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Whey Based Culture Media for The Production of Selenium Nanoparticles Rich Product by Three Lactic Acid Bacterial Strains

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THE ABILITY of three LAB strains namely, *L. brevis*, *L. plantarum* and *P. acidilactici* to grow and produce SeNPs in MRS or whey media supplemented with 100 or 200 ppm of Se(IV) as sodium selenite (Na_2SeO_3) was investigated. The presence of Se(IV) in the media retarded the bacterial growth in the first six hours of incubation thereafter, the bacterial growth gradually increased up to 72 hr. *P. acidilactici* was more tolerable to the inhibitory effect of Se(IV) than the other two strains. Although MRS media was more suitable for the growth of the three LAB strains, as estimated by the reduction of media pH and increase of absorbance compared to whey media, both media showed significantly similar conversion rate (98-100%) of Se(IV) to SeNPs at the end of incubation period (72 hr). *L. brevis*, produced smaller SeNPs (125.7 nm) followed by *L. plantarum* (140.7 nm) and *P. acidilactici* (176.6 nm). The obtained data demonstrate the possibility of using the whey as a source of low-cost culture media for the production of milkwhey based product rich in SeNPs with the assistant of the environmental friendly LABfor different usage, i.e. as a feed supplement for livestock production.

Keywords: *L. brevis*; *L. plantarum*; *P. acidilactici*; Sodium selenite; Whey; MRS; Nanoselenium

Introduction

Selenium (Se) is a trace mineral found in the soil. It naturally appears in water and some foods. Although people need a very small amount of selenium, it plays a key role in the metabolism. It plays critical roles in reproduction, thyroid hormone metabolism, DNA synthesis, and protection from oxidative damage and infection (Sunde, 2012, Terry and Diamond, 2012). The recommended daily allowance of Se for male and female is set to be 15 µg for babies from birth up to 6 months old, 20 µg from 7 months up to 3 years old, 30 µg from 4-8 years old, 40 µg from 9-13 years old and 55 µg for more than 14 years old. However, an increase of 15 µg / day is recommended for pregnant and lactating women (FNB,2000). Selenium exists in two forms, inorganic (selenate and selenite) and organic (selenomethionine and selenocysteine) (Sunde, 2012). Both forms can be good dietary sources of selenium (Elkholy et al., 2019 and Terry & Diamond, 2012). Soils contain inorganic selenites and selenates that plants accumulate and convert to organic forms, mostly selenocysteine and selenomethionine and their methylated derivatives (Ježek, et al., 2012).

Nano-Se (nano-elemental Se) is another form of inorganic Se made for utilize as food supplements and in therapeutic treatment. It is bright red, highly stable, and of nano-size in the redox state of zero (Se^0). There are several methods to obtain selenium nanoparticles (SeNPs). It can be chemically synthesized (Zhang et al., 2004b) or through physical procedures (Quintana*et al*., 2002) or by biological way, the so-called green synthesis, using microorganisms or plant extracts, (Prokisch and Zommara, 2011, Ramamurthy et al., 2013, Shoeibi and Mashreghi, 2017). The green synthesis of SeNPs using microorganisms takes more attention for its simplicity, high purity, producing of a uniform and stable SeNPs.

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Several species of lactic acid bacteria (LAB) are able to convert the potentially poisonous selenite, as sodium salt (Na_2SeO_3), to the form of selenium nanospheres (SeNPs) within the cell and in the surrounding media (Eszenyi et al., 2011, Prokisch and Zommara, 2011). Nanoselenium was found to be non-toxic and has better bioavailability (Zhang et al., 2008, Shi et al., 2011 a&b). *Pediococcus acidilactici* is a species of Gram-positive cocci that is often found in pairs or tetrads. *P. acidilactici* is a homofermentative bacterium that can grow in a wide range of pH, temperature, and osmotic pressure, therefore being able to colonize the digestive tract. *Lactobacillus brevis* is a gram-positive, rod shaped species of lactic acid bacteria which is heterofermentive, creating CO_2 and lactic acid during fermentation. *L. brevis* is a microaerophilic, obligately heterofermentative lactic acid bacterium isolated from many different environments. *Lactobacillus plantarum* is a widespread member of the genus L actobacillus, commonly found in many fermented food products as well as anaerobic plant matter. It is also present in saliva (from which it was first isolated). *L. plantarum* is Gram positive, bacilli shaped bacterium occurring singly, in pairs or in short chains. Several potential health benefits have been attributed to the consumption of products containing probiotic strains of *P. acidilactici* (Barbosa et al., 2015), *L. brevis* (Ronka et al., 2003) and *L. plantarum* (Manzoora and Tayyeb, 2019).

The aim of the present study was to investigate the ability of the three previously mentioned LAB strains cultivated in MRS or whey media to produce SeNPs from sodium selenite Se(IV). The production efficiency and SeNPs shape and size were also investigated.

Materials and Methods

Bacterial strains

Pure lyophilized culture of *Pediococcus acidilactici* (*P. acidilactici*), *Lactobacillus brevis* (*L. brevis*) and *Lactobacillus plantarum* (*L. plantarum*) strains was obtained from Microbiological Resource Centre, Ain Shams University (MIRCEN), Cairo. The bacterial strains were confirmed by API identification kit using VITEK 2® compact systems, USA and also by16S ribosomal RNA gene sequence analysis.

Cultivation of bacterial cultures with selenium

The bacterial cultures (2%) with about 10^5cfu/ml were individually cultivated in MRS broth medium (De-Man et al., 1960) or milk whey (Kar and Misra, 1999) medium fortified with 1% yeast extract and 0.1% skim milk powder (Parente and Zottola, 1991). The media was amended with 100 or 200 ppm of filter sterilized (Sartorius AG, Germany) sodium selenite, Na_2SeO_3. $5H_2O$ [Se(IV)] (Sigma-Aldrich, Switzerland) and incubated at 37°C up to 72 hr.

