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Full Length Research Paper

Effect of extracellular calcium chloride on sporangiospore-yeast transformation of *Rhizopus stolonifer*

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Previous studies showed that zinc and myoinositol, which are intracellular, serve as components of signalling elements in many eukaryotes, and participate in stimulating the induction and proliferation of yeast cells from sporangiospores of fungi. It was thought that a transmembrane-pH-gradient, similar to what is obtained in bacterial mitochondrion or proton-substrate symport in yeasts, permitted influx/efflux of materials into the cell that triggered the induction and subsequent proliferation of yeast cells. To examine this model further, this study evaluated the ability of sporangiospores of *Rhizopus stolonifer* to undergo morphogenetic transformation in the presence of different levels of extracellular calcium (0.0, 0.20, 0.25, 0.50, 1.0, 1.5 and 1.8 mM). It was found that calcium supported yeast induction and proliferation to varying extent. Ca^{2+} at 0.50 and1.8 mM, which was outstanding, was compared with broth treatment at 0.25 mM $ZnSO_4$: pH 4.5 which gave optimum biomass in a previous study. Medium pH was further varied: pH 4.2, 4.5 and 5.0. The results showed that there was interaction between the level of divalent cations and pH. At all levels tested, Ca^{2+} was better stimulatory than Zn^{2+}. Direct induced yeast cell count, which eliminated possible impact of protoplasts and calcium phosphate precipitate on optical density measurements, made these results more valid. Treatment at 0.50 mM Ca^{2+}: pH 5.0 gave the most profound result. This was therefore chosen for further biochemical work.

Key words: *Rhizopus stolonifer*, synthetic broth, dimorphism, extracellular Ca^{2+}, sporangiospores, induced yeast cells.

INTRODUCTION

Dimorphism is the conversion of a microorganism from one growth habit to another, an effect caused by changes in environmental conditions (Romano, 1966). In a report presented over a decade ago, it was shown that sporangiospores of a mucoraceous strain is transformed to terminal budding yeast cells (Omoifo, 1996). This was opposite the multipolar budding yeast-like morphology that has been reported for many mucoraceous species including *Mucor rouxii* (Bartnicki-Garcia, 1968; Bartnicki-Garcia and Nickerson, 1962a, b, c, d; Lubberhusen et al., 2003; McIntyre et al., 2002), *Mycotypha* (Hall and Kolankaya, 1974; Schulz et al., 1974; Price et al., 1973).

The preceding report showed that several distinct morphologies can be induced from the fungus, *Rhizopus stolonifer*. These include yeast cells, coenocytic hyphae, moniliform hyphae and septate hyphae. This is in contrast to the multipolar budding yeast-like cells that have been reported for other members of the Zygomycetes. It is also in sharp contrast to the study of Bartnicki-Garcia and Nickerson (1962b) whereby it was shown that *R. stolonifer* was incapable of morphogenetic interconversion.

Conversion of sporangiospores to terminal budding yeast cells require structural modifications that involved transient morphologies, including growth spheres and cytosolic nucleates that are released when cell wall is lysed, and protoplasts till the yeast is formed. This becomes the stable form, which is proliferated by terminal budding. These various forms have been shown in several publications (Omoifo, 1996, 1997, 2003; Omoifo and Omamor, 2005; Omoifo et al., 2006) including the research monograph “development of yeast cells from sporangiospores” (Omoifo, 2005).

In an attempt to explain the cryptic biophysical changes that occurred leading to the formation of yeast, a system model known as the sequential sporangiospore-yeast

transformation (SSYT) hypothesis was formulated (Omoifo, 2003). This was based on electrochemical gradient which permits transport processes and energy metabolism in mitochondria or proton-substrate symport through plasma membranes (Alderman and Hofer, 1981; Albert et al., 1994; Dawes, 1986; Mitchell, 1967; Slayman and Slayman, 1974; Voet and Voet, 1995; West and Mitchell, 1972; 1973). It holds that the multiple conversions are possible because the individual entities are bathed in a milieu whereby a transmembrane-pH-gradient is set up and this encourages influx/efflux of materials with consequent fundamental biochemical and biophysical changes in cytosolic components and cell wall integrity. It was shown that monovalent elements like H^+, K^+ and Na^+, play important roles in the induction of terminal budding yeast cells from sporangiospores of muco-raceous strains. H^+ was involved in the maintenance of the transmembrane-pH-gradient; K^+ was absolutely necessary for the formation of protoplasts from spora-ngiospores of *Mucor circinelloides* Tieghem (Omoifo, 2005). On the other hand, accumulation of Na^+, which is antiported with K^+ (Tonomura, 1986) was necessary for the protoplasts to convert to the yeast form (Omoifo, 2005). The effect of K^+ was confirmed in another study where protoplast formation peaked at a high level of K^+ 1.0 g/l, and yeast induction was optimal at 0.10 g/l Na^+. In a combinational manner under various treatments, the interaction between 1.0 g/l K+ and 0.1 g/l Na^+ had the greatest impact on yeast induction, notwithstanding the occurrence of other anamorphic subtypes including enterothallic and holothallic conidia, which were scanty. However, these anamorphs were eliminated when 100 mM uracil was incorporated into the medium of growth (Omoifo, 2006a).

Subsequent study showed the effect of intracellular signalling precursors on yeast induction from sporrangiospores. Although, myoinositol and zinc were not the primary initiators, they individually enhanced yeast induction from sporangiospores (Omoifo and Aruna, 2006; Omoifo et al., 2006). When the cyclic hexitol and divalent ion were used in combination, induced yeast sigmoid growth pattern ensued and biomass was upped by 63 to 65% in comparison with control cultures (Omoifo, 2006b). Calcium is a primary element in signal transduction in eukaryotic cells where it's binding to proteins triggers changes in conformation as well as altering local electrostatic fields, thereby controlling cellular processes (Kretsinger, 1979; Albert et al., 1994; Berridge, 1987; Berridge et al., 2000; Voet and Voet, 1995; Clapham, 2007). Since zinc which plays key role in catalytic and structural component of many proteins (Eide, 2006) has been shown to enhance yeast induction from sporangiospores, perhaps calcium, which functions as an intracellular messenger (Albert et al., 1994) and further involved in cytosolic Ca^{2+} homeostasis (Forster and Kane, 2000; Ton and Rao, 2004), would also promote yeast induction from sporangiospores.

