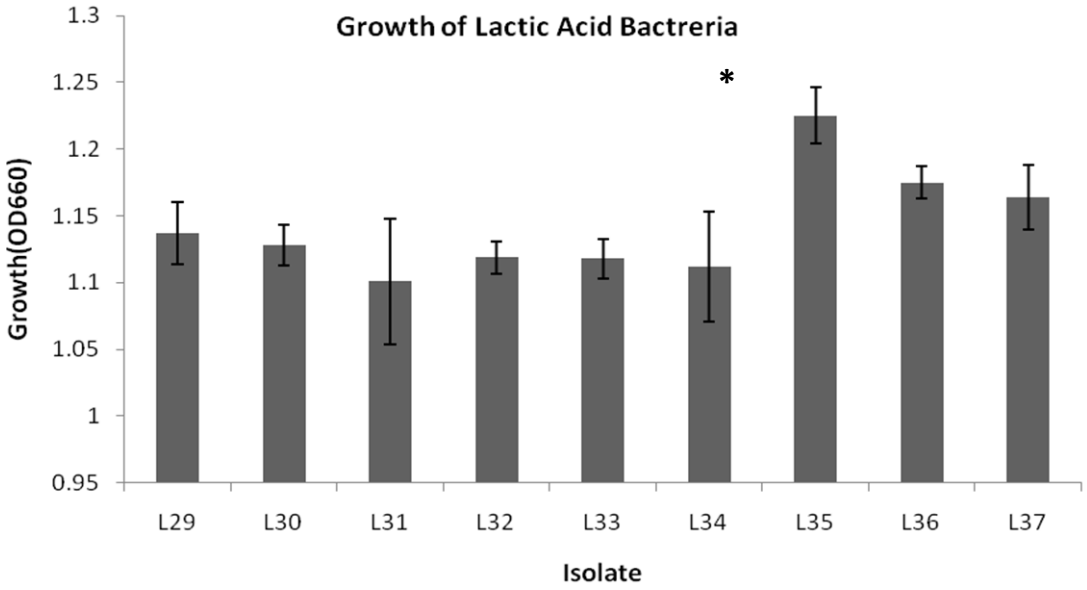

Figure 1. Growth of the 9 LAB isolates (Group 3) that grew well in MRS broth containing 0.25% NaCl under static condition after 24 h incubation. Each value is given as a mean ± standard deviation of triplicate determinations. The asterisk indicates the selected strain L35 with the best growth ($p<0.05$).

Table 1. Effects of NaCl and incubating conditions on maximum specific growth rate (μmax) of lactic acid bacteria (L35), photosynthetic bacteria (tk123) and co-culture between LAB (L35) and PB (tk123) in TSB broth with and without supplementation of 0.25% NaCl.

	Aerobic dark conditions, μmax (h^{-1})			Microaerobic light conditions, μmax (h^{-1})		
	L35	**tk123**	**Co-culture**	**L35**	**tk123**	**Co-culture**
TSB	0.36^{A}	0.54^{D}	0.43^{C}	0.39^{B}	0.72^{G}	0.59^{E}
TSB+0.25%NaCl	0.35^{A}	0.53^{D}	0.43^{C}	0.38^{B}	0.67^{F}	0.59^{E}

The different letters in the same row indicate a significant difference ($p < 0.05$) for the effect of incubating conditions while in the same column for the effect of NaCl.

significantly higher than the growth rate in TSB with dark aerobic conditions (0.54 h^{-1}) when the presence of 0.75% NaCl (0.53 h^{-1}) had no significant effect. The numbers of viable cells in TSB after 30 h incubation with microaerobic light reached 9.00 log cfu/ml and this was statistically significantly different from the other treatments (8.26-8.65 log cfu/ml) (Figure 3a). There were small but significant increases in pH values after the growth of PB tk123 from an initial pH of 6.42-6.48 to 6.71 and 6.65 in the absence and presence of extra 0.25% NaCl with aerobic dark conditions and with microaerobic light conditions to 7.36 and 7.02 in the absence or presence of extra 0.25% NaCl, respectively (Figure 3b). This again corresponded well with the observed differences in the rates of growth.