Bacterial growth measurement

The bacterial growth in the cultured media was monitored at 2 hr. intervals for 12 hr. and then after 24, 48 and 72 hr. of incubation. The bacterial growth was estimated by measuring the absorbance at 650 nm (Ayad et al., 2004) and determination the pH value (3020 Jenway, England) of the cultured media.

Selenium determination

Selenium determination was carried out according to the method previously described by Zommara et al. (2007). One ml Se containing medium or five ml of medium supernatant (after centrifugation at 7000 rpm for 20 min.) samples were used for total selenium determination in heat-resistant glass digestion tubes. To each tube, 10 ml of 65% nitric acid were added and heated at 60°C for 30 min. using digestion block (KJELDATHERM®, Gerhardt, Germany). Then, 3 ml of 30% hydrogen peroxide (Merck, Germany) were added and digestion was continued at 120 °C for 90 min. and then cooled to room temperature. Samples were diluted with Milli-Q water, filtered using filter paper (Macherey-Nagel, Germany) and quantitatively transferred to 25 ml volumetric flasks. Selenium concentration in the diluted digested samples was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Prodigy7, Teledyne Leeman Labs, USA).

Conversion rate of Se(IV) to SeNPs

The conversion rate (%) of Se(IV) to SeNPs by different bacterial strains was estimated by measuring the residual Se(IV) in the cultured media supernatant after centrifugation at 7000 rpm for 20 min. as the following:

% of conversion = (Initial Se conc. in the medium - Se conc. in the supernatant) / Initial Se conc. in the medium X 100

Purification of SeNPs

To obtain purified SeNPs, cultured media (after 72 hr. of incubation) was subjected to centrifugation at 7000 rpm for 20 min. to separate the bacterial cells along with SeNPs. The separated pellets were washed twice with distilled water and finally suspended in suitable volume of distilled

water. Then 1.5x concentrated hydrochloric acid (37% HCl) was added to the obtained suspension (i.e. 15 ml acid to 10 ml sample) and kept for 5 days at room temperature to digest the bacterial cell walls. To get rid of the acid, the samples were centrifuged at 7000 rpm for 20 min. and washed several times with distilled water until its pH returns to neutral (Eszenyi et al., 2011).

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and SeNPs size determination

The SEM (JSM-IT100, JEOL Co. Japan) and TEM (JEM-2100, JEOL Co. Japan) photos of the cultured media after 72 hr. of incubation and purified SeNPs size determination were carried out according to Nagy et al. (2016).

Statistical analysis

The Duncan's test (at $P<0.05$) were carried out using the SPSS program (version 16) (2007), SPSS Inc., Chicago, IL, USA.

Results and Discussion

Bacterial growth and SeNPs production in MRS medium

Data illustrated in Fig. 1 show the effect of Se(IV) concentration (100 or 200 ppm) in MRS media on growth of LAB strains as estimated by the media reduction of pH and increase of absorbance during cultivation period of 72 hr. at 37°C. Addition of Se(IV) to the media suppressed the acidity development estimated as pH in all bacterial strains compared to control media (free of Se(IV)) and this effect increased by increasing the concentration of Se(IV) from 100 to 200 ppm. However, the *P. acidilactici* strain was more resistant to the inhibitory effect of Se(IV) compared to *L. brevis* and *L. plantarum* (Fig. 1. A, C & E). On the other hand, the media increase of absorbance (Fig. 1 B, D & F) showed somewhat different results. Although the media with added Se(IV) resulted in lower absorbance compared to control during the incubation period, the media with added 200 ppm had finally higher absorbance than that with added 100 ppm Se(IV). These results may be attributed to the developed red SeNPs in the cultivation media. The illustrated data also show that, although all media pH and absorbance had reached the plateau after 24 hr. of incubation in both of *L. brevis* (B) and *L. plantarum* (D) media, the *P. acidilactici* (F) cultivated media continued to have lower pH and higher absorbance values up to 72 hr. of incubation. These results may suggest the ability of *P. acidilactici* to withstand the inhibitory effect of Se(IV) compared to the other two strains.

Data illustrated in Fig. 2, derived from that in Fig. 1, is a comparison between the bacterial strains growth rate and SeNPs production in MRS media as affected by Se(IV) concentration.There was no significant decrease in all media pH up to 6 hr. of incubation (Fig. 2A & C) indicating inhibitory effect of Se(IV) at 100 and 200 ppm on growth of bacterial strains. Thereafter, the medium pH gradually decreased up to 24 hr. of incubation with no significant change till the end of incubation period (72 hr). However, it was found that *P. acidilactici* media had the lowest pH values than that of other strains which indicate its ability to tolerate and withstand the inhibitory effect of Se(IV) to a greater degree than that of two other strains. On the other hand, the medium supplemented with 100 ppm Se(IV) resulted in little absorption change in the media during the first six hours of incubation with respect to *L. brevis* (B) and *L. plantarum* (D) strains and then increased gradually until 24 hr without noticeable change till the end of the incubation period. However, the increase in *P. acidilactici* media absorbance started after 4 hr of incubation and gradually increased till the end of incubation period, with significant increase compared to other strains. Almost same trend was found in the media supplemented with 200 ppm Se(IV) except that the absorbance started to raise after 6 hr in all examined strains. The increase in media absorbance may be attributed to the bacterial growth (multiply) and formation of the red SeNPs inside the bacterial cells and excretion it in the cultured media.

Bacterial growth and SeNPs production in whey medium

The bacterial strains growth rate in whey media is illustrated in Fig. 3. There was gradual decrease in all media pH during the incubation period (Fig. 3. A, C & E). The whey medium was suitable for the LAB bacterial strains growth although the pH values were higher at the end of incubation period compared to MRS media (Fig. 2). In general, addition of Se(IV) to the media suppressed the bacterial growth that commensurate with Se(IV) concentration in the media. This effect was predominant in the media cultivated with *L. brevis* (A) followed by *L. plantarum* (C) and to some extent in *P. acidilactici* (E) media. The media absorbance resulted in opposite trend that was consistent with the decrease of pH. A slight increase of media absorbance was found up to 12 hr of incubation, and then tremendously increased till the end of incubation period (72hr.).