The objective of this study was to evaluate the effect of $CaCl_2$ on the ability of *R. stolonifer* to convert to terminal budding yeast cells. Since exogenous culture pH influence Ca^{2+} uptake through Ca^{2+} channels in plasma membrane (Youatt, 1990), the effect of pH on the stimulatory ability was also evaluated.

MATERIALS AND METHODS

Chemicals

Reagent grade chemicals were obtained from BDH Laboratory supplies (Poole, UK) except Bacto peptone (Sigma), myoinositol (Sigma) and yeast extract (Oxoid).

Fungal strain

R. stolonifer used in this study was isolated at the Microbial Type Culture Collection Laboratory 1, Institute of Microbial Technology, Chandigarh India as reported in the preceding report. It was maintained on glucose-yeast extract-peptone agar (GYPA: 10-0.3-5.0-15g/l) slants. Fresh culture was prepared on Petri dish unto which 20 ml of agar was poured and used after 7days of growth.

Inoculum preparation for growth studies

A 7 day old culture of *R. stolonifer* was flushed with sterile deionized distilled water and a sterile glass rod was gently passed over the fungal growth so as to dislodge the spores. The suspension, poured into centrifuge bottles, was washed with 3 changes of sterile deionized distilled water in an MSE 18 centrifuge at 5000 rpm for 7 min at 25°C. Spore count was taken with a Neubauer hemocytometer and was made up to 10^6/ml in sterile deionized distilled water.

Reagents and culture media

Procedure for preparing the base medium has been described (Omoifo, 1996) and has been used in subsequent studies (Omoifo, 2003, 2006a, b; Omoifo and Aruna, 2005; Omoifo et al., 2006). It contained per litre: 10 g glucose, 5 g $(NH_4)_2SO_4$, 5 g K_2HPO_4, 5 g KH_2PO_4, 0.10 g NaCl, 0.10 g $FeSO_4$, 2.0 g $MgSO_4$, 0.060 g $CuSO_4$, 0.060 g $ZnSO_4$, 0.065 g $MnCl_2$, 100 mg uracil and 2 Mm myoinositol. Where the effect of Zn^{2+} was to be tested, zinc sulphate was not added to the basal preparation. The initial culture was buffered at Ph 4.5 with 0.2 M Na_2HPO_4: 0.1 M citrate. When Ph level was the test factor, culture was then buffered with equivalent weights of the buffer species. Since levels of calcium were to be tested, these were prepared separately. Each of the duplicate flasks of the synthetic broth was incorporated with the various concentrations of calcium chloride at the following levels: 0.0, 0.20, 0.25, 0.50, 1.0, 1.5 and 1.8 Mm. The Ph was adjusted with 2 N NaOH or 1 N HCl using a Cole-Parmer Ph Tester model 59000. The solution in each flask was made up to 100 ml with glass distilled deionized water and sterilized at 121°C for 15 min.

Inoculation, growth conditions and sample collection

A 1 ml spore suspension was inoculated into each broth flask using Eppendorf micropipette in an operation done in a laminar flow chamber model CRC HSB-60-180. Before each inoculation, the

Table 1. Treatments and biomass estimates with their respective standard errors, and form of growth during the cultivation of *R. stolonifer* in synthetic broth for 120 h at Ph 4.5 and temperature 28°C.

Treatment: Calcium chloride (Mm)	Mean biomass at 620 nm	Form of growth
Control	0.1399 ± 0.0075	Yeast cell
0.2	0.1361 ± 0.0062	Yeast cell
0.25	0.1462 ± 0.0107	Yeast cell
0.5	0.1666 ± 0.0095	Yeast cell
1.0	0.1381 ± 0.0153	Yeast cell
1.5	0.1360 ± 0.0086	Yeast cell
1.8	0.1854 ± 0.0418, turbidity	Yeast cell

spores were kept in suspension by shaking for 30 s and the broths were similarly shaken after each inoculation before transferring to a laboratory side bench for incubation at 28 ± 1°C. At 24 h intervals, the culture flasks were brought to the chamber and 10 ml were withdrawn from each culture flask with sterile pipettes, one for each flask, into factory-sterilized plastic sample tubes. The culture flasks were thereafter returned for further incubation. The samples were kept at -18°C until further analysis. Slides were prepared for viewing by putting one or two drops of lactophenol-in-cotton blue on a glass slide and with the aid of wire loop, one drop from the culture flask properly shaken was then added and covered with a glass cover slip and viewed under a binocular microscope at x1000 magnification using the microscope Olympus CHB14. The observed morphologies were recorded.

Biomass determination

Culture samples were thawed up to room temperature before biomass determination. The absorbance was obtained at 620 nm, using a Camspec M105 spectrophotometer (Cambridge, UK).

Statistical analysis

Results were subjected to a 2-way analysis of variance (ANOVA) test for the single factor, or a split-plot format for combined factors and considered significant if $p < 0.05$ and comparison between means was performed using the Genstat 5 package.

RESULTS AND DISCUSSION

The effect of various levels of Ca^{2+} on morphological expression of *R. stolonifer* cultivated in still cultures is shown in Table 1. Yeast cell was the predominant morphology. In other dimorphic fungi, *Ceratocystyis ulmi* (Paranjape et al., 1990) and *Ophiostoma ulmi* (Gadd and Brunton, 1992), exogenous Ca^{2+} stimulated germ tube formation in media designated for yeast production, while it led to a reduction of mycelia production in *Aureobasidium pullulans* (Madi et al., 1997). Madi and colleagues attributed this difference to the use of 'restrictive medium' in the former, as compared to their use of what they termed 'general medium' for the growth of *A. pullulans*. The preceding report illustrates the fact that multiple morphologies could be induced from sporangiospores in buffered synthetic broth, but this could be streamlined to obtain specific morphology, depending on the environmental factor incorporated into the medium of growth. For example, multipolar budding yeast like cell was obtained in complex nitrogen sourced medium incubated at elevated temperature (Omoifo, 1996, 2005) and terminal budding yeast cells from simple nitrogen sourced-medium (Omoifo, 1996), or myoinositol-incorporated medium (Omoifo, 1997, 2003, 2005; Omoifo et al., 2006).

In the present study, proliferation of terminal budding yeast cells induced from sporangiospores of *R. stolonifer* occurred at all concentration levels tested. Growth profile did not describe a sigmoid pattern (Figure 1). Mean biomass showed differences in growth between cultures where control broth apparently had higher values than 0.2, 1.0 and 1.5 mM Ca^{2+} (Table 1). Analysis of variance of O.D. readings showed that only time had significant impact on yeast induction and growth (Table 2). However, on separation of means, LSD 0.04889 $p < 0.05$, the aforementioned concentration levels were not distinct except for 0.5 and 1.8 mM Ca^{2+}, which were in separate subsets (Table 3).