When the 2 cultures were incubated together (Table 1 and Figure 4). there was in general a synergistic effect. The growth of both organisms in TSB alone or supplementation with 0.25% NaCl with microaerobic light (μ_{max}, 0.59 h^{-1}) was still significantly higher than that with the aerobic dark conditions (μ_{max}, 0.43 h^{-1}) and this pattern of bacterial growth was similar with the pure cultures. There was no effect of NaCl on μ_{max} of a mixed culture as the μ_{max} in TSB alone or TSB supplemented with 0.25% NaCl was equal. This was in agreement with results of viable cells as there was no significant difference in the growth of either organism under aerobic dark in TSB with supplementation of 0.25% NaCl (L35, 8.96 and tk123, 8.73 log cfu/ml) and TSB alone (L35, 8.89 and tk123, 8.70 log cfu/ml). However, the most significant effects of a co-culture were that the inhibition on growth in the presence of 0.75% NaCl was abolished. Strain tk123 achieved a population of 8.91 log cfu/ml with microaerobic light conditions, the highest recorded value. There was no significant difference in the initial pH value for all sets (6.45-6.53) but after 30 h of bacterial growth the pH values of a co-culture under aerobic dark significantly increased to 6.75 and 6.72 in the absence and presence of extra 0.25% NaCl, respectively. In contrast the pH values of a co-culture slightly decreased under

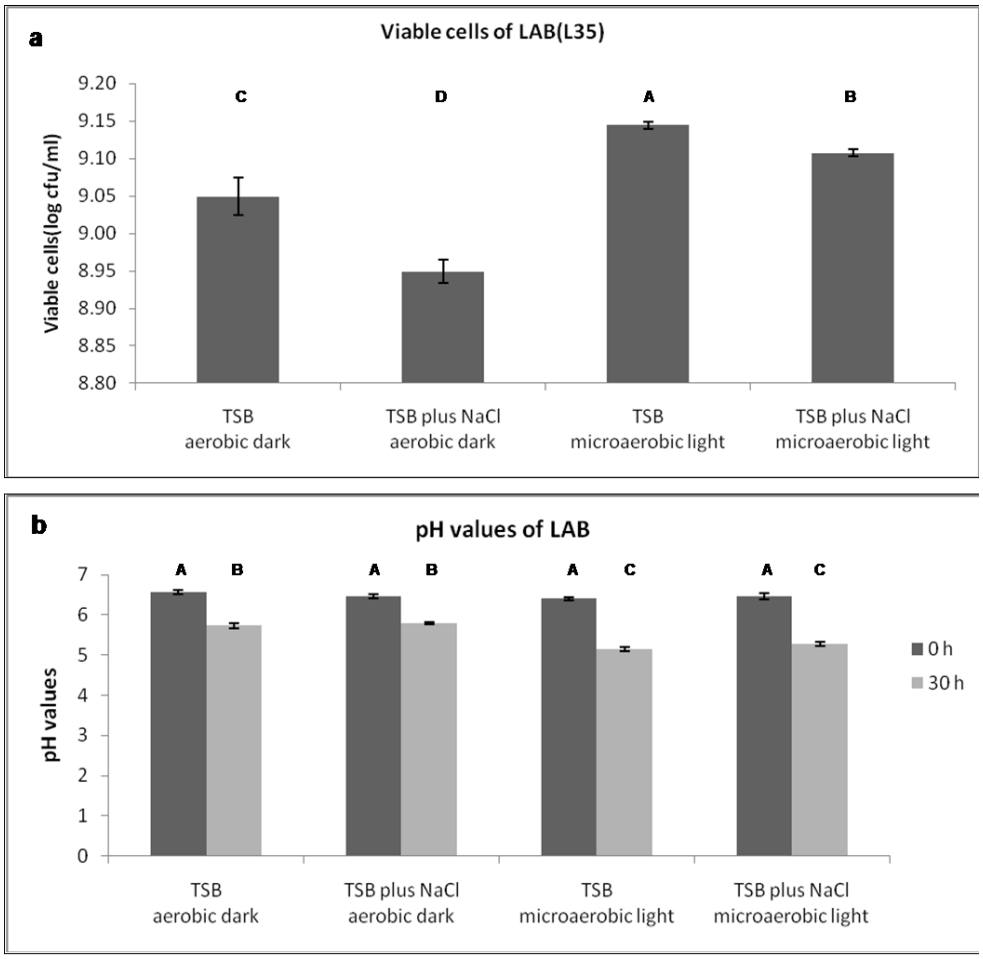

Figure 2. Effects of incubating conditions and addition of extra 0.25% NaCl into TSB on viable cells of LAB strain L35 after 30 h incubation (a) and pH values in culture broths (b). Values are given as a mean ± standard deviation of triplicate determinations. Different letters indicate significant differences ($p<0.05$) among treatments.

microaerobic light conditions in the absence and presence of extra 0.25% NaCl to 6.33 and 6.36, respectively (Figure 4b).