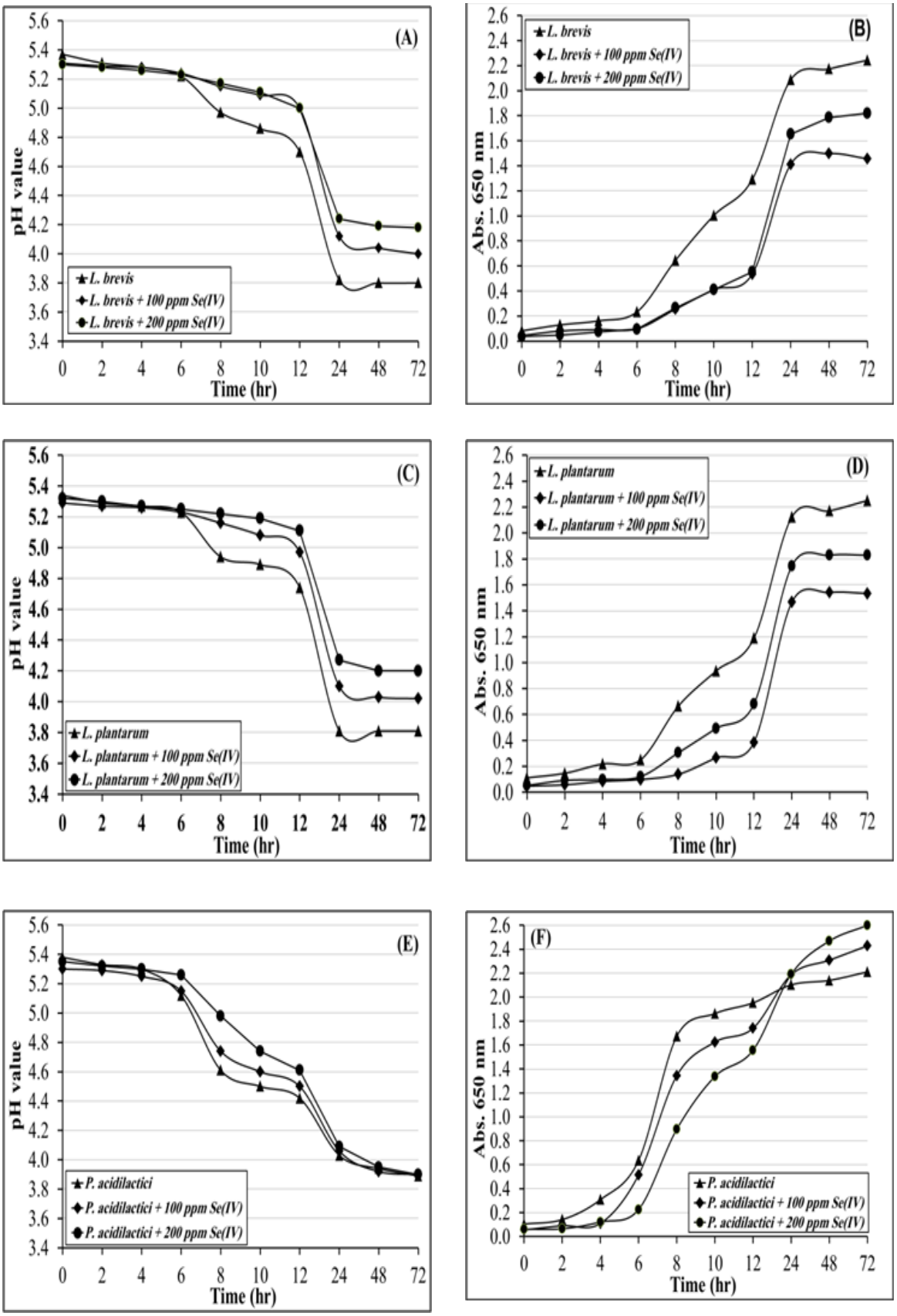

Fig. 1. Effect of Se(IV) concentration on growth of LAB strains cultivated in MRS media at 37°C for 72 hr