In this study, control culture or cultures with low level Ca^{2+} made had no difference with yeast proliferation, except when there was high level challenge, 0.5 or 1.8 mM Ca^{2+}. But the magnitude of yeast cells formed in the 1.5 mM Ca^{2+} supplemented broth compares with that of control although protoplasts were part of entities in the latter culture. This study decided to subject the Ca^{2+} levels with the higher O.D. values to different pH treatments.

The effect of extracellular pH levels on sporangiospore-yeast transformation had earlier been shown (Omoifo, 1996). In synthetic broth, pH 4.5 was found to be most ideal for conversion of sporangiospores to yeast cells (Omoifo, 1996). A subsequent report showed that buffered synthetic broth pH 4.5, supplemented with 0.25 mM $ZnSO_4$ further enhanced conversion of sporangiospore of *M. circinelloides* to yeast (Omoifo, 2006a). In the present study, Zn^{2+} was contrasted with Ca^{2+} under the optimum conditions previously determined but also varied this with a ≤0.5 unit pH differences in either direction. Cumulative mean values showed that the highest biomass was obtained at 0.5 mM Ca^{2+}

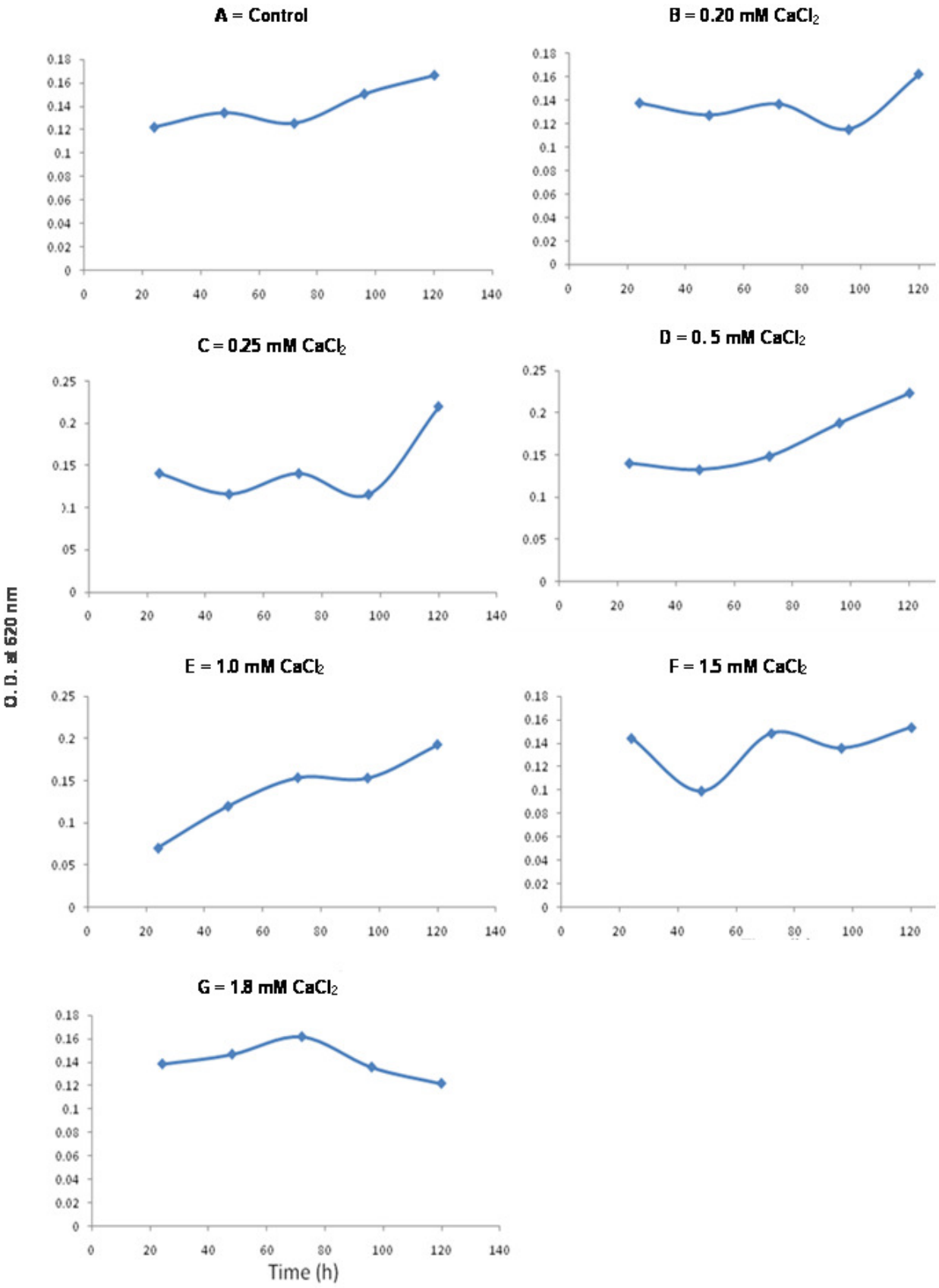

Figure 1. Biomass profiles of *R. stolonifer* cultivated in buffered synthetic broth supplemented with different levels of calcium chloride for 120 h at pH 4.5 and temperature of 20°C, ambient.

Table 2. Analysis of variance of optical density readings (620 nm) of induced yeast cells of *R. stolonifer*.

Source of variation	DF	SS	MS	F-pr.
Concentration	6	0.043517	0.007253	0.316
Time	4	0.068225	0.017056	0.029*
Concentration x time	24	0.187666	0.007819	0.192
Residual	105	0.638335	0.006079	
Total	139	0.937743		

*Significant at $p < 0.05$ probability level; DF, degree of freedom; SS, sum of square, MS, mean sum; F-pr, frequency of probability.

Table 3. Homogenous subsets of mean biomass estimates obtained during sporangiospore-yeast transformation of *R. stolonifer* cultivated in synthetic broth for 120 h with pH 4.5 at 28°C, ambient.