DISCUSSION

Isolation and selection of lactic acid bacteria

Lactic acid bacteria are widespread in most ecosystems in soil, water, plants, animals (Calo et al., 2008) and silage fermentation (Contreras-Govea et al., 2011; Ennahar et al., 2003). The investigation of Gao et al. (2008) about the efficient use of rice straw as a soil conditioner showed that an LAB community, SFC-2 developed naturally using the fermentation substrates of rice straw that had been continuously enriched with MRS broth to accelerate the fermentation process in air dried straws. In the study reported here, 46 LAB strains were isolated from 70 samples of SSPs that we had previously incubated with microaerobic light conditions with the aim to enrich for isolating PB as the dominant organisms although the MRS may have encouraged enrichment of LAB (Kantha et al., 2010). Of the 46 LAB isolates 65.22% grew to a moderate level in MRS containing 0.25% NaCl (average concentration of organic saline soil) and 19.57% of the LAB isolates grew well. This indicates that the LAB that originated from rice straw and soil samples collected from organic saline soil had the potential for use in paddy fields. Consequently, strain L35 was selected for further studies due to its growth being significantly higher than the other 8 LAB strains in the best growing group (Figure 1).

Effects of NaCl and incubating conditions on the growth of pure cultures and a co-culture

Based on Figure 2a and Table 1, the incubating conditions had a bigger effect on the growth of strain L35