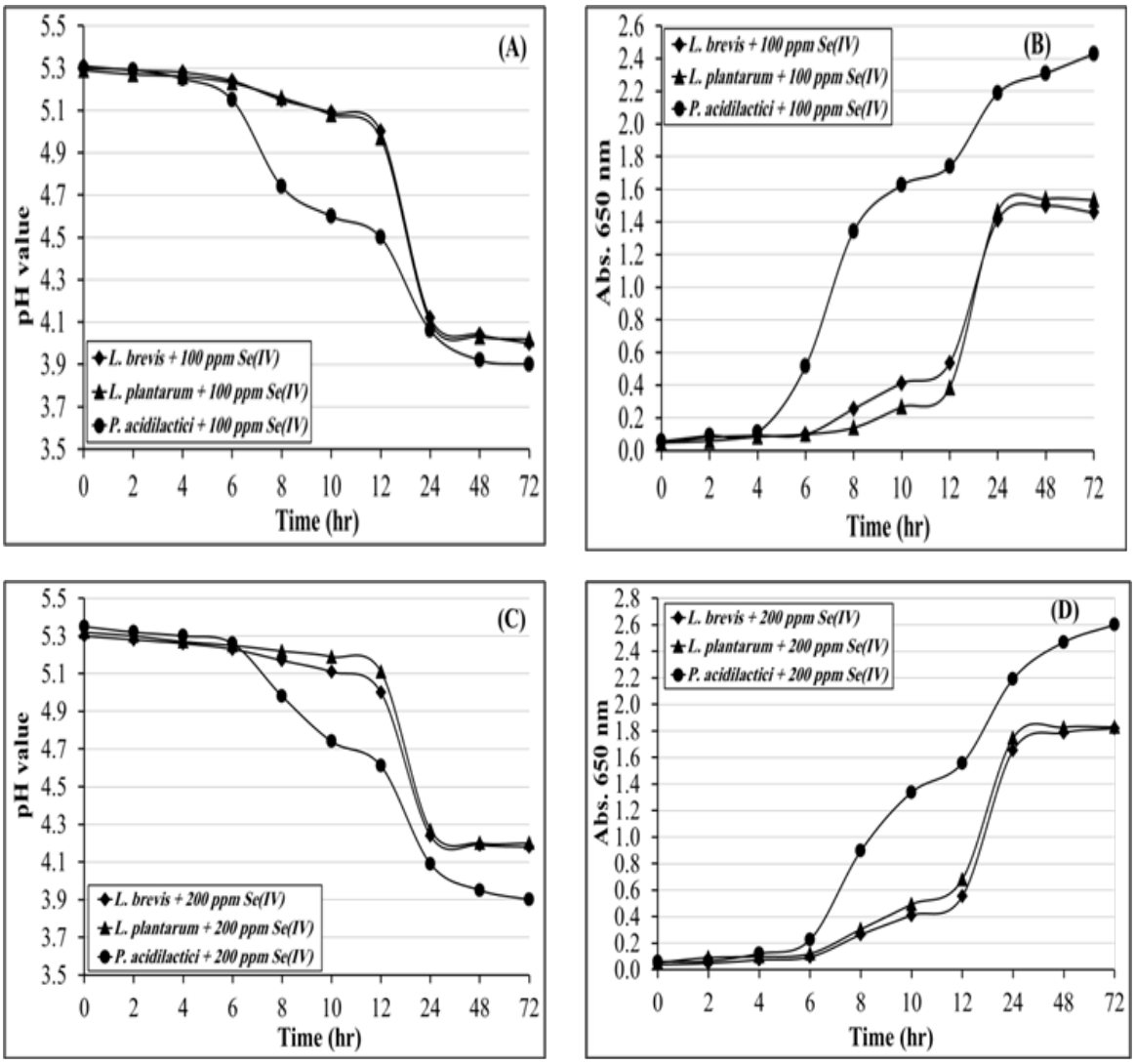

Fig. 2. Comparison of LAB strains growth rate and SeNPs production as affected by Se(IV) concentration in MRS media incubated at 37°C for 72 hr

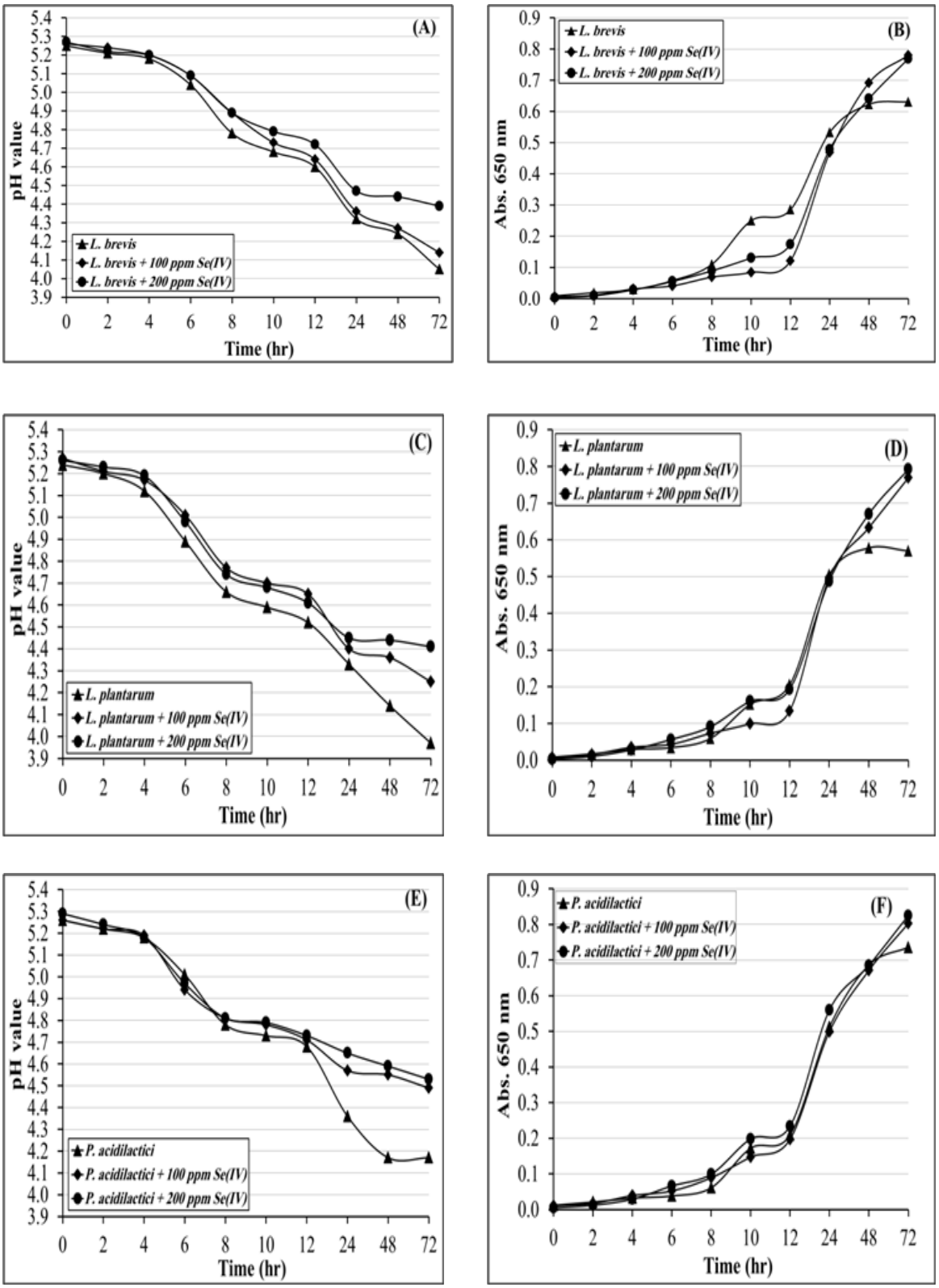

Fig. 3. Effect of Se(IV) concentration on growth of LAB strains cultivated in whey media at 37°C for 72 hr

The media cultivated with Se(IV) resulted in higher absorbance at the end of incubation period compared to Se(IV) free media (control) that may be attributed to the formation of red SeNPs during bacterial growth.