Treatment : Calcium chloride (mM)	Mean
Subset 1	
1.8	0.1854
Subset 2	
0.5	0.1676
Subset 3	
Control	0.1399
0.2	0.1361
0.25	0.1462
1.0	0.1381
1.5	0.1360

Means separation based on L.S.D. 0.04889 at $p < 0.05$.

supplementation but was the least with the 0.25 mM Zn^{2+} challenge which was barely lower than 1.8 mM Ca^{2+} insult (Table 4). This indicated that Ca^{2+} ion at 0.5 mM concentration was more effective in promoting yeast induction/proliferation.

To know the contribution of the various factors incorporated into the growth medium, a split-plot analysis of variance of growth data was done. It was shown that the main factors including ionic concentration, pH and time, as well as their interactions made significant impact ($p < 0.1$) on growth of *R. stolonifer* as yeast cells (Table 5). The specific contribution of pH levels to yeast proliferation is shown in Figure 2. The pH influenced yeast proliferation. At 0.5 mM Ca^{2+} concentration where optimum growth occurred, maximum growth was at pH 4.5, whereas it was at pH 5.0 when the 1.8 mM Ca^{2+} concentration (which had lower optimum values than the 0.5 mM Ca^{2+} concentration) was used. While the least biomass was recorded at 0.25 mM Zn^{2+} concentration, maximum growth with this ion also occurred at pH 5.0. But at pH4.2 where the least growth occurred for all the ionic concentrations, the 0.25 mM Zn^{2+} had higher growth value than the 1.8 mM Ca^{2+} concentration. While growth was compromised at pH 4.2 in the 1.8 mM Ca^{2+} culture, the pressure was relieved as the external pH rise. This indicated that at the cytosolic level, there was interaction between the type of ion and pH on the one hand, and the ionic concentration and pH level on the other. A comparison of biomass of the Ca^{2+}challenge at all the pH levels tested suggested that yeast induction and proliferation is more sensitive to higher level Ca^{2+} insult.

Since there is interest in conditions where determinable growth phases (lag, log and stationary) occurred, a time-course pattern should illustrate this. Induced proliferating yeast cells of *R. stolonifer* assumed sigmoid curve with 0.25 mM Zn^{2+} supplementation at the lower pH values, whereas at pH 5.0 where the biomass was higher (Figure 2), there appeared to be a resurgence of growth after the initial rapid log growth and wind-down 96 h from commencement of experiment (Figure 3a). The occurrence of sigmoid growth of yeast cells induced from sporangiospores of *M. circinelloides* cultivated in 0.25 mM Zn^{2+} supplemented pH 4.5 minimal cultures has been previously reported (Omoifo, 2006a). In that study, other

Table 4. Mean biomass of induced yeast cells from sporangiospores of *R. stolonifer* cultivated in buffered synthetic broth at different levels of exogenously applied Ca^{2+} and Zn^{2+} ions.

Parameter	Optical density at 620 nm		
	0.25 mM $ZnSO_4$	0.50 mM $CaCl_2$	1.80 mM $CaCl_2$
Mean	0.3795	0.8252	0.3941
S.E.	0.003	0.010	0.006
Fold-increment	2.2	←	→ 2.1

Table 5. Analysis of variance of growth data of *R. stolonifer* cultivated in buffered synthetic broth at different pH levels and exogenously applied ions.

Source of variation	d.f.	Sum of square	F-value
Concentration	2	7.693008	<0.001
pH	2	1.861680	<0.001
Time	4	19.055131	<0.001
Concentration x pH	4	1.028543	<0.001
Concentration x time	8	7.840491	<0.001
pH x time	8	0.354672	<0.001
Concentration x pH x time	16	1.014106	<0.001
Residual	135	0.541972	
Total	179	39.389656	

The table shows that the effect of the main factors and their interactions on the induction and proliferation of yeast cells from sporangiospores of *R. stolonifer* was statistically significant at $p < 0.001$.

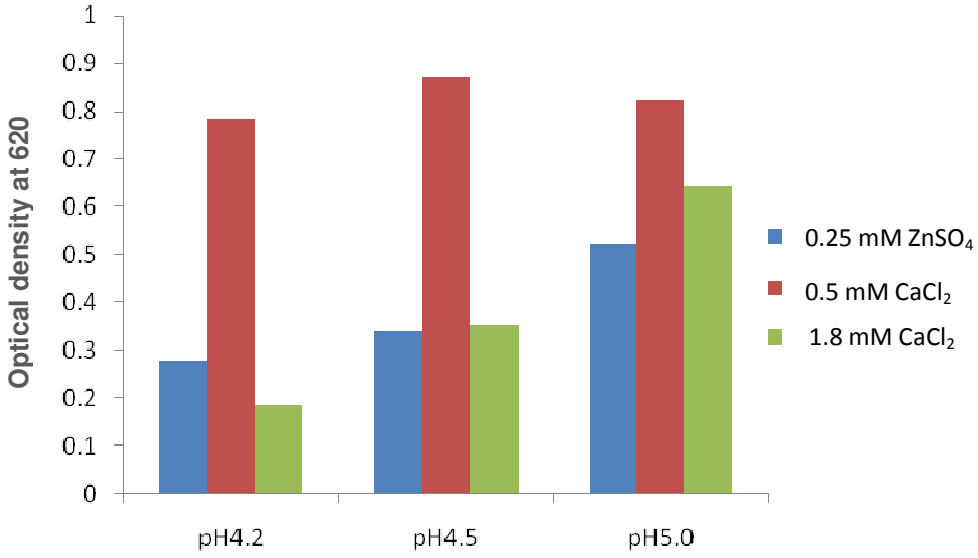

Figure 2. Growth of transformed yeast cells of *R. stolonifer* in calcium chloride and zinc sulphate incorporated buffered synthetic media as affected by different levels of pH.

determinate anamorphic features including holothallic and enterothallic subtypes were also induced especially at other levels of Zn^{2+} supplementation and this appeared to distance the growth profile from the sigmoid pattern. In the present study, although holothallic and enterothallic conidia were not observed, protoplasts and unbudded yeasts occurred at pH 5.0 in the 0.25 mM Zn^{2+} cultures. Perhaps after the initial rapid growth, these entities