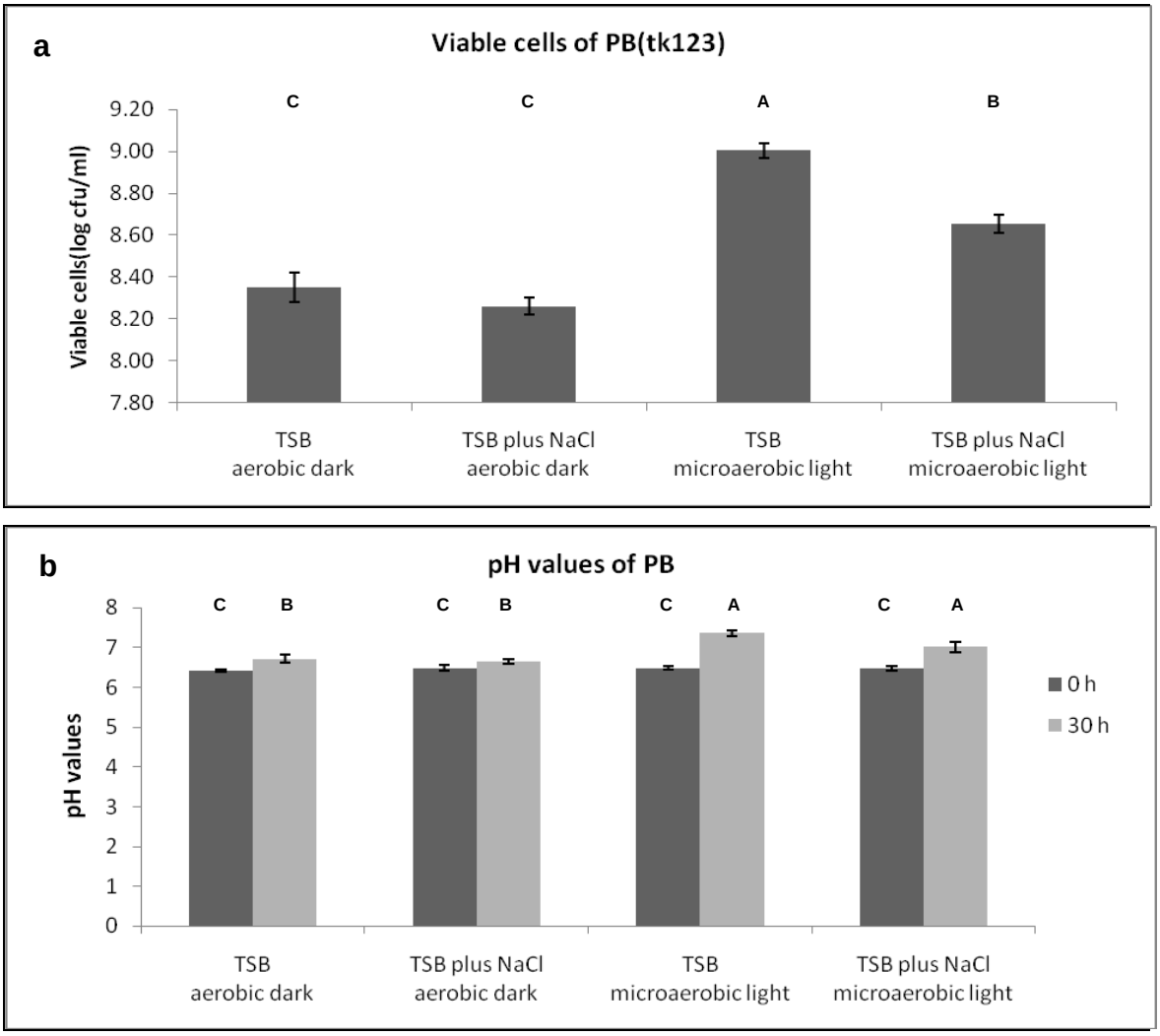

Figure 3. Effects of incubating conditions and addition of extra 0.25% NaCl into TSB on viable cells of PB strain tk123 after 30 h incubation (a) and pH values of culture broths (b). Values are given as a mean ± standard deviation of triplicate determinations. Different letters indicate significant differences ($p<0.05$) among treatments.

than did NaCl. The strain L35 grew better in microaerobic light conditions, presumably because of the low amounts of oxygen compared to the dark aerobic conditions (Axelsson, 1998; Ennahar et al., 2003; Gao et al., 2008). Additional NaCl that gave a total concentration of 0.75% in the TSB broth had little effect on the growth rate of strain L35 in both incubating conditions (Table 1). In contrast, over 30 h of incubation, there was a significant reduction in viable counts that was abolished when grown in the presence of the PB (Figure 4a). It is possible that the reduction of L35 viable counts when grown singly was caused by the reduction of pH which did not occur with the mixed culture. LABs can grow well in the presence of 0.75% NaCl but their survival may be compromised when the pH falls. This indicates lower extracellular pH was harmful to L35 and it may be attributed to acidification of cytoplasmic pH below a threshold value and consequent inhibition of cellular functions (Kashket, 1987).

The growth rate of the PB strain was significantly better in the light with a μmax (h^{-1}) of 0.72 compared to a μmax (h^{-1}) of 0.54 in the dark. This is normal for PB (Imhoff and Triiper, 1989; Panwichian et al., 2010a). The presence of NaCl at 0.75% inhibited the growth rate in the light (μmax, 0.67 h^{-1}). This was also reflected in the considerable reduction of viable counts after 30 h of growth (Figure 3a). However when grown in the presence of the LAB, this inhibition was abolished (Figure 4a). Growth of the mixed culture obviously provides a situation where both organisms benefit. There is no evidence for any negative interactions. Therefore, culturing LAB and PB together will produce advantages such as maintaining the pH at slightly acid or neutral, allowing PB to grow better in the presence of salt and to provide optimal growth condition for both organisms, including the optimum condition for ALA production by PB (Choi et al., 2004).