Data illustrated in Fig. 4, derived from that in Fig. 3, is a comparison between the bacterial strains growth rate and SeNPs production in whey media as affected by Se(IV) concentration. *L. brevis* and *L. plantarum* strains were more resistance to the inhibitory effect of Se(IV) than *P. acidilactici* as estimated from the decrease of media pH. However, *L. brevis* was more tolerant of the inhibitory effect of 100 ppm but not at 200 ppm Se(IV) than *L. plantarum* (Fig. 4 A &C). Parallel to the reduction of pH, the whey media absorbance slowly increased until 12 hr. of incubation and then steady increased at a higher rate till the end of incubation period. There were no significant differences in media absorbance during the incubation period among all bacterial strains in the media supplemented with 100 ppm Se(IV) (Fig. 4 B). However, the media supplemented with 200 ppm Se(IV) resulted in higher absorbance for *P. acidilactici* followed by *L. brevis* and *L. plantarum*at the end of incubation period (Fig. 4 D).

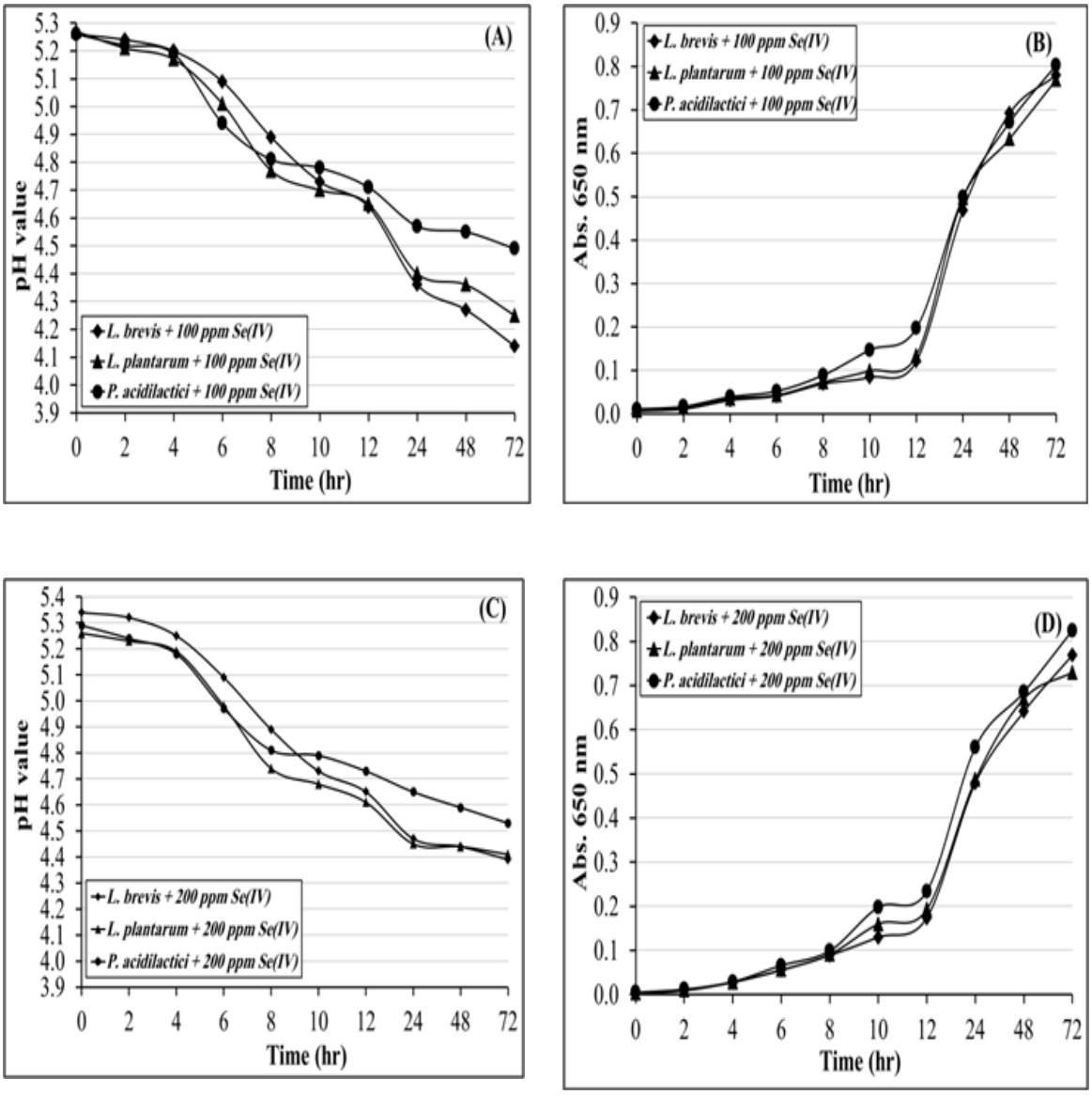

Fig. 4. Comparison of LAB strains growth rate and SeNPs production as affected by Se(IV) concentration in whey media incubated at 37°C for 72 hr

When comparing the suitability of the used two media for LAB strain growth and nano-selenium production, one can observe the superiority of MRS over the whey media (Fig. 5). All strains showed less ability to grow in whey media compared to MRS as indicated by the media reduction of pH values at the end of incubation period (4.05 vs 3.82, 3.97 vs 3.81 and 4.17 vs 3.89) for control media of *L. brevis*, *L. plantarum* and *P. acidilactici*, respectively. The whey media with added Se(IV) resulted in higher pH values (Fig. 5 A, B & C) compared to MRS media. An opposite trend was found for media absorbance. The control whey media resulted in lower absorbance values compared to MRS media (0.631 vs 2.244, 0.570 vs 2.251 and 0.836 vs 2.211) for *L. brevis*, *L. plantarum* and *P. acidilactici*, respectively (Fig. 5 D, E & F). Addition of Se(IV) significantly reduced the absorbance of whey media compared to MRS (Fig. 5 D, E & F) either in the 100 ppm Se(IV) supplemented media (0.780vs1.457, 0.769vs1.534 and 0.803vs2.429) or in the 200 ppm Se(IV) supplemented media (0.769vs1.820, 0.688vs1.829and 0.825vs2.606) for *L. brevis*, *L. plantarum* and *P. acidilactici*, respectively. These data also clearly demonstrate that the inhibitory effect of Se(IV) concentration on the ability of LAB to grow was dose dependent as indicated by obstructing the progress of acidity (pH reduction) and absorbance in the media during cultivation period.