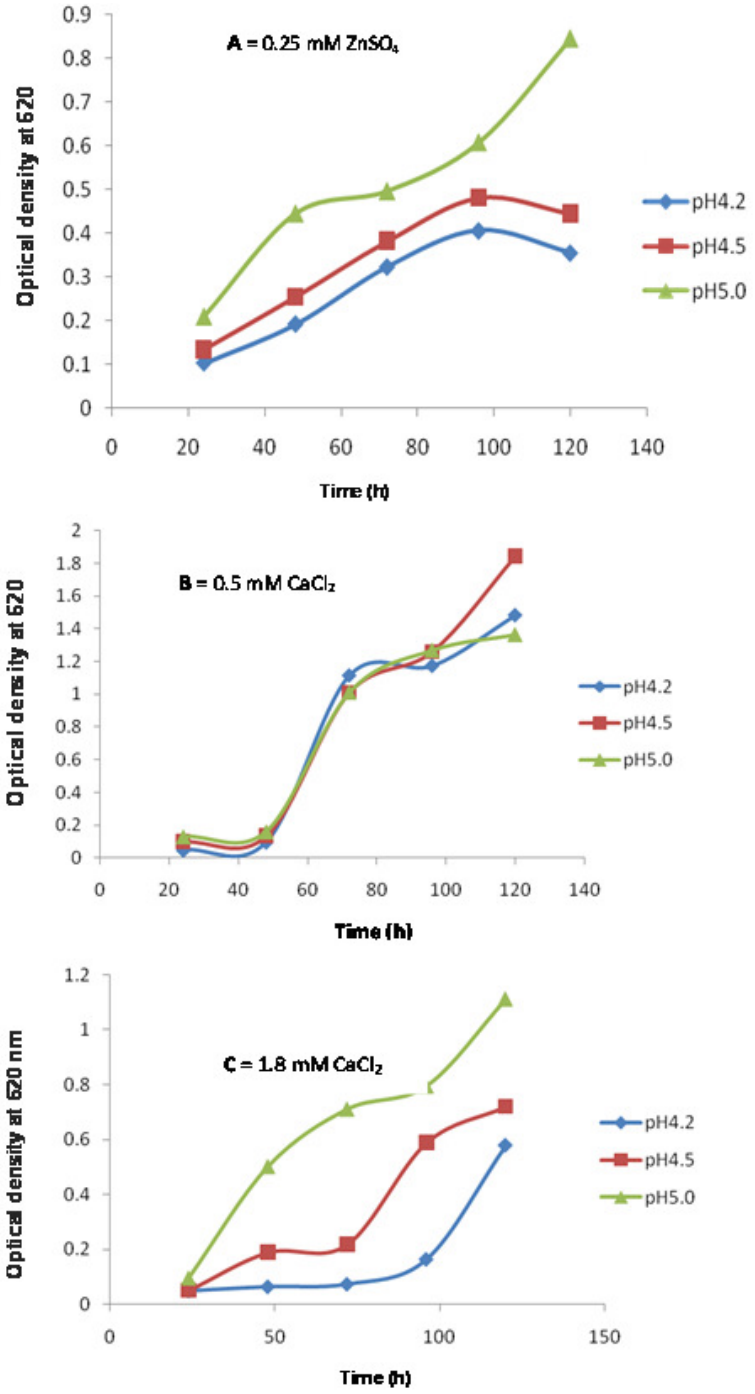

Figure 3. Time-course pattern in the growth of induced yeast cells of *R. stolonifer* at different combinations of ions and pH levels.

overcame inherent repression and assumed proliferation, and hence the resurgence of growth prior to termination of experiments. In contrast, in the 0.5 mM Ca^{2+} challenge, a sigmoid growth pattern was described in the buffered pH 5.0 culture. However, a resurgence of growth occurred after sigmoid pattern when the cultures were buffered at the lower levels of pH (Figure 3b). Perhaps similar explanation for this phenomenon stated earlier is here forth attributable. Growth profiles at the 1.8 mM Ca^{2+} concentration showed greater variability from the sigmoid pattern (Figure 3c). Growth had extended lag: 96 h at pH 4.2 and 72 h at pH 4.5; but it was very short at pH 5.0

Table 6. Overall yield of yeast cells induced from sporangiospores of *R. stolonifer* cultivated in buffered synthetic broth at different levels of exogenously applied Ca^{2+} and Zn^{2+} ions.

Parameter	Unicellular count (x10^6)		
	0.25 mM $ZnSO_4$	0.50 mM $CaCl_2$	1.80 mM $CaCl_2$
Mean	8.99	87.31	53.56
Standard error	0.149	1.629	1.64
Fold-increment	9.71 ←	→	1.63

Comparing tables 4 and 6, the difference that exists between 0.25 mM $ZnSO_4$ and 0.5 mM $CaCl_2$ on one hand and 0.5 mM $CaCl_2$ and 1.8 mM $CaCl_2$ on the other are also reflected in the direct cell count data, although the magnitude vary. As the $CaCl_2$ level increases, growth of induced yeast cells is suppressed.

where a resurgence of growth also occurred after 96 h. The higher profile description at pH 5.0 is a reflection of the magnitude of growth shown in Figure 2 and shows similarity with the pattern in the 0.25 mM Zn^{2+} supplementation (Figure 3a) although its magnitude was greater (Figure 2). Microscopic examination revealed that morphological expression in the pH 5.0 buffered cultures was protoplast and yeast cell, except when the ionic concentration was 0.5 mM Ca^{2+}, which was of mainly terminal budding yeast cell.

In all experiments, except with control and 0.25 mM Zn^{2+} supplementation, turbidity appeared to occur probably resulting from precipitation of calcium phosphate and this appeared to increase with Ca^{2+} levels. Similar observation was made in the study which showed the effect of exogenous Ca^{2+} on the growth of *Aureobasidium pullulans* (Madi et al., 1997). In order to validate the findings of this study, especially where sigmoid curves were obtained for optical density readings, direct cell count was do. This would eliminate any discrepancy in O.D. readings that could arise from calcium phosphate precipitation.

Unicellular population size in the 0.5 mM Ca^{2+} culture was 9.7-fold that of the 0.25 mM Zn^{2+}, while it was 1.6-fold the magnitude of the 1.8 mM Ca^{2+} broth (Table 6). In contrast, when biomass estimation was done through measurement of O.D., the value of 0.5 mM Ca^{2+} broth was 2.2-fold that of 0.25 mM Zn^{2+} and 2.1-fold the magnitude in 1.8 mM Ca^{2+} culture. Earlier experiment had shown that protoplast, cytosolic units that is, nucleates in earlier studies, are released when cell wall lysed, and thallic subtypes and conidia were induced in Zn^{2+} supplemented broths (Omoifo, 2006a). However, thalli were absent in the presence of 100 mM uracil (Omoifo, 2006b). In the present study, protoplasts and yeast cells contributed to the O.D. readings in the Zn^{2+} supplemented medium, whereas only yeast cells were herein counted. Calcium phosphate precipitated only in Ca^{2+} supplemented media. Thus, the contributors to the O.D. readings included the induced cellular entities and calcium phosphate precipitate. Since the value differential between the 1.8 and 0.5 mM Ca^{2+} estimate was larger, as reflected in the greater than 2-fold increase when O.D. was used in biomass assessment in contrast to the use of direct unicellular count (less than 2-fold increase), it was difficult to assume that calcium phosphate precipitation was the sole cause of the reduction in the biomass estimate in the 1.8 mM Ca^{2+} supplemented medium. Perhaps, the high Ca^{2+} concentration suppressed yeast cell induction and proliferation.