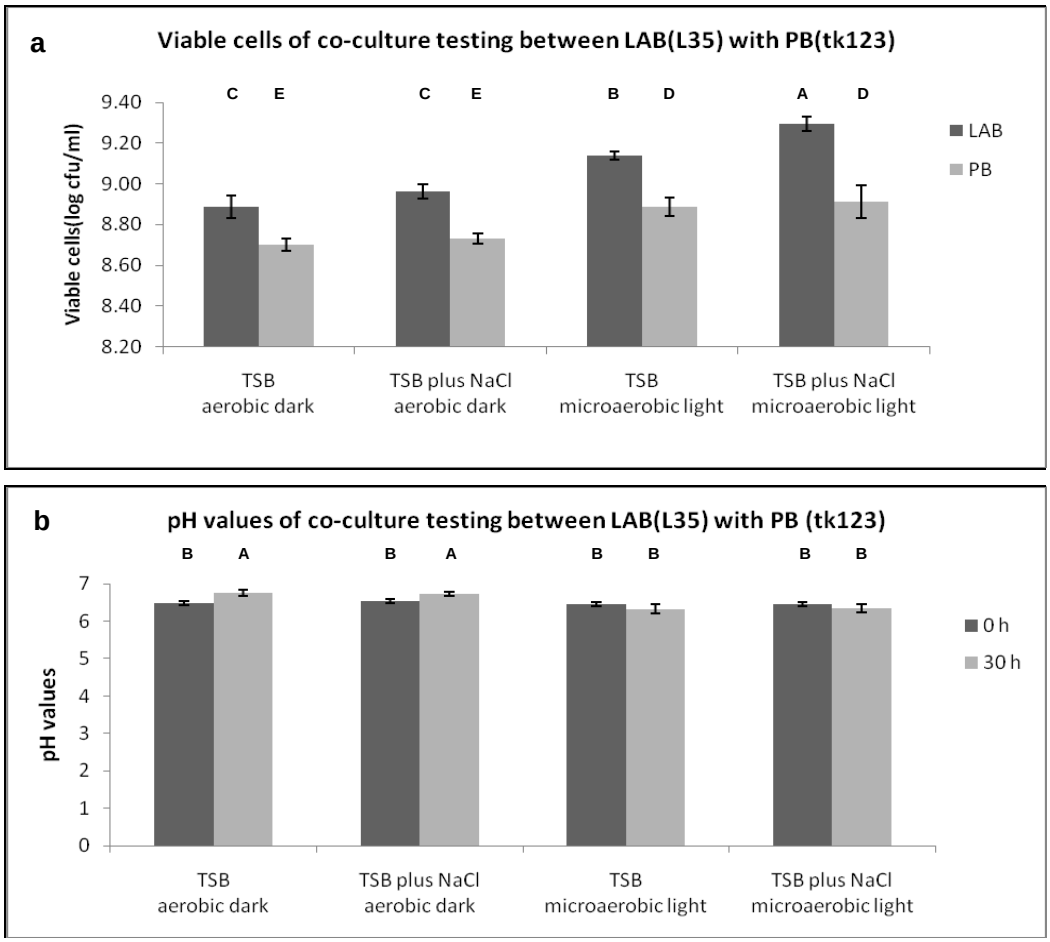

Figure 4. Effects of incubating conditions and addition of extra 0.25% NaCl into TSB on viable cells of co-culture between LAB (L35) and PB (tk123) after 30 h incubation (a) and pH values of their culture broths (b). Values are given as a mean ± standard deviation of triplicate determinations.

That leaves two questions unanswered. The first, why does the presence of PB restrict the lowering of the pH produced by the LAB? This result was in agreement with Kantachote et al. (2005); Kantachote et al. (2010), who reported that the pH values in rubber sheet wastewater significantly increased after treatment by PB. The most likely explanation is that the organic acids produced by the LAB were quickly consumed by the PB as has been previously mentioned. However, there was no evidence that this led to a significant increase in the viable counts of PB in the mixed culture after 30 h growth in the absence of extra NaCl but there was a significant increase in the presence of extra NaCl (Figures 3a and 4a). It was also of interest that both cultures produced almost the same amount of growth when grown together as when they grew separately in the same medium. The second question, is perhaps more difficult to answer, why did the presence of the LAB abolish the inhibitory effect of the extra NaCl on the growth of PB? Perhaps some compound present in the growth medium becomes more inhibitory to PB when the NaCl concentration is increased and the LAB removes this compound. Further work would be required to identify the reasons.

Conclusion

It seems that there could be a number of advantages in cultivating the LAB and PB as a co-culture. The metabolic products produced by the LAB would not reduce the pH and the PB would grow much better in saline conditions. We are now further investigating if this can be applied to producing SSPs from rice straw inoculating with L35 and tk123 for use as bio-fertilizers, particularly in saline soil.

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