Although the data in Fig. 5 clearly demonstrated that the preference of MRS medium over the whey medium for growth of LAB strains and formation of SeNPs, there were no significant differences between the two medium in the conversion rate of Se(IV) to SeNPs by the used strains (Table 1). As shown in Table 1, at the end of incubation period, the estimated conversion rate of Se(IV) to SeNPs ranged from 98.2%-100% and 97.6%-99.5% for 100 and 200 ppm of Se(IV) in MRS media, respectively. On the other hand, these figures were 100% and 98.3-99.3% for the whey media with no significant differences among all strains. Therefore, it could be concluded that whey media can be used effectively to obtain a SeNPs rich product containing non or traces of the inorganic Se(IV).

The high economic cost of using the MRS medium compared to whey medium should also be taken into consideration. Huge amounts of unsalted milk whey produced as a by-product in cheese industry. Using such whey in the green production of SeNPs by lactic acid bacteria will reduce the environmental pollution on one hand and produce a substance of great economic importance at a lower cost on the other hand. These findings also give the advantage to use the one-step spry-drier technique rather than centrifugation and drying process to obtain concentrated dry SeNPs rich whey products for commercial usage, i.e. as feed supplement for animal and poultry nutrition (Shi et al., 2011a&b, Cai et al., 2012, El-Deep et al., 2016, Sarkar et al., 2015, Zommara, et al., 2018, Dawood et al., 2020).

The data in Table 2 show the average particle size of the obtained SeNPs by the examined bacterial strains. *L. brevis* produced the smallest particles ranged from 47-250 nm with an average of 125.7 nm followed by *L. plantarum* 65-244 nm with an average of 140.4 nm and *P. acidilactici* 90-278 nm with an average of 176.6 nm. It is clear that the *Lactobacilli* strains produce smaller SeNPs than the cocci shaped *P. acidilactici* (Table 2). There are several factors that affect SeNPs size, i.e. bacterial strain, cultivating medium and pH. Eszenyi et al. (2011) stated that *Lactobacillus* sp., *Bifidobacterium* sp. and *Streptococcus thermophiles* produces SeNPs in the range of 100-200, 400-500 and 50-100 nm, respectively when cultivated in milk.

Table 3 shows the SeNPs size distribution (%) as affected by the used strain type. The data clarify that the strains produced different sizes of SeNPs. However, 39.6 % of the size of SeNPs produced by *L. brevis* was less than 100 nm followed by *L. plantarum* (22.6%) and *P.* acidilactici (8.6%). The nanoparticles size plays a crucial role in their bioactivity, the smaller size nanoparticle the more active one (Shegokar, 2015). However, Zhang et al. (2004a) stated that no significant size effect of SeNPs (5-200 nm) on the induction of some seleno-enzymes in mice and liver HepG2 cell line. Therefore, SeNPs size may not be the factor that limits its usage as a supplement in animal nutrition. Feeding Japanese quails on diet supplemented with SeNPs based whey product prepared by yoghurt culture, had no deleterious effect on bird's growth parameters and had biological and nutritional properties comparable to that obtained by the commercially available organic selenium Selplex® (Zommara et al., 2018).

The SEM and TEM photos of LAB strains cultivated in MRS media are shown in Fig. 6. The Se(IV) free cultures are shown in Fig. 6 A, however, Fig. 6 B shows the accumulation of SeNPs inside the bacterial cells and the photos of purified SeNPs produced by different strains are shown in Fig. 6 C.