This was probably confirmed at pH 5.0 where yeast count in the 1.8 mM Ca^{2+} supplementation was significantly less than that of 0.5 mM Ca^{2+} (Figure 4). Furthermore, the 1.8 mM Ca^{2+} medium contained terminal budding yeast cells, unbudded yeast cells as well as protoplasts, whereas 0.5 mM Ca^{2+} broth contained terminal budding yeast cells only. Apparently, excess Ca^{2+} influenced the ability of protoplasts to convert to yeast form on the one hand, and of unicellular yeast form to undergo mitogenesis and or proliferation on the other. Similar trend, higher values for 0.5 mM Ca^{2+} as against 1.8 mM Ca^{2+}, occurred at pH 4.2 or 4.5 and further supports the results obtained when optical density was used for biomass estimation (Figure 2). These results illustrate the effect of interaction between pH level and exogenous Ca^{2+} supply.

To further test all such interactions, an analysis of variance of induced yeast count data was done (Table 7). There was significant ($p < 0.001$) difference for the induction and proliferation because of treatments, indicating the existence of variability in the transformation process.

The existence of variation in the treatments is in agreement with such findings when optical density was the measure of biomass, as shown earlier. This also agrees with the finding of Omoifo (2006a) when *M. circinelloides* Tieghem was the test microorganism and Zn^{2+} and myoinositol were incorporated into the growth medium. In the present study, the mean performances of treatments are presented in Table 8.

Of the treatments, the least induced yeast cell count was obtained in the 0.25 mM $ZnSO_4$: pH 4.2 medium and progressively increased with pH until the optimum for this ion in 0.25 mM $ZnSO_4$: pH 5.0 broth. The 0.5 mM Ca^{2+}

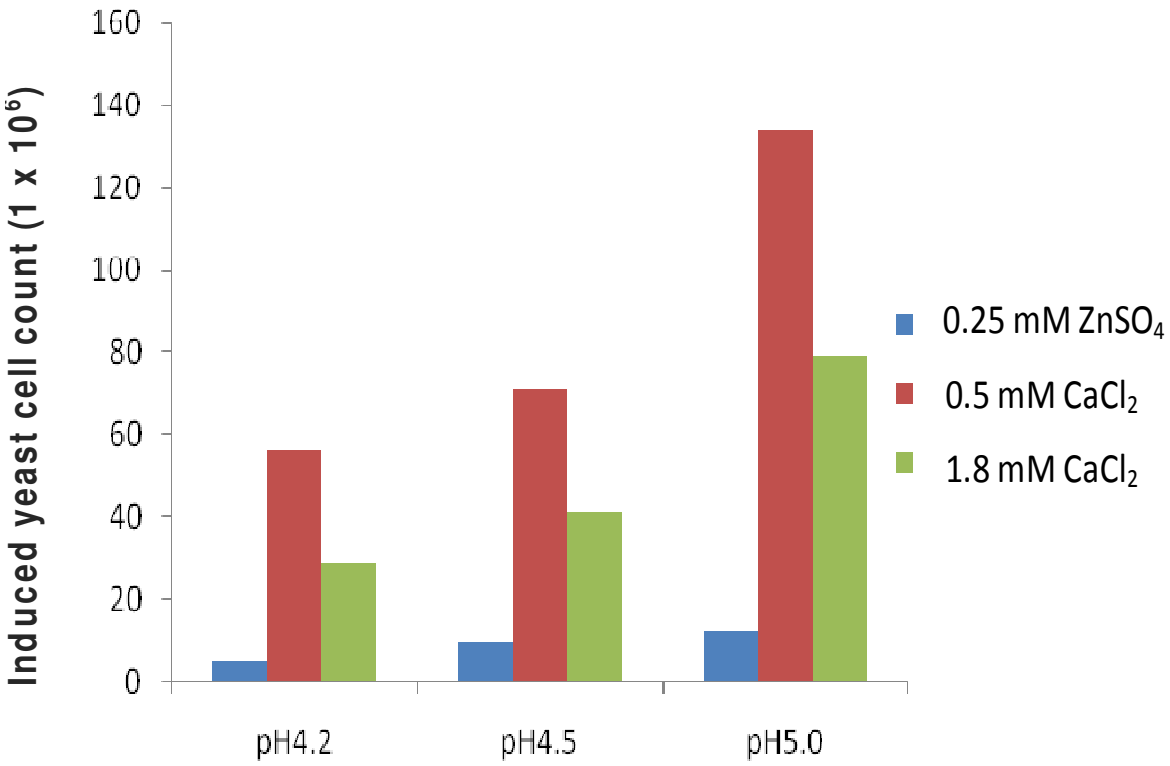

Figure 4. Microscopic cell count of induced yeast cells of R. stolonifer in Ca^{2+} and Zn^{2+} incorporated buffered synthetic broth as affected by different levels of pH.

Table 7. Treatment and induced cell estimates with their respective standard errors and form of growth during the cultivation of *R. stolonifer* in synthetic broth for 120 h at temp28°C.

Treatment	Mean cell count (x10^6)	Form of growth*
0.25 mM $ZnSO_4$: pH4.2	4.94±2.04	Y,P
0.25 mM $ZnSO_4$: pH4.5	9.68±4.47	Y,P
0.25 mM $ZnSO_4$: pH5.0	12.42±4.83	Y,P
0.50 mM $CaCl_2$: pH4.2	56.60±29.87	Y, P
0.50 mM $CaCl_2$: pH4.5	71.02±33.99	Y
0.50 mM $CaCl_2$: pH5.0	134.08±62.67	Y
1.80 mM $CaCl_2$: pH4.2	24.24±14.21	Y, P
1.80 mM $CaCl_2$: pH4.5	41.38±19.61	Y, P
1.80 mM $CaCl_2$: pH5.0	80.15±35.84	Y, P

*Y, yeast cell; P, protoplast.

concentration on average had higher growth value. The buffered pH 4.2 culture had mean count of 56.60 ± 29.87 and this increased as the medium pH until the maximum at 134.08 ± 62.27 at pH 5.0. Comparatively, 0.5 mM $CaCl_2$ supported higher level of growth than 1.8 mM $CaCl_2$. The increase in growth value also followed the same trend as those at the other levels. Therefore, the results of treatment means confirmed the influence of extracellular pH on the induction from, and subsequent proliferation of yeasts from sporangios pores of *R. stolonifer*.

Figure 5 shows yeast proliferation profiles in the different

Table 8. Analysis of variance of induced yeast cell count of *R. stolonifer* cultivated in buffered synthetic broth with different pH levels and exogenously applied ions.

Source of variation	Degree of freedom	Sum of square	F-value
Concentration	2	184113.5	<0.001
pH	2	66765.5	<0.001
Time	4	372095.5	<0.001
Concentration x pH	4	29373.5	<0.001
Concentration x time	6 (2)	177476.0	<0.001
pH x time	8	57370.1	<0.001
Concentration x pH x time	12 (4)	28381.7	<0.001
Residual	117 (18)	22439.1	
Total	155 (24)	875854.6	

As it was with optical density determination (Table 5), this table showed that the effect of the main factors and their interactions on the induction and proliferation of yeast cells from sporangiospores of *R. stolonifer* was statistically significant at $p < 0.001$.

ionic concentration: pH combinations. In the 0.25 mM $ZnSO_4$ treatment and with each pH level, the profile exhibited an extended lag phase, 96 h. This contrast the sigmoid pattern exhibited when O.D. measurements was made (Figure 3a). The difference probably resulted from the fact that in the 0.25 mM Zn^{2+} supplemented broth, protoplasts and nucleates, which are transient morphologies in the transformation process (Omoifo, 1996, 2003, 2005), were not counted except yeast cells.

At the 0.5 mM $CaCl_2$ level, apparently perfect sigmoid pattern was described at pH 4.5, whereas at the other pH levels, cell count was exponential at termination of experiments (Figure 5b). Similar trend was observedforthe various pH levels when the concentration was 1.8 mM Ca^{2+} (Figure 5c).

Zinc forms part of the zinc finger motif of the PKC where it's tetrahedral coordinates 2 histidine residues of the regulatory domain and responds stoichiometrically to DAG (Lalli and Sassone-Corsi, 1994). Since it plays critical role in activating PKC for its catalytic activity (Korichnieva et al., 2002), and serves as signalling molecule (Eide, 2006), a role for which calcium is known, being primary ion in signal transduction (Charp and Whitson, 1982; Berridge, 2005; Kretsinger, 1979; Clapham, 2007) and intracellular Ca^{2+} homeostasis (Cunningham and Fink, 1996; Clapham, 2007), the more effective divalent ion as exhibited in this experiment was determined. Results of a paired t-test for population means in Ca^{2+} and Zn^{2+} incorporated broths are shown in Table 9. The two test means are significantly ($p < 0.05$) different; $t = 6.87839$, $p = 4.34432E-9$. It is hereby concluded that Ca^{2+} is a better stimulatory cation to induction of and proliferation of yeast cells from sporrangiospores of *R. stolonifer.*

Growth profiles from log transformed induced yeast count data was obtained at the concentration levels of exogenously applied calcium. Profiles at the various pH levels displayed sigmoid growth pattern. This is shown in Figure 6. It was then possible to calculate the lag period and specific growth rate at the different pH levels. This is shown in Table 10. The lag periods at the 0.50 mM Ca^{2+} was considered short (24 h). Since a sporangiospore had to transit through composite biophysical stages, including growth sphere, nucleates (released cytosolic units) and distinct protoplasts prior to the yeast formation (Omoifo, 1996, 1997, 2003, 2005, 2006a, b; Omoifo et al., 2006), it possibly meant that the progress through these transient morphologies was very rapid. Contrastingly, lag phase at the 1.8 mM Ca^{2+} supplementation was prolonged (48 h). Perhaps biophysical and biochemical processing through the various stages of the transient morphologies became more tenuous, which possibly resulted from repression imposed by the presence of excess Ca^{2+}. But when inchoate yeast forms overcame the repression, growth became very rapid. As Table 10 showed, higher specific growth rates were achieved with 1.8 mM Ca^{2+} supplementation. This notwithstanding, the longer lag period at this level of concentration is a significant hindrance to achieving rapid growth and product formation. Therefore, 0.5 mM Ca^{2+} supplementation could be the choice for further biochemical work.

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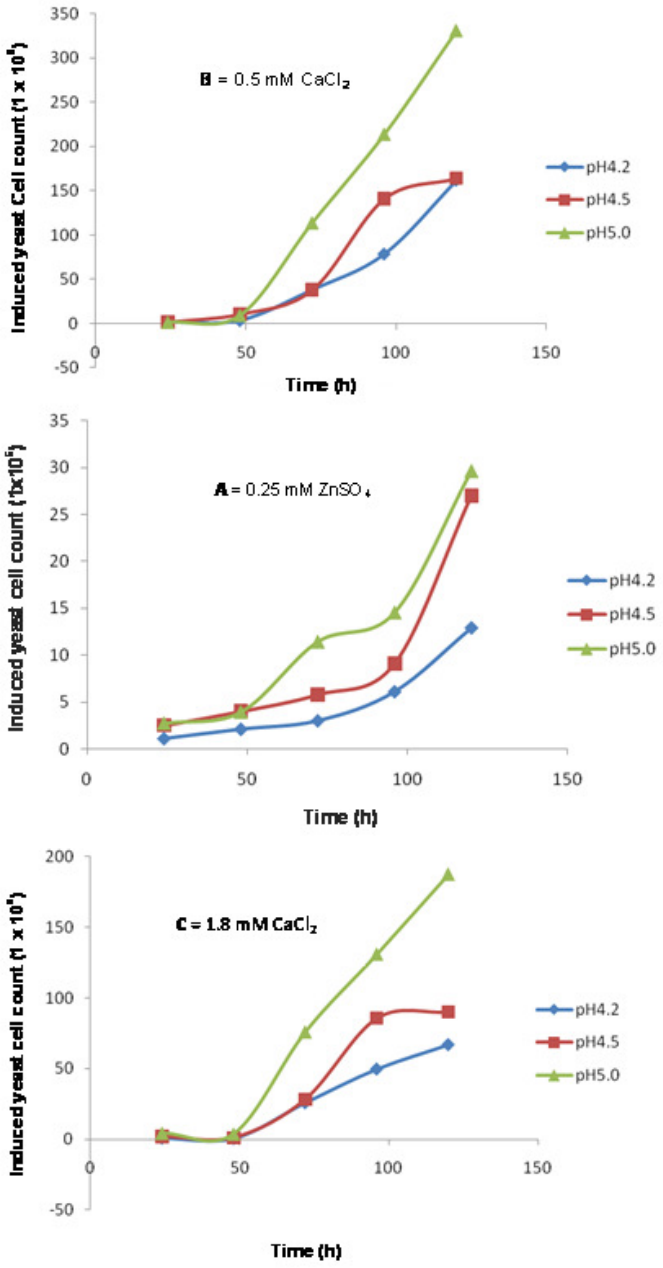