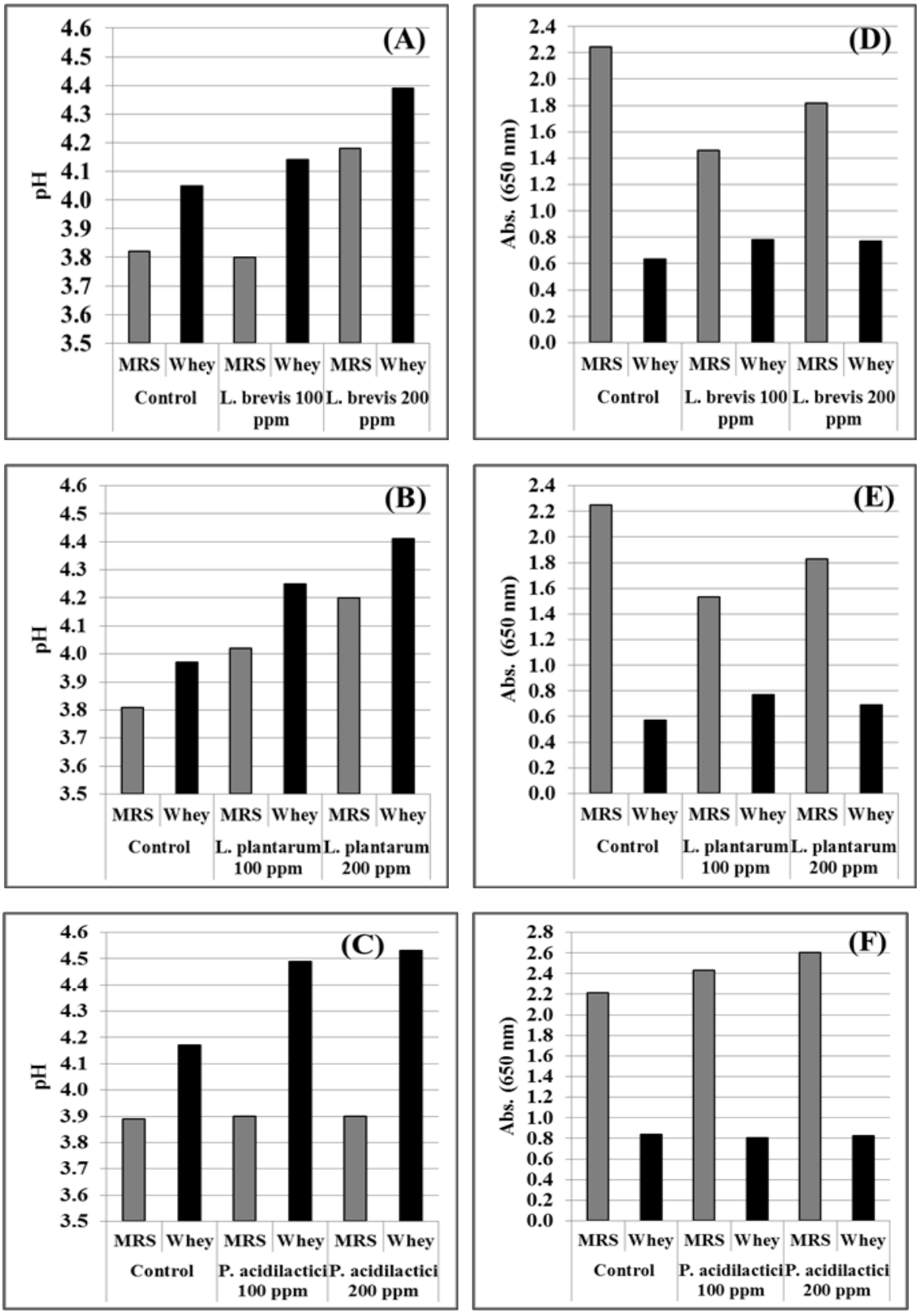

Fig. 5. Effect of culture media on LAB bacterial strains growth as estimated by pH reduction and media absorbance after 72 hr. of incubation at 37°C

TABLE 1. Conversion rate of selenite Se(IV) to SeNPs by LAB strains in different cultivation media after 72 hr of incubation at 37 °C

Cultivation medium	Bacterial strain	Medium Se(IV) concentration (ppm)	Conversion rate (%)
MRS	*Pediococcusacidilactici*	100	100
		200	99.5
	Lactobacillus brevis	100	98.2
		200	97.6
	Lactobacillus plantarum	100	100
		200	99.3
Whey media	*Pediococcusacidilactici*	100	100
		200	99.3
	Lactobacillus brevis	100	100
		200	98.3
	Lactobacillus plantarum	100	100
		200	99.2

TABLE 2. SeNPs size (nm) produced by LAB strains cultivated in MRS media with 100 ppm of Se (IV) after 72 hr of incubation at 37 °C

LAB strain	Min.	Max.	Average	SD	SE
L. brevis	47	250	125.7[a]	45.5	4.9
L. plantarum	65	244	140.4[b]	48.3	6.1
P. acidilactici	90	278	176.6[b]	49.7	6.5

Data are mean ± SE for 3 replicates with 20 SeNPs each

a,b,c Means with unlike superscripts within column are significantly different at $P< 0.05$.

TABLE 3. Size distribution (%) of SeNPs produced by LAB strains cultivated in MRS media with 100 ppm of Se (IV) after 72 hr of incubation at 37 °C

LAB strain	SeNPs size distribution (%)				
	≤ 100 nm	> 100 nm	100-150 nm	150-200 nm	>200 nm
P. acidilactici	8.6	91.4	31.1	36.2	24.1
L. brevis	39.6	60.4	33.7	20.9	5.8
L. plantarum	22.6	77.4	35.5	29	12.9

The photos show the ability of LAB strains to produce uniform monoclonal crystal of SeNPs. Although the SeNPs produced by the so called green synthesis using different LAB strains gives larger particle size compared to the chemically produced one, it still has priority for its uniform monoclonal crystal shape, abundance of production and safety (Prokisch et al., 2008, Benko et al., 2012, Nagy et al., 2016, Moreno-Martin et al., 2017). In our previous studies we found that several species of LAB were able to accumulate organic Se along with SeNPS within their cells or in the cultured media when cultivated with different concentrations of Se(IV) in suitable media or milk (Prokish and Zommara, 2011, Zommara and Prokisch, 2015, 2019. Eszenyi et al (2011) found that "LactomicroSel" a nanoselenium rich milk product produced by a mixture of *L. acidophilus*, *L. casei* and *S. thermophiles* cultivated in skim milk supplemented with 200 ppm sodium hydrogen selenite ($NaHSeO_3$) contains >95% of Se in the form of nanoparticles and <5% as organic Se.

In conclusion, we have found that certain species of LAB can accumulate spherical elemental Se nanospheres having a median diameter within the range of about 50-280 nm when cultivated in MRS or whey medium with added 100 or 200 ppm selenium in the form of selenite ions Se(IV). The bacteria reduce selenite and excrete Se intracellularly as elemental form through detoxification processes. The obtained SeNPs rich milk whey product could be a promising feed supplement. Although several studies recommended the use of SeNPs in livestock nutrition, further investigations with different experimental animal models still in need to confirm its safety and physiological effects.

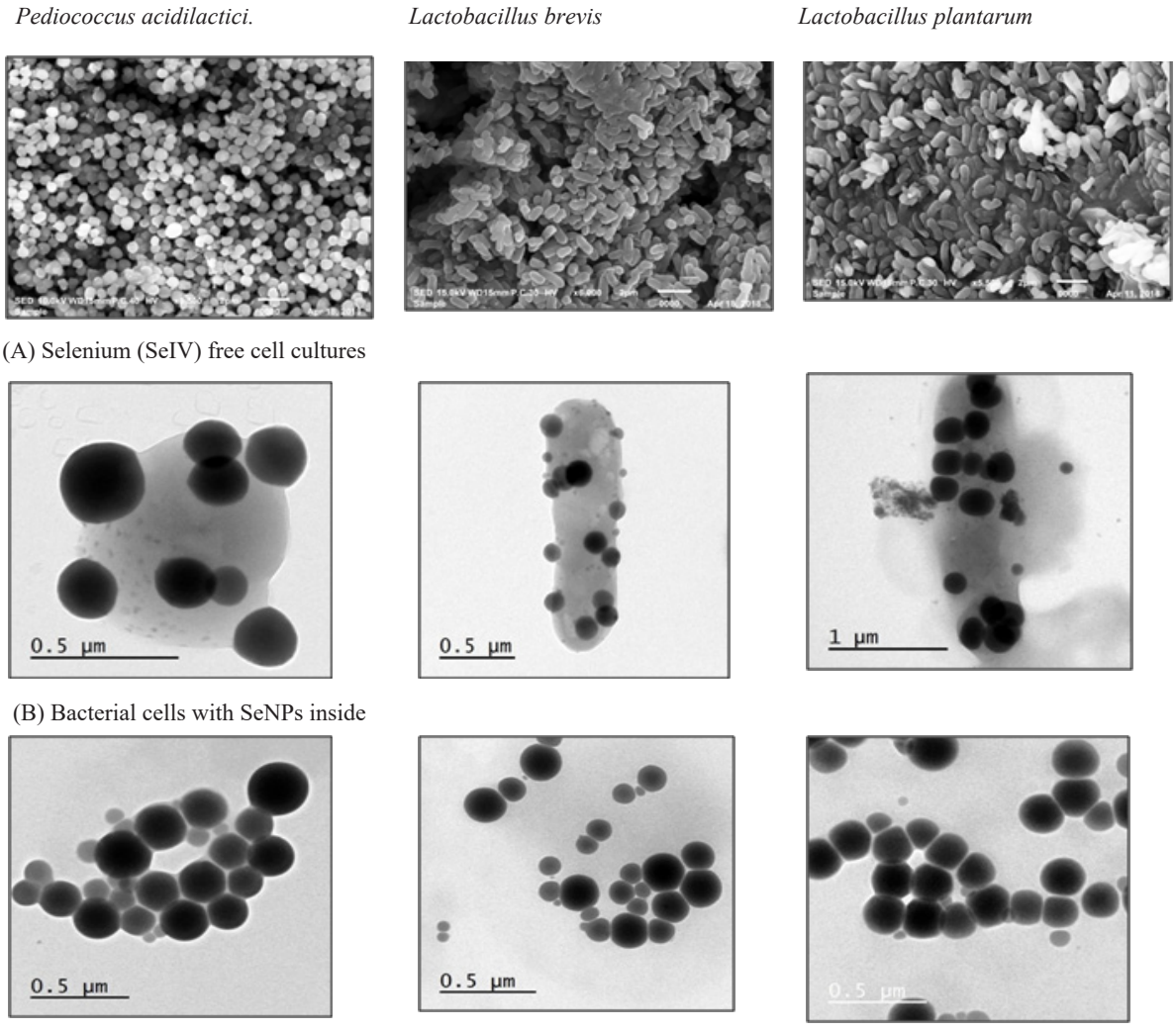

Fig. 6. Scanning electron microscope (SEM) photos of LAB bacterial strain cultures (A) and transmission electron microscope (TEM) photos of a single bacterial cell with SeNPs inside (B) and purified SeNPs produced by different bacterial strain cultivated in MRS media at 37°C for 72 hr

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بيئة الشرش لأنتاج منتج غنى بحبيبات السيلنيوم النانوميترية بواسطة ٣ سلالات من بكتريا حامض اللاكتيك

فى هذه الدراسة تم التحقق من مقدرة ثلاث سلالات من بكتريا حامض اللاكتيك *L. brevis* و *L. plantarum* و *P. acidilactici* على النمو وإنتاج حبيبات السيلنيوم النانوميترية في بيئات MRS أو شرش اللبن المضاف لها ١٠٠ أو ٢٠٠ جزء في المليون سيلنيوم على هيئة سيلينيت الصوديوم (Na_2SeO_3). أدى وجود السيلنيوم في البيئات إلى إعاقة النمو البكتيري خاصة في الساعات الست الأولى من التحضين، بعد ذلك زاد النمو البكتيري تدريجيًا حتى ٧٢ ساعة، كما هو مقدر من انخفاض رقم الـ pH وزيادة الامتصاصية (Absorbance) فى البيئات. أوضحت النتائج أن السلالة *P. acidilactici* أكثر تحملاً للتأثير المثبط للسيلنيوم عن السلالتين الأخريين. على الرغم من أن بيئة MRS كانت أكثر ملاءمة لنمو السلالات الثلاثة مقارنة ببيئة شرش اللبن ، أظهرت كلا البيئتان معدل تحويل مشابهًا بشكل ملحوظ (٩٨-١٠٠٪) للسيلنيوم إلى حبيبات السيلنيوم النانوميترية في نهاية فترة الحضانة (٧٢ ساعة).أنتجت السلالة *L. brevis* حبيبات سيلنيوم نانوميترية أصغر بمتوسط بلغ ١٢٥,٧ نانومتر من تلك المنتجة بواسطة السلالة) *L. plantarum* ١٤٠,٧ نانومتر) والسلالة *P. acidilactici* (١٧٦,٦ نانومتر). توضح النتائج التي تم الحصول عليها إمكانية استخدام مصل اللبن كمصدر لبيئة نمو منخفضة التكلفة لإنتاج منتج من شرش اللبن غني بحبيبات السيلنيوم النانوميترية بمساعدة سلالات من بكتريا حامض اللاكتيك الصديقة للبيئة لاستخدامات مختلفة ومنها على سبيل المثال كمكمل غذائى للعلائق فى مجال الأنتاج الحيوانى.