Figure 5. Time-course pattern in induced yeast cell count of R. stolonifer at different combinations of ions and pH levels. Growth pattern was sigmoid in the buffered pH 4.5 synthetic broths when Ca2+ was exogenously supplied, and at pH 4.2and 5.0, growth was still at the logarithmic phase when the experiments were terminated. Growth magnitude was reduced at pH 4.2.

Table 9. Paired t-test on total unicellular count in media incorporated with Ca^{2+} or Zn^{2+}

Ionic concentration	Mean	Variance	N
0.5 mM Ca^{2+}	9.31567E7	1.05997E16	60
0.25 mM Zn^{2+}	8.10667E6	6.52549E13	60

t = 6.87839, p = 4.34432E-9. At the 0.05 level, the two means are significantly different

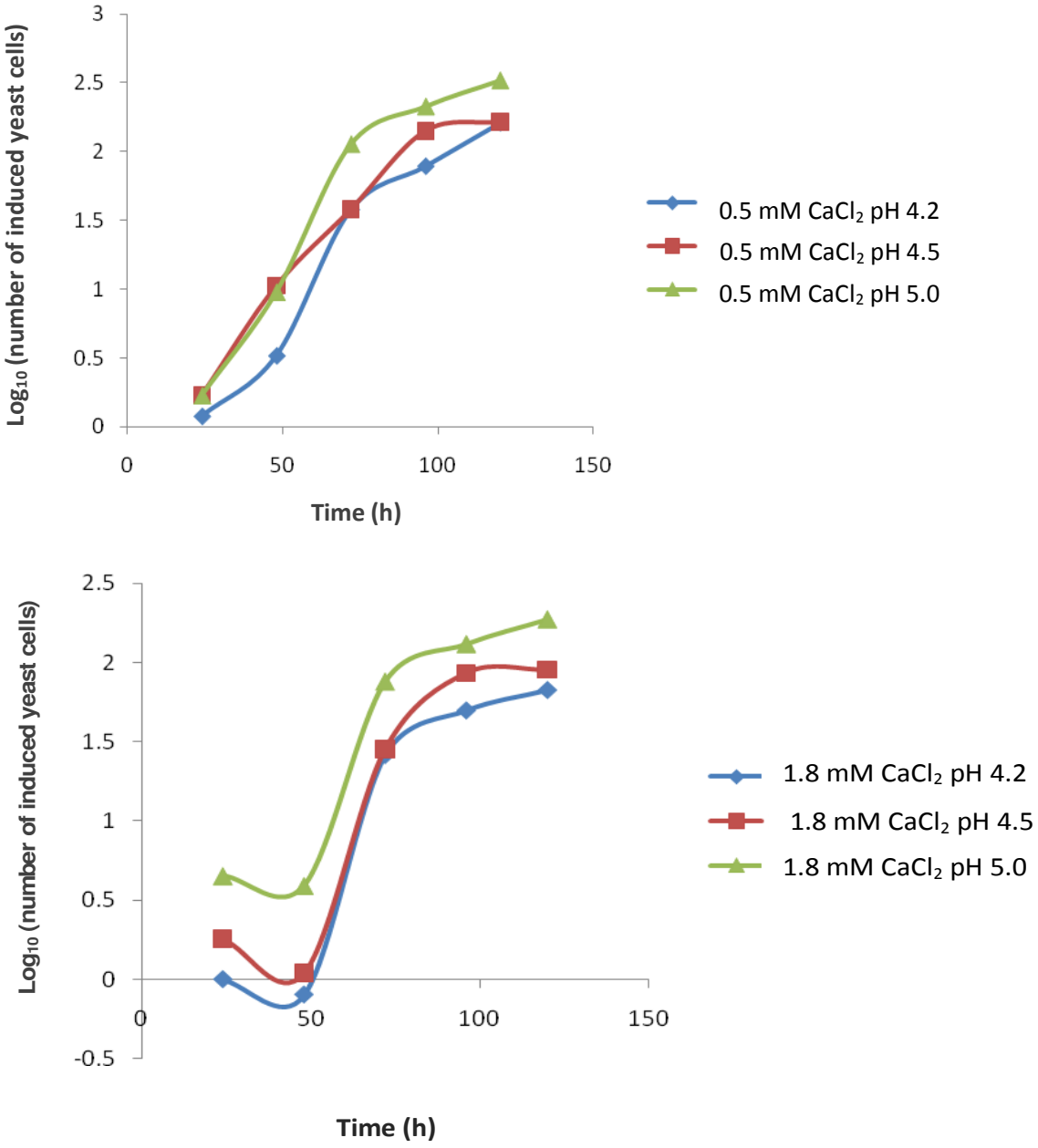

Figure 6. Log transformed induced yeast cell count of *R. stolonifer* in cultures where sigmoid growth was exhibited

Table 10. Lag phase and specific growth rate in cultures where sigmoid growth pattern was exhibited.

Extracellular concentration	0.5 mM Ca^{2+}			1.8 mM Ca^{2+}		
Extracellular pH	4.2	4.5	5.0	4.2	4.5	5.0
Lag phase (h)	24	24	24	48	48	48
μ	0.025	0.026	0.029	0.037	0.039	0.031

The longer lag possibly means that growth was repressed at that concentration/pH level resulting in lack of cell number increase. Conceivably, the eventual increase in cell number may then have been due to a minority of cells in the medium not initially suppressed, which could continue to grow and divide. In this situation, the lag would represent the time required for the subpopulation of cells not repressed to overgrow the arrested population. Alternatively, the cells could overcome the repressive effect of Ca^{2+}, by genetic adaptation and subsequently proliferate, thus achieving higher specific growth rate.